

ATTACHMENT AND SURVIVAL OF VIRUSES ON LETTUCE
(*Lactuca sativa* L. var. *capitata* L.): ROLE OF
PHYSICOCHEMICAL AND BIOTIC FACTORS

A Dissertation

by

EVERARDO VEGA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2006

Major Subject: Food Science and Technology

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Approved by:

Chair of Committee,
Committee Members,

Suresh D. Pillai
Ellen W. Collisson
Luc R. Berghman
Huaijun Zhou

Chair of Food Science
and Technology,

Rhonda Miller

August 2006

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ABSTRACT

Attachment and Survival of Viruses on Lettuce (*Lactuca L. sativa* var. *capitata* L.): Role of Physicochemical and Biotic Factors (August 2006)

Everardo Vega, B.S., University of Texas at El Paso

Chair of Advisory Committee: Dr. Suresh D. Pillai

Enteric viruses are responsible for a significant amount of foodborne disease in the United States. Foodborne disease associated with enteric viruses has been increasing within the last few years due to technological advances and raised awareness. Salads and salad crops are the principal vector for transmission of enteric viruses. The objective of this study was to determine if viruses are able to attach non-specifically to the surface of lettuce and to determine the forces responsible for non-specific viral adsorption to lettuce. Additionally, the impact of the microbial flora on viral persistence was studied to determine the effect on viruses. The four viruses studied were echovirus 11, feline calicivirus, MS2 and ϕ X174. The viruses were chosen based on their varying isoelectric points and similar physicochemical attributes. The isoelectric point was not the main factor determining virus attachment to lettuce. Viruses had varying attachment efficiencies, with echovirus 11 having the highest affinity to lettuce and ϕ X174 the least. Viral adsorption to lettuce was mediated by electrostatic forces due to the removal of virus adsorption at pH 7 and 8 with the addition of 1 M NaCl to the buffer solutions. Microcosm studies indicated that the microbial flora did not

have a negative impact on virus survival. The bacteriophages had the highest survival rate. Virus survival in the microcosm studies was not indicative of virus survival on the surface of the lettuce. The animal viruses exhibited survival rates greater than or equal to the survival of bacteriophages at 4° C, but at room temperature viable animal viruses rapidly declined compared to the bacteriophages. Additional studies also indicated that the microbial flora was not able to degrade the viruses for aerobic microbial growth. Overall, these results indicate that viruses are able to attach to the surface of lettuce, providing a possible explanation for the high incidence of virus associated disease involving salads and fresh produce. More importantly the use of surrogates for virus studies involving fresh produce must be re-evaluated, because of the lack of correlation between animal viruses and bacteriophages. Appropriate viral surrogates, if used, have to be carefully chosen based on viral physicochemical properties as well as the infectious route of the virus.

DEDICATION

This work is dedicated to my family

ACKNOWLEDGMENTS

I would first of like to acknowledge my wife, Noemi, who has helped me keep my goals in sight and who has never wavered in her support of me. I would not be the person I am today without her. I would also like to acknowledge my son, who in two years has shown me how important I am and how unimportant I am. I would also like to thank my mom and dad for giving me the confidence and values I have today and showing me the importance of family and hard work. I would also like to acknowledge my brothers for giving me great memories and their love.

I would like to acknowledge my advisor and mentor Suresh Pillai. He is responsible for my intellectual upbringing and has given me the skills and confidence to face the world. I thank him for changing my life.

I would also like to acknowledge my past and present lab mates, especially, Ken Widmer, Lingeng Lu, Nicholas Garcia, Jeannon Smith and Jim Totten for their work, the sharing of ideas and laughs. I would like to additionally thank Ken for helping me out with all the “real world” mundane problems and most of all for his help when my son was born. A favor I will always remember.

I would like to thank Jay Garland and Anabelle Matos for their support in my research project and their mentoring. Additionally, I would like to thank Jay for standing behind my work and his suggestions in improving manuscripts and in the direction of my overall research. I would like to thank my committee

members: Ellen Collisson, Luc Berghman, Huaijun Zhou and James Zhu. I would like to thank Dr. Collisson and her lab for providing valuable information for the completion of my work as well as Dr. Collisson for supporting me and having faith in my abilities. I would like to thank Dr. Berghman for helping me out when I most needed him and Dr. Zhou for stepping into my committee as soon as arriving at TAMU.

I would also like to thank the Poultry Science and Food Science and Technology departments and the administrative support staff for their work. I would also like to thank Robert Pottberg for getting things done when they needed to be done and Patty Horseman for everything from help with the printer to providing all kinds of valuable information. In addition, I would also like to thank Chris Horseman and all the other workers who have shared his position for keeping the lab functioning and allowing me to get my research done.

I would also like to give my gratitude to the NASA graduate student researchers program who had the foresight to fund this project to meet the demands of future space flight and help in the prevention foodborne disease here on earth. I have been honored to be a NASA-GSRP fellow.

TABLE OF CONTENTS

		Page
ABSTRACT		iii
DEDICATION		v
ACKNOWLEDGMENTS		vi
TABLE OF CONTENTS.....		viii
LIST OF FIGURES.....		xi
LIST OF TABLES		xiv
CHAPTER		
I	INTRODUCTION	1
	Relevance of Research.....	2
	Rationale	3
	Overall Objective.....	5
	Specific Objectives	5
II	LITERATURE REVIEW.....	6
	Enteric Viruses: Morphological and Physicochemical Characteristics	6
	Enterovirus Background and Replication	8
	Calicivirus Background and Replication.....	14
	Microvirus Background and Replication	24
	Leviridae Background and Replication.....	25
	Impact of Enteric Viruses on Semi-enclosed Systems.....	26
	Biases in Viral Associated Foodborne Disease	27
	Impact of Enteric Viruses on Foodborne Illness.....	31
	Enteric Virus Contamination Routes of Fruits and Vegetables	36
	Survival of Enteric Viruses on Fruits and Vegetables.....	39
	Attachment Mechanisms of Enteric Viruses onto Surfaces: Aquifer Material, Membranes and Plant Surfaces	45

CHAPTER	Page
	Decontamination Strategies for Removing Enteric Viruses from Surfaces 50
III	THE EFFECT OF CRITICAL pH ON THE ADSORPTION OF VIRUS TO BUTTERHEAD LETTUCE..... 53
	Overview 53
	Introduction 54
	Materials and Methods 57
	Results 60
	Discussion 66
IV	IDENTIFICATION OF FORCES CRITICAL FOR VIRUS ADSORPTION TO LETTUCE 71
	Overview 71
	Introduction 72
	Materials and Methods 74
	Results 80
	Discussion 93
V	THE SURVIVAL OF VIRUSES ON LETTUCE AND THE IMPACT OF THE MICROBIAL FLORA ON VIRAL PERSISTENCE 98
	Overview 98
	Introduction 99
	Materials and Methods 100
	Results 105
	Discussion 124
VI	SUMMARY 129
	The Effect of Critical pH on the Adsorption of Virus to Butterhead Lettuce 131
	Identification of Forces Critical for Virus Adsorption to Lettuce..... 131
	The Survival of Viruses on Lettuce and the Impact of the Microbial Flora on Viral Persistence 132
	Conclusions 133

	Page
REFERENCES	134
APPENDIX	
A	
PROTOCOLS AND PROCEDURES	155
Cell Culturing and Virus Assay Procedures	155
Harvesting and Purification of Viruses	159
Fluorescence Microscopy Protocol	161
PCR and Denaturing Gradient Gel Electrophoresis.....	163
B	
SUPPORTING EXPERIMENTS.....	173
Characterization and Identification of Lettuce Microbial <i>Flora</i>	173
Summary of the Microbial <i>Flora</i> Supporting Experiments	187
VITA	190

LIST OF FIGURES

FIGURE		Page
3.1	MS2 Adsorption to Lettuce	60
3.2	Feline Calicivirus Adsorption to Lettuce	61
3.3	Echovirus 11 Adsorption to Lettuce.....	64
3.4	ϕ X174 Adsorption to Lettuce	65
4.1	Echovirus 11 Adsorption to Lettuce with 1M NaCl.....	81
4.2	Echovirus 11 Adsorption to Lettuce with 1% Tween [®] 80	82
4.3	Echovirus 11 Adsorption to Lettuce with 1M NaCl and 1% Tween [®] 80	83
4.4	Feline Calicivirus Adsorption to Lettuce with 1M NaCl.....	84
4.5	Feline Calicivirus Adsorption to Lettuce with 1% Tween [®] 80	85
4.6	Feline Calicivirus Adsorption to Lettuce with 1M NaCl and 1% Tween [®] 80	86
4.7	MS2 Adsorption to Lettuce with 1M NaCl.....	87
4.8	MS2 Adsorption to Lettuce with 1% Tween [®] 80	88
4.9	MS2 Adsorption to Lettuce with 1M NaCl and 1% Tween [®] 80	89
4.10	ϕ X174 Adsorption to Lettuce with 1M NaCl.....	90
4.11	ϕ X174 Adsorption to Lettuce with 1% Tween [®] 80	91

FIGURE		Page
4.12	ϕ X174 Adsorption to Lettuce with 1M NaCl and 1% Tween [®] 80	92
5.1	ϕ X174 Microcosm Survival.....	106
5.2	MS2 Microcosm Survival	106
5.3	Feline Calicivirus Microcosm Survival.....	108
5.4	Echovirus 11 Microcosm Survival.....	109
5.5	ϕ X174 Lettuce Surface Survival	110
5.6	MS2 Lettuce Surface Survival.....	111
5.7	Feline Calicivirus Lettuce Surface Survival	112
5.8	Echovirus 11 Lettuce Surface Survival	114
5.9	Bibb Lettuce Microbial Flora Carbon Utilization	115
5.10	Iceberg Lettuce Microbial Flora Carbon Utilization	117
5.11	Hydroponic Bibb Lettuce Microbial Flora Carbon Utilization	118
5.12	Amino Acid Control Microbial Flora Utilization	120
5.13	Microbial Flora Virus Utilization	121
5.14	Washed Microbial Flora Virus Utilization.....	122
5.15	Background Microbial Respiration	123
A.1	Fluorescence Microscopy Slide Assembly	162

FIGURE		Page
A.2	Representative Gel of 16s rDNA PCR Product	166
A.3	DGGE Perpendicular Gel	168
A.4	DGGE Parallel Gel	169
B.1	Comparison of Microbial Elution and Growth Media	174
B.2	Comparison of Microbial Elution Second Experiment	175
B.3	Lettuce Microbial <i>Flora</i> 16s rDNA Cluster Analysis	180
B.4	Generic Cross Section of Leaf	184
B.5	ESEM Surface of Lettuce.....	186
B.6	Antiviral Activity of Lettuce Exudate	188

LIST OF TABLES

TABLE		Page
3.1	Physicochemical Properties of Viruses Used in the Study	56
3.2	Obtained <i>P</i> -values Between Virus Attachment at Each pH	63
4.1	Medians of Control Virus Adsorption to Lettuce	74
4.2	Obtained <i>P</i> -values Comparing Treatments	79
B.1	Total Microbial Counts Using Fluorescence Microscopy	177
B.2	Total Microbial Culturable Counts on Iceberg Lettuce	177
B.3	Identification of Microorganisms by FAME	182
B.4	Statistics of Antiviral Lettuce Exudate	189

CHAPTER I

INTRODUCTION

There has been considerable interest in a continuous salad crop production for long duration space mission flights to help maintain air quality within spacecraft and as edible food. The benefits of growing salad crops in space include wastewater processing, CO₂ elimination, O₂ production, and psychological benefits to astronaut (50). Studies involving lettuce production in a biomass production chamber have already been initiated and production of lettuce in the International Space Station (ISS) may be expected in the future (50, 124). Even though growing and consuming salad crops are beneficial to the over all well being of space mission crews, it can also be a source of human pathogens. Studies have indicated that bacteria and viruses are able to be and attach to the external surfaces and be absorbed into edible plants (143, 166). Because the health of space mission crews are vital to space missions, foods treatments, like thermostabilization, irradiation or dehydration, are utilized by NASA to ensure food safety (124). Fresh salad crops pose a problem because they cannot be treated as other foods without affecting food quality. Conditions aboard ISS, like scarcity of water and cramped living conditions coupled with minimal processing of salad crops makes contamination of salad vegetables a strong possibility. These conditions can lead to foodborne disease outbreaks among astronauts.

This dissertation follows the style of Applied and Environmental Microbiology.

Lettuce (*Lactuca sativa*) is a cause of concern because it is primarily eaten raw, has a large surface area, and grows adjacent to the growing substrate thereby increasing the probability of contamination. Of all the foodborne pathogens, none are as prevalent as enteric viral pathogens. In the year 2000, enteric viruses accounted for 28% of outbreaks and it is estimated that 80% of all foodborne gastroenteritis is caused by enteric viruses (22, 113). Viral outbreaks have a high incidence of occurrence in enclosed and semi-enclosed conditions, like daycare centers, cruise ships, campgrounds, military installations and hospitals. Furthermore, properties inherent to enteric viruses, like structural stability, resistance to disinfectants, and a low infectious dose make enteric viruses far more infectious than bacteria. Enteric viruses may also infect individuals asymptotically and are also capable of reinfecting individuals that have been previously exposed (19). Therefore, the primary goal of this study was to determine the forces responsible for attachment of enteric viruses to Butterhead lettuce (*Lactuca sativa* L. var. *capitata* L.) and to determine the effect of the lettuce normal flora on enteric virus persistence.

Relevance of Research

The results from this study will have important implications in food safety by elucidating a knowledge gap. Salad crop food safety during extended space missions will be improved by identifying conditions under which enteric viruses will be able to adsorb to butterhead lettuce. Additionally, this study will be relevant for food safety in general. Producers will be aware of conditions that

will induce viral adsorption, thereby provide a method to implement a critical control point in fresh produce processing. If viral adsorption cannot be prevented, the microbial flora can be harnessed to deactivate enteric viruses. This work will also provide information for optimal processing of fresh produce to remove enteric viruses from the surface. This information is important in conducting risk analysis, investigating foodborne outbreaks and conducting studies with viruses and fresh produce.

Rationale

In order to reduce the variability of enteric viruses, only non-enveloped viruses with icosahedral structures were used. To further remove variability, viruses were chosen based on their icosahedral similarities. The viruses that will be assayed will include MS2 bacteriophage, echovirus 11, feline calicivirus and ϕ X174 bacteriophage. Feline calicivirus (FCV) is the only non-enteric virus that was included in the study. Feline calicivirus have been routinely used as a surrogates for enteric caliciviruses because they are genetically and structurally similar to norovirus and are culturable, unlike norovirus (3, 4, 59, 60). Possible problems in using FCV include that FCV is a respiratory not an enteric virus, therefore the evolutionary pressure to maintain a stable structure in order to traverse the gastrointestinal tract is not present. Furthermore, it is possible because of differing tissue tropisms that the adsorption properties may be entirely different than in norovirus. MS2 and echovirus 11 are both non-enveloped, enteric viruses. MS2 phage is a T=3 virus with a pI of 3.9 (61).

Echovirus 11 is a pseudo T=3 virus. The pI of echovirus 11 has not been experimentally determined, but a closely related strain Echovirus 1 has a pI of 5.7 (173). The pI of echovirus 11 based solely on the amino acid sequence is 5.9 [<http://www.embl-heidelberg.de/cgi/pi-wrapper.pl>]. Bacteriophage ϕ X174 is a T=1 virus and has a pI of 6.6 (39). Thus, the experimental range of isoelectric points used in this study was in the range of 3.9-6.6.

Butterhead lettuce was used as the adsorber for attachment assays and as the source of the epiphytic microbial community. The isoelectric point (pI) of the viruses is important because if the pH of a solution is below the isoelectric point of a virus, it will cause the virus to have a positive charge, and if the pH of the solution is above the isoelectric point of the virus, it will cause the virus to have a negative charge. There are no published reports on the surface charge of butterhead lettuce, but the waxy cuticle of a plant is believed to have a pI of approximately 3 (56).

The pH range used in this study was 3-8. The pH range of 3-8 is important for two reasons. 1) The pH range covers the isoelectric points of all the viruses tested. 2) The pH range is biologically important because fruits and vegetables and irrigation or wash water can be within the range of 3-8. Furthermore, previous studies have used acids or base washes to try to increase the shelf-life of fruits and vegetables by decreasing the normal flora or as a possible treatment for reduction of pathogens (7, 73, 78, 141).

Overall Objective

The overall objective of this work was to elucidate how enteric viruses are able to contaminate salad crops and once contaminated the subsequent persistence of enteric viruses on salad crops.

Specific Objectives

1. Determine if the critical pH of viruses is the overriding factor in virus adsorption to butterhead lettuce.
2. Determine the forces that mediate non-specific viral adsorption to lettuce.
3. Determine if viruses are negatively impacted, in terms of survival, in the presence of the butterhead lettuce microbial flora.

CHAPTER II

LITERATURE REVIEW

Enteric Viruses: Morphological and Physicochemical Characteristics

Enteric viruses are viruses that propagate themselves in the gastrointestinal tract of animals. Propagation of enteric viruses may arise from infecting the animal host or a microbial colonizer in the gastrointestinal tract (GI) of an animal. In general, enteric viruses lack a lipid envelope and are thus termed naked viruses. Naked viruses are more resistant to desiccation stress. Desiccation stress is the main environmental condition affecting survival of viruses in the environment. Because naked viruses do not have a viral envelope, receptor specificity is located in the protein structure surrounding the viral nucleic acid or capsid. The protein capsids in naked viruses serve two main functions, protection and receptor specificity. Viral nucleic acid is more resistant to DNA or RNA nucleases if located within a capsid (84). The capsid of an enteric virus has evolved to withstand environmental and GI tract stresses. Environmental stresses include predation, nucleases, proteases, deactivation by UV light, desiccation and chemical deactivation. Gastrointestinal stresses include large fluctuations of pH, digestive enzymes, mechanical shearing, and predation in the lower GI tract (109).

Because of the severe stresses placed on enteric viruses, many enteric viruses have structurally stable capsids. Structural stability is due in part to the icosahedral shape. A true icosahedron has 20 polyhedral faces, which is a

solid bounded by polygons, composed of 60 equilateral triangles. Thus, a true icosahedral virus will have 60 identical capsomeres. A true icosahedral virus will have a $T=1$ structure, or a triangulation number of 1. Virus structure is described as a function of its icosahedral structure. The equation $T \times 60 = N$, where T is the triangulation number and N is the number of subunits in the viral icosahedral structure, provides the relationship between triangulation number and the number of capsomeres in an icosahedral virus. Viruses with triangulation numbers above one, are described as quasi-equivalent to an icosahedral. In viruses that have triangulation numbers greater than one, the virus capsomeres interact with each other much like an icosahedral. A $T=1$ virus will have 60 identical subunits making up its capsid and each capsomere is made up of 1 protein. A $T=3$ virus will have 180 subunits making up its capsid and 3 proteins making up each capsomere. Pseudo $T=3$ viruses are viruses with interactions within the capsomere that functions like a $T=3$, but are composed of 4 proteins within a capsomere.

The icosahedral shape also provides 2-3-5 fold symmetry. Two-fold symmetry refers to the point between any two capsomeres along the whole length of a single capsomere. Three-fold symmetry refers to a complete capsomere face and its interactions between other capsomers along all three sides. Five-fold symmetry refers to five capsomeres interacting together to form a pentagon. Icosahedral symmetry simplifies viral attachment because only three stereotypic conformations are available for attachment. Furthermore, the

interaction between a virus and surface is further simplified because the icosahedral is composed of identical reoccurring protein faces. Therefore, the icosahedral shape of an enteric virus is geometrically stable and is made up from reoccurring protein faces (capsomeres) allowing for 1) minimal structural encoding in the nucleic acid, 2) large internal surface area, 3) quasi-equivalent capsid structure requiring minimal energy for assembly and 4) 2-3-5 fold symmetry (53).

Enterovirus Background and Replication

Enterovirus Background. The picornaviruses are a diverse group of viruses comprising of six virus groups: 1) aphthovirus, which includes the foot and mouth disease virus, 2) cardiovirus, which includes the encephalomyocarditis virus and theiler's murine encephalomyelitis virus, 3) enteroviruses, which include echoviruses and coxsackie viruses, 4) hepatovirus, which includes hepatitis A virus, 5) parechovirus, which includes the parechovirus, and 6) rhinovirus, which includes one type of the viruses responsible for the common cold, rhinovirus. Structurally, all picornaviruses are similar in genome length, 7.2-8.5 kbp, shape, icosahedral, and size, about 30 nm in length. Though picornaviruses are structurally similar, there exist fundamental differences in capsid integrity which are related to infectious route. The enteric picornaviruses, i.e. viruses that have a fecal-oral route as part of their infectious route, are stable viruses. Studies have shown that hepatitis A virus, poliovirus and other enteroviruses are environmentally stable viruses (90, 93, 153, 168). Polioviruses infect cells by

translocating their genomic RNA into the cell, thus the capsid remains outside of the cell. Other picornaviruses, like foot and mouth disease virus and rhinoviruses, infect the cell by undergoing endocytosis. After endocytosis, a decrease of pH within the endocytic vesicle causes the viral capsid to dissociate with the viral genomic RNA (18, 118). Thus, even though all picornaviruses are similar, they have evolved into divergent cellular infectious route, which ultimately effect the stability of the capsid. This is an important point that must be considered when choosing surrogate viruses for stability or attachment studies.

Enteroviruses are a large group of viruses within the picornavirus family of viruses. The human enteroviruses are comprised of four groups 1) human enterovirus A, 2) human enterovirus B, 3) human enterovirus C and 4) human enterovirus D, known as HEV-A, HEV-B, HEV-C, HEV-D, respectively. These four enterovirus groups are known to cause a wide range of diseases, like mild cold-like symptoms, conjunctivitis, a multisystemic hemorrhagic disease of newborns and are the principle cause of aseptic meningitis (66, 104). It is believed that enteroviruses are responsible for about 90% of the cases of aseptic meningitis (66). The large variety of diseases caused by enteroviruses also cause a large variation in incubation times of 2 days - 1 month depending on the disease (66). In addition to a varying incubation times, the majority of infections are sub-clinical or inapparent. Only 1-2% of infections develop significant disease (66). Even though the rate of severe sequela is low, the vast amount of

infected individuals, 50 million in the United States (est.) and at least 1 billion worldwide (est.) each year translate to a large disease burden (121).

The enterovirus, coxsackie virus, was the first enterovirus, after poliovirus, to be isolated. This virus was first isolated in 1948, in New York State during a paralytic poliomyelitis investigation in the town of Coxsackie. The coxsackie virus isolated was pathogenic to newborn suckling mice, whereas other enteroviruses, like echovirus isolated in 1955 were not pathogenic to newborn suckling mice. This was the initial basis of separating the coxsackie and echoviruses. Echoviruses were originally unclassified, thereby their name reflects their status when first isolated, enteric cytopathogenic human orphan viruses, echovirus. In 1962, the committee on enteroviruses determined to classify enteroviruses using a numerical system to avoid confusion (34). At the present, new enteroviruses use the numerical system, ex: enterovirus 70, whereas the older enteroviruses are still commonly referred to by their original names, ex: coxsackie A1, poliovirus type 2, or echovirus 11.

Enteroviruses exist as a collection of circulating enteroviruses. A predominant strain will emerge and cause the majority of disease. After immunity is developed a new species, arising from either mutation or recombination, will circulate causing disease. Enteroviruses, like many RNA viruses, have a high mutation rate, for poliovirus its $1 \text{ bp}/10^3 \text{ bp}$ (167). Studies with a high fidelity RNA dependent RNA polymerase in poliovirus shows that a high mutation rate is important for immune evasion within the host (163).

Enterovirus Replication. The enterovirus genome is about 7.5 kbp and is a plus strand RNA. The RNA of enteroviruses is infectious and structured as follows: 5'- NTR-VP4-VP2-VP3-VP1-2A-2B-2C-3A-3B-3C-3D-3'-NTR (66). The proteins are translated into a single polyprotein, which is then post-translationally processed into the precursor proteins (P1, P2, and P3) and mature proteins. The 5'- non-translated region (NTR) contains secondary structures that are important in viral replication and protein translation (66). The P1 region encodes the virus capsid structural proteins VP4-VP2-VP3-VP1. Enteroviruses, like all picornaviruses, are membrane associated when they are being assembled. During assembly the VP4 and VP2 protein (VP0) are fused until the genomic RNA is packaged into the capsid. After packaging, VP0 is cleaved into the mature protein VP4 and VP2. The capsid, as is true for all non-enveloped viruses, contains the receptor site for initiating the infection of the host. Picornavirus receptor sites are located in “canyons” on the capsid. These sites are hidden from the immune system thereby making antibody neutralization difficult. Some picornaviruses, like poliovirus, have the lipid sphingosine within the receptor site.

The P2 and P3 regions encode proteins that are involved in shut-down of host cell protein synthesis, as well as enzymes that replicate the viral RNA and process viral proteins (99, 107, 119). The 2A and 3C (or 3CD) proteins contain protease activities that are responsible for cleavage of the viral polyprotein into mature proteins. The P2 region consists of 2A-2B-2C genes. The protein 2A has

been shown to be responsible for limiting host cell translation. The main target for 2A is the eukaryotic initiation factor GI or eIFGI. The host protein eIFGI is a translational initiation co-factor responsible for translation initiation. The cleavage of eIFGI by 2A does not completely inhibit cellular translation. Other cellular co-factors eIFGII can also function in the initiation of translation. Subsequently, 2A can also cleave eIFGII, though not as efficiently as eIFGI (64). An additional target of 2A is the poly-A binding protein (PABP) which is involved in translation of mRNA by interacting with the 5' end (82). The end effect of eIFGI and II cleavage is that 2A stops cellular translation and helps induce apoptosis.

The other viral protease is the 3C protease. Viral protein 3C is responsible for processing of the capsid proteins (76). Much like 2A, 3C is able to cleave the PABP. The difference seems to be that 2A cleaves PABP to limit initiation of translation and 3C cleaves PABP to prevent the recycling of the PABP (91). In the latter, 3C seems to process PABP much more efficiently than 2A. Also, like 2A, 3C also induces apoptosis. Together 2A and 3C are able to effectively shut down cellular translation and thus induce apoptosis of the cell.

Viral protein 2B has been shown to form pores in the endoplasmic reticulum (ER) and golgi apparatus (GA) (159). Studies have shown that 2B is able to cause a decrease in Ca^{2+} in the ER and GA. This is believed to prevent a massive release of Ca^{2+} causing mitochondrial damage and the subsequent release of cytochrome c. The release of cytochrome c is involved in a positive

feedback loop in apoptosis. Thus the main function of 2B is to be antagonistic to the effect of the 2A and 3C proteases (159). The viral protein 2B though cannot prevent apoptosis, but slows down the process sufficiently for the virus to complete its infectious cycle of the cell. An additional function of 2B is to create favorable conditions within the cell. Protein 2B also causes aggregation of vesicles derived from the GA and ER which function in viral replication (159). All picornaviruses replicate in virus associated membrane complexes. Viral protein 2C is a viral regulatory protein, and has been reported to have a variety of effects. Banerjee *et al.* has reported that protein 2C down regulates the effect of 3C and 2A proteases on the cellular transcription factors (6). In addition, 2C has shown an effect on viral RNA minus strand replication. The 2C protein has also been shown to have GTPase and ATPase activity. In addition, 2C has membrane-binding ability though it does not have a hydrophobic domain, but is believed to bind to a membrane bound protein (6). The exact role of 2B and 2C though has not been fully elucidated and their exact role in the viral replication has of yet be fully explained.

Protein 3D contains the viral RNA-dependent RNA polymerase that replicates the viral RNA. The viral replicase is usually complexed with the 3C protease and is released from the protease during replication. RNA synthesis is primed by a uridylylated form of 3AB, resulting in covalent linkage of 3B (VPg) to the 5'-end of the genomic RNA.

Calicivirus Background and Replication

Calicivirus first came to attention in the 1930's with an outbreak of a Foot and Mouth Disease (FMD) like disease. Symptoms were similar to FMD, but during the outbreak only swine were infected indicating a new agent was involved (140, 147). For about 10 years, the FMD-like disease was sporadically reported in California, but outbreaks were controlled by the culling of herds and outlawing the feeding of uncooked pork to other pigs (140, 147). In an effort to further control the disease, export of pigs or pig products to other states in the United States was not allowed. Ultimately, the law was unsuccessful in containing the disease (140, 147).

Investigation of the FMD-like disease identified a viral agent that also infected sea lions. Infected sea lions had vesicular lesions on their flippers and had abortive pregnancies (140). The agent responsible for the FMD-like disease was named San Miguel Sea Lion Virus (SMSV) or Vesicular Exanthema Swine Virus (VESV) (140). The virus was isolated from a variety of animals including, birds, reptiles, insects, fish and nematodes (140, 147). The virus, VESV, has up to date been the only virus capable of infecting such a large range of different hosts. The epidemiology of the VESV indicated that sea lions could not have spread and propagated the disease. Further studies indicated that the sea lions were only part of the virus life cycle. The isolation of SMSV from opal eye perch fish provided evidence that SMSV is actually a fish virus (140). Furthermore, SMSV was isolated from a lungworm parasite, which uses the opal eye perch fish

as an intermediary host for its main host, the sea lion (32). Thus, the VESV outbreak is believed to have started from a calicivirus that infects opal eye perch, or the lungworm, then the virus infected sea lions by either consumption of the infected fish or by the lungworm. The VESV outbreak has been the only reported case in which a land disease outbreak has its main reservoir in the ocean (140).

In the 1970's volunteers were fed an unknown agent prepared from a rectal swab of an infirmed volunteer from a school in Norwalk, Ohio, where half of all the students and teachers were infected with a viral agent (45). Using electron microscopy, the diseases agent was identified as a virus with a diameter of approximately 27 microns with cuplike depressions. The caliciviruses were thus named after their morphology, calix, meaning cup. The agent responsible for the school outbreak was termed Norwalk virus. Ever since then, caliciviruses have been recognized as the leading cause of non-bacterial gastroenteritis in the United States. Mead *et al.* estimated that caliciviruses are responsible for about 74% of all viral gastroenteritis (113).

Aside from the burden on human morbidity, caliciviruses also cause disease in other animals. The rabbit hemorrhagic disease virus (RHDV) was first identified in a large rabbit outbreak in China (147). Rabbit hemorrhagic disease virus is notable because this virus caused greater than 99% infection rate accompanied with greater than a 90% mortality rate, additionally only rabbits older than two months were susceptible to infection (32). After exposure,

rabbits became depressed and had a fever 16-24 hours after infection, then died rapidly. Pathology of the rabbits indicated hemorrhagic lesions all throughout the body organs but was most evident in the liver and spleen (147).

The disease caused by RHDV spread rapidly and almost eliminated rabbit industries in many countries. The infectious route of RHDV was determined to be fecal-oral. In Europe, a related virus that causes the same symptoms but is avirulent was discovered. The virus was called European Brown Hare Syndrome Virus (EBHSV). It is believed that RHDV evolved from EBHSV (32, 147, 151). A vaccine for RHDV was developed and was found to be effective against infection to RHDV (147). The virus RHDV was used as a biocontrol agent for wild rabbit populations in Australia and New Zealand. Rabbit populations initially declined but most rabbits are now resistant to RHDV(147).

A respiratory disease of domestic cats was determined to be caused by feline calicivirus (FCV) (147). Further studies indicated that FCV could infect domestic felines, wild felines and dogs (110, 147). Though its virulence was mostly connected to domestic cats, most members of the family *Felidae* are susceptible (32, 116, 147). Disease symptoms include respiratory problems along with lameness, and ulcers in the tongue and mouth (147). Infected felines exhibited a low-grade infection for up to two years (128). During the infection FCV is constantly produced. The low-grade infection has been attributed to the generation of viral quasi-species, where the immune system is unable to effectively clear the infection from the body (128). The severity of the disease

ranges from respiratory distress to a hemorrhagic disease, much like RHDV (2). An effective vaccine has been developed for FCV, but due to the generation of quasi-species, boosters and reconfiguration of vaccine is needed for effectiveness.

The virus diseases caused by VESV, FCV, RHDV and human viral gastroenteritis has increased awareness of caliciviruses. Caliciviruses have been isolated from most farm animals, domestic pets, birds, and a variety of other animals (140). Caliciviruses are ubiquitous in nature. The outbreaks involving caliciviruses has generated interest but due to a lack of a cell culture system research has been limited.

The calicivirus family of viruses comprise of four genera: lagovirus, which includes RHDV, vesivirus which includes FCV and SMSV, norwalk (renamed norovirus) which are primarily human caliciviruses (HuCV), and sapporo-like virus which are human and animal caliciviruses (69). The sapporo-like viruses are also human pathogens, but seem to affect mostly children, and some share homologies to both animal and human caliciviruses (30). The norovirus group can be divided into two main genogroups, though there are 5 genogroups, which are important for human viral gastroenteritis (68).

The calicivirus FCV has been the only calicivirus amenable to a cell culture system, with the exception of RHDV, which can only be replicated in a primary rabbit hepatocyte cell culture. Recently a canine calicivirus (CaCV) has been used for studies but the virus is not as easy to work with as FCV, therefore

FCV remains the most common calicivirus amenable to research (42). A porcine calicivirus and a murine calicivirus have recently been identified that will propagate itself in a cell culture system, but if it is as easy to use as FCV remains to be seen (27, 172). The culture of the porcine calicivirus, the first enteric calicivirus, in cell culture was made possible with the addition of bile salts to cell culture which was shown to make cells permissive to the enteric calicivirus.

Outbreaks of HuCV indicate that HuCV are highly infectious with a rapid onset of morbidity. Human caliciviruses are excreted in the feces of infected individuals. Symptoms reported include vomiting, diarrhea (non-mucous, watery, non-bloody), nausea, headache, and malaise (45). There have also been some HuCV reports that indicate the virus is able to become systemic (24). Persons infected with HuCV are resistant to the same inoculum for up to a year (111). If exposed after two years, infection occurs again. Furthermore, the antibody immune response did not correlate with the susceptibility to infection. Persons with a high antibody response were susceptible, whereas persons without an antibody response were not susceptible. The persons with a high antibody response were later found to have non-neutralizing antibody to HuCV (111). Furthermore, some people were completely recalcitrant to infection, irrespective of the dose of virus administered. More recent studies have surfaced that may elucidate the infectivity of HuCV.

Studies show that a person's secretor status, the ability of a person to secrete blood carbohydrates in body fluids, and blood type may be important in HuCV susceptibility (96). Non-secretors, 20% of people of European ancestry, are believed to be resistant to many HuCV. Furthermore, the blood type of a person may also be a factor of the status and carrier state of HuCV infections. In a study by Lindesmith *et al.*, Type O individuals were resistant to infection, but in other study by Meyer *et al.* Type O individuals were more susceptible (96, 115). Other studies since then have indicated that distinct HuCV have different affinities to different blood types, Lewis antigens and even the density of certain carbohydrates on the surface of cells (149). In effect, HuCV have evolved to infect defined populations of people. Unlike Influenza virus which has a predominate strain circulating at one time, HuCV has many strains that circulate at one time. The affinity of HuCV to blood types and secretor status may explain the high diversity of strain circulation at one time.

Structural and molecular studies of HuCV have shown that the capsid is unlike many animal viruses. The calicivirus capsid is made up of one major structural protein, a trait seen in viruses of plants, bacteria and insects, not in animals. The capsid protein contains three domains, S, P1, and P2 (162). The S domain is the core capsid and fuses to form one contiguous core. The P1 domain is located after a hinge region where the capsid is able to accommodate its structure to fit into a T=3 structural configuration. The hinge region is also believed to act as an area of flexibility to allow the outer capsid domains to

interact with each other or with the receptor. The P2 domain shows variability between genogroups. The P1 region is a globular region at the outer most end of the capsid protein. The P1 region is also known as the hypervariable region (29). The hypervariable region is believed to allow caliciviruses to evade the immune system and possibly provide the host diversity common to some caliciviruses (29). The hypervariable region also explains the high rate of mutation and the large diversity of calicivirus in circulation at one time. It is now obvious that HuCV utilizes the same strategy that many viruses, like hepatitis C virus and influenza virus, use to evade immune antibody neutralization.

Replication of Calicivirus. Caliciviruses encode for 8 proteins: 5'-2B-2C (helicase-like)-3A-3B (Vpg)-3C (protease)-3D (polymerase)-VP1 (major capsid protein)-VP2 (minor capsid protein)-3'. All caliciviruses have the same genomic structure with variations in ORFs (open reading frame). Norovirus has one large ORF, which includes all the non-structural proteins, and an ORF that encodes the major capsid protein, and a third ORF that encodes the minor capsid protein (33, 45, 69). Sapporo-like viruses encode for another protein, of unknown function, that is located within the capsid protein and is offset by a -1 bp frame shift. Rabbit hemorrhagic disease virus has two ORFs, the first ORF encodes the non-structural and major capsid protein and the second ORF encodes for the minor structural protein.

Calicivirus proteins, are believed to be homologs of enterovirus proteins, and are located in the same area during replication as the enterovirus proteins

and in the same order. The genomic structure of enteroviruses and caliciviruses are similar. The main difference being that the enterovirus structural proteins are located before the non-structural proteins whereas in caliciviruses the non-structural proteins are located before the structural proteins. The order and function of the non-structural proteins appear to be similar.

Much of the replication data for calicivirus has not been determined. Until recently, most of the proteins had unknown functions and much information is still unknown. Most of the data involving calicivirus has been determined with FCV and RHDV because of their ability to replicate in cell culture. More recently, recombinant baculovirus systems have been used for identifying protein functions.

The 3C protease, 2C helicase-like protein, and 3D polymerase have similarities to picornaviruses. Studies have indicated that the 3C protease in RHDV and FCV is able to cleave the viral proteins in trans but maintains some activity in cis (15). More recently, the protease has been shown to cleave poly-A binding protein and cleaves translation initiation co-factors (91). Cleavage of translational factors is much like the strategy that picornaviruses utilize to stop host cell translation. Additionally, the protease has been found in high concentrations in membrane bound replication centers (a replication strategy that picornavirus also utilizes) and still fused to the polymerase, which indicates that the pro-pol complex may be involved in replication, like picornaviruses (9). Studies with the 2C helicase-like protein have not found a helicase activity. Even

though the helicase-like protein has high homology to the picornaviral helicase, it uses NTPs indiscriminately whereas the poliovirus helicase uses ATP predominantly (125). The importance of this is unknown. Only a few studies have been initiated with the 2B protein. In the study by Ettayebi *et al.* the 2B protein was membrane bound and was shown to disrupt a glycoprotein expression at the cell surface (46). In a FCV homolog, the 2B protein was associated with the membrane replication complexes (81). Much like poliovirus 3A protein, calicivirus 3A has been found to disrupt golgi trafficking by disassembly (51). Calicivirus 3A has also been found to be associated in the replication complexes with the 2B-like protein, thus calicivirus-3A also may be involved in replication (69, 81).

The Vpg protein in caliciviruses is larger than enterovirus Vpg. A comparison between enterovirus and feline calicivirus Vpg indicates that they have different functions. Enterovirus RNA is infective without a Vpg and only functions in replication and in RNA absorption into the host cell (see enterovirus replication). Furthermore, the start of translation of enteroviruses is more than 100 bp downstream of the Vpg, whereas the start of translation for calicivirus ranges from 9-14 bp from the Vpg (10, 74). This difference is probably due to the IRES in enteroviruses, whereas caliciviruses do not have an IRES. Additionally, the Vpg in feline calicivirus is necessary for infection, and has also been shown that FCV RNA translation is not effected by high potassium concentrations, which inhibit normal cellular translation. Calicivirus Vpg is also able to bind

translation initiation factors (36, 65). All this information indicates that calicivirus Vpg is needed for translation initiation. Thereby, even though picornavirus and calicivirus have similar genome structure and proteins, calicivirus translation is different than picornavirus translation, which utilizes an internal ribosome entry site (IRES).

The replication of genomic RNA for caliciviruses has not been fully elucidated, but the function and similarity of many non-structural proteins to enterovirus does indicate a similarity in genome replication. Both poliovirus and caliciviruses form membrane bound replication centers and their homologous proteins are also present at these replication centers, indicating a similar function in replication.

It was believed that caliciviruses had only one structural protein. Crystallization of the capsid had failed to show more than one protein (162). At the same time it was known that the protein at the 3' end was transcribed on a sub-genomic RNA along with the major capsid protein (151). The function and even its expression were not known. It was discovered by Glass *et al.* that the minor protein (VP2) was present in low numbers in the virion (63). Further work has elucidated the function of this protein in the calicivirus structure. The minor structural protein is translated at about 20% of the major capsid protein. Its function is part regulatory and part structural. The presence of VP2 induces translation of VP1 (11). Furthermore, VP1 dimers are protected from degradation by proteases and virion capsids are more stable in the presence of

VP2 (11). Thus, it is believed that VP2 “ties” together VP1 and is the main contributor to the stability of many caliciviruses. The translation of VP2 is controlled by RNA secondary structure at 3' end of the genomic RNA.

Microvirus Background and Replication

The virus family microviradae has four genera: 1) microvirus, 2) spirovirus, 3) bdellovirus and 4) chlamydia virus. All viruses in this family are single stranded circular DNA bacteriophages. The microviradae are non-enveloped and are icosahedral with a T = 1 structure (38). They are 25-27 nm in diameter.

The bacteriophage ϕ X174 belongs to the genus microvirus and its natural host is *E. coli*. The genome length is 5.4 kbp and is made up of 11 genes: 5'-A-A*-B-K-C-D-E-J-F-G-H-3'. Bacteriophage ϕ X174 was the first DNA genome to be completely sequenced, this was accomplished by Sanger *et al.* in 1977 (134). The ability of viruses to have overlapping genes was first discovered in ϕ X174 bacteriophage. Since then, ϕ X174 has been the most intensely studied virus in the family microviradae. The bacteriophage ϕ X174 digested genome is also commonly used as a DNA marker. Bacteriophage ϕ X174 has also been extensively used as a viral surrogate for human viruses and as a viral indicator for fecal contamination (105, 176).

The genome of ϕ X174 has 11 genes. Genes F-H are virion structural capsid proteins. Gene J is also found within the virion, but its function is two fold. It serves as a DNA packaging protein and as a DNA binding protein to

neutralize the negative charge of the single stranded DNA genome (38). Gene products A and C are replication modulators. Gene product A functions in stage II and III DNA synthesis, whereas gene product C is involved in the switch from stage II to stage II DNA synthesis (38). Bacteriophage protein A* inhibits host DNA synthesis and prevents re-infection of host. Bacteriophage genes B and D are scaffolding proteins, which function in pro-capsid (immature virion) morphogenesis (38). Bacteriophage gene E is responsible for cell lysis, by destroying the cell wall leading to the rupture of the cell membrane with the subsequent release of bacteriophage progeny (38). Gene K is believed to somehow enhance phage yields though its exact function is not known.

Leviviridae Background and Replication

The virus family leviviridae are single stranded positive sense RNA bacteriophages. The leviviridae are icosahedral viruses with a diameter of 26 nm with a T = 3 structure. The leviviridae have two virus genera the Levivirus and Allolevirus. The prototype virus for the leviviridae is Ms2 bacteriophage and the prototype allolevirus is Qbeta. Ms2 bacteriophage and Qbeta have been routinely used for as human viral surrogates and as viral fecal indicators (3, 37, 39, 146, 154).

The bacteriophage Ms2 is a male-specific phage, which infects the pilus of F⁺ *E. coli*. The MS2 genome consists of 4 genes: 5'-A-C-L-P-3'. The viral capsid is comprised of the C gene product, 180 copies in the capsid and 1 A protein (158). The A protein is involved in the attachment of the virion to the pilus.

Attachment of the A protein causes the A protein to cleave into two fragments (158). The cleavage of the A protein causes the viral genomic RNA to be released from the capsid. As the pilis is retracted, the 5'-end of the viral RNA is internalized into the host cell, this step outside of the cell is the RNase sensitive step (158). Translation of the A protein is dependent on the secondary structure of the MS2 RNA genome.

The P gene encodes the polymerase and its timing, i.e. ability to be translated is controlled by the amount of C protein (158). The presence of C protein inhibits translation of the polymerase gene P. The L gene is the lysis gene but it does not have a ribosomal binding site. Translation of the L gene is dependent on a frameshift from the C gene.

Mutations in the Ms2 genome are not common, mostly because the secondary structure of the viral genome is vital in the viral infectious cycle (158). Removal of the secondary structure will eliminate many of the translational controls of the MS2 infectious cycle.

Impact of Enteric Viruses on Semi-enclosed Systems

Enteric viruses are egregious for causing disease in enclosed and semi-enclosed systems. Semi-enclosed conditions have limited access to the outside environment, as well as have a high density of persons within a confined area. Crowded conditions facilitate transmission of viral diseases by maximizing the number of people a single person can infect. Some examples of semi-enclosed environments are daycare centers, hospitals, campgrounds and barracks. As a

result, outbreaks have been reported in youth camps, military installations, hospitals, naval vessels, daycare centers, and college dormitories (23-25, 47). In addition, many semi-enclosed conditions also have poor sanitary conditions, like daycare centers and elderly homes. This fact, coupled with the robustness of enteric viruses in the environment, causes a high incidence of enteric virus infections. The use of hand sanitizers in homes with children attending day-care centers has been shown to greatly decrease the amount gastrointestinal illness in these settings (133). The efficacy of the hand sanitizers though has not been shown to be effective with enteroviruses or caliciviruses (135). In semi-enclosed settings, immediate intervention is important to stanch new viral infections. In cruise ships, removal of passengers and personnel is recommended to thoroughly disinfect a vessel (25). In hospitals, studies have indicated that afflicted wards must be closed and all patients and staff must be removed for proper disinfection to halt the duration and spread of the outbreak (14).

Biases in Viral Associated Foodborne Disease

Biases in the foodborne viral disease abound in the medical community, sentinel programs and research community. A survey of physicians by Jones and Gerber (2001) shows that physicians in Tennessee are largely unaware of the disease burden of viruses (80). In this survey 90% of respondents believed that *Salmonella* was one of the top three causes of foodborne disease, followed by *E. coli* (56%), *Staphylococcus* spp. (36%) and *Shigella* spp. (32%). Though *Salmonella* is an important foodborne pathogen responsible for an estimated

10% of foodborne disease in the United States, the other three together amount to no more than 3.2% of the total foodborne disease illness in the United States (113). In this survey, 5% of the physicians surveyed listed norwalk and an additional 4% included viruses as one of the top three causes of foodborne disease. In actuality viruses cause 67% of foodborne illness in the United States (113). Because physicians are the front lines of many sentinel programs and are often responsible for identifying and reporting foodborne disease outbreaks, this presents itself as an important bias.

The main sentinel program for foodborne disease in the United States is Foodnet. Foodnet incorporates cohort studies, surveys of both physicians and people as well as sample analysis. Even though surveys of physicians do include a questionnaire for viral analysis of stool, viruses are not included in the active surveillance system (Foodnet) though viruses comprise the majority of foodborne diseases causing microorganisms. Hepatitis A is the only viral associated reportable disease, but it is reportable under the national notifiable disease surveillance system.

The lack of knowledge on the importance of viral diseases in foodborne illness at both the surveillance and physician level can be traced back to the scientific community. Even though enteric viruses are recognized as causing significant morbidity in foodborne associated gastroenteritis, there is disagreement on the route of infection or a false perception on the foods associated with viral gastroenteritis. Epidemiology studies on the cases of

foodborne illness shows differences within different surveillance systems. A European study of viral gastroenteritis outbreaks between 1992-2000 in England and Wales indicated that 85% of viral outbreaks are due to person to person spread whereas foods were implicated in 10% of the outbreak cases (102). In contrast, two more recent studies in the United States show that between the years 2000-2004, 30% of calicivirus outbreaks were attributed to food and 35% were attributed to person to person spread (16). Another study in the U.S. by Widdowson *et al.* looked at norovirus disease between 1991-2000 (170). This study, the largest and most complete to date, included 8,271 foodborne outbreaks reported to the CDC. In this study, 48% of the outbreaks implicated a foodhandler, whereas 52% of the outbreaks a foodhandler was not implicated. Of the unknown causes of foodborne disease, 9% implicated a foodhandler and 91% did not implicate a foodhandler. It is generally believed that the majority of unknown foodborne disease outbreaks fit the epidemiologic pattern of a viral pathogen. These results are contrary to popularly held beliefs in the scientific community.

A review article by O'Brien *et al.* studied the publication bias of infectious intestinal disease in England and Wales for the years 1992-2003 (117). O'Brien *et al.* found publication biases in a variety of areas. Certain locations like shop caterers were 9.2 times more represented in the literature than in actual data. Likewise unknown outbreak types were almost 10 times more underrepresented in the literature than in the outbreak data. Certain food products also enjoyed

an overrepresentation. Milk and milk products were overrepresented almost six times more than their actual occurrence. This study is important for the scientific community to be aware of the occurrence of biases in the literature and for risk analysis of certain disease agents or foods. Consequently, publication bias is the most likely source of the belief that foodborne transmission of viruses is primarily associated with a 1) foodhandler and 2) seafood. The data compiled from the CDC clearly does support these beliefs.

In addition to investigator biases, there are technological advances that can overcome certain biases. Foodborne disease attributed to viruses is an example of an underestimation due to technology. The study by Widdowson *et al.* also showed that norovirus outbreaks increased from 11 in 1996 to 164 in 2000 (170). This increase coincided with the state technical support program in 1997 where certain states confirmed outbreaks by RT-PCR. Even then, the CDC did not use RT-PCR to confirm outbreaks until 1993.

The vehicle associated in viral foodborne disease also has considerable bias. Shellfish a commonly believed vector for viruses accounted for 3% of the total norovirus outbreaks (170). The top two vectors of foodborne disease for norovirus was salads at 26%, followed by produce and fruit at 17% (170). These two vectors are not commonly thought to be responsible for viral outbreaks even though the CDC foodborne outbreak data indicates the importance of these vectors in norovirus transmission.

Recent work has shown that viruses do not adsorb to lettuce at the same amounts. This is an important point to consider when sampling salads of fresh produce for the presence of viral pathogens. If pathogenic viruses are not present in high numbers on the produce, then detection will be difficult. This may be the reason why there is a great disparity between data from England and Wales (9.9% foodborne transmission) and from the United States (52% foodborne transmission). England and Wales surveillance is based on electron microscopy, a difficult and insensitive method to detect viruses, whereas the United States uses RT-PCR, somewhat difficult but very sensitive. Electron microscopy favors identifying viruses in stool samples, a large concentration of viruses per gram of sample, than food which would have much less virus load per gram of sample.

Impact of Enteric Viruses on Foodborne Illness

The Centers for Disease Control (CDC) foodborne disease data from the year 1993-1997 indicates that viruses accounted for 6% of foodborne disease outbreak cases (120). The majority of foodborne disease outbreaks, 68%, are of unknown origin (120). Many of the outbreaks due to unknown agents are considered by the CDC to be caused by viral agents, but due to the lack of resources and technology, many of these outbreaks were not identified (120). The next five years, 1998-2002, viruses as a percent of foodborne disease cases increased to 40.6%, a nine fold increase in foodborne disease cases. More recently from 2003-2004, viral foodborne disease cases now comprise 51.5% of all foodborne

disease cases, an 11 fold increase, of the foodborne disease cases in the United States. The increase of viral agents associated with foodborne disease is due to technological advances and raised awareness (79, 170). Calicivirus outbreaks, for example, are initially identified epidemiologically by “wildfire” outbreaks, a rapid increase in infected individuals. Viral gastroenteritis symptoms are sufficiently different from other foodborne disease agents that epidemiology criteria can be used to identify viral gastroenteritis symptoms. The recognition that it is difficult to identify viral gastroenteritis because of technology and monetary issues, as well as the slow dissemination of these technologies to the public health laboratories, have led to a non-laboratory based identification of viral gastroenteritis outbreaks.

There has been a call for a reevaluation of epidemiological criteria for identifying viral gastroenteritis caused by norovirus. Turcios and other scientist from the CDC have proposed the use of Kaplan criteria for identifying outbreaks of acute gastroenteritis caused by norovirus (155). The Kaplan guidelines for identifying outbreaks of gastroenteritis due to norovirus includes four criteria: 1) vomiting in more than half of affected person, 2) median incubation period of 24-48 hours, 3) median duration of illness of 12-60 hours and 4) the lack of bacterial pathogens in a stool culture. When this criteria was applied to a subset of foodborne outbreaks between 1998-2000 from the foodborne disease outbreak data maintained by the CDC, the Kaplan criteria was able to identify 68% of the outbreaks correctly, and specifically (99%) (155). This criteria has

already proven useful in Sweden and Denmark, where Kaplans criteria is already applied to certain outbreaks (103).

Previously, with the lack of technology and awareness, Mead *et al.* attempted to estimate illness due to foodborne disease outbreaks. Mead *et al.* estimated that viral gastroenteritis due to foodborne disease accounted for 67% of all foodborne disease outbreaks in the United States (113). Other studies since the Mead *et al.* study has reported a lower number. Widdowson *et al.* estimated that 50% of all foodborne outbreaks are attributable to norovirus. Blanton *et al.* studied the molecular and epidemiological trends of calicivirus associated outbreaks, and they determined that 35% of the outbreaks was person to person and 30% was foodborne (16). Because of low population coverage, the true amount of foodborne disease is difficult to verify, nevertheless current data from the CDC indicates that viruses are the primary agent responsible for foodborne disease (47, 170).

Recent years have identified major outbreaks, which would have otherwise not been connected without the aid of sequencing. In 1997, 258 persons were reported to be infected with Hepatitis A Virus (HAV) in 5 states. Genetic relatedness indicated that the same strain of HAV was responsible for the multistate outbreak. Frozen strawberries from the same producer were implicated in the outbreak (77). In 1998, frozen raspberries were involved in a calicivirus outbreak across 5 countries and in 2003, over 500 persons were sickened in the U.S. by HAV due to contaminated green onions (2, 4). In 1988,

202 cases of HAV was caused by contaminated lettuce in Kentucky (53). A problem with identifying viral foodborne outbreaks is the low infectious dose of the major enteric viral pathogens. The infectious dose of caliciviruses, Hepatitis A virus, and rotavirus is between 10-100 virus particles (10, 17, 32). Because viruses are able to reproduce in humans, clinical identification is not difficult, but identification of environmental sources is difficult to detect. As a result, many outbreaks, may not be identified as foodborne because of the lack of a common food source (see Biases in Viral Associated Foodborne Disease).

Data collected from the CDC and other public health agencies indicate that enteric viruses are responsible for a significant cause of morbidity. Mortality associated with viral foodborne disease is of concern especially with the ageing of industrialized nations. Elderly, neonates and other immune-compromised individuals are especially susceptible to enteric viruses. For norovirus, data from England and Wales indicate that hospitalization occurred in 1 out of every 40 outbreaks and mortality occurred in 1 out of every 50 outbreaks (102). In the United States, Mead *et al.* estimated that the proportion of hospitalization and death was 10%, more recent data from Widdowson *et al.* indicated that 9.8% of persons afflicted with norovirus required medical care and 1% required hospitalization. Considering the large pool of morbidity caused by norovirus (2.3 outbreaks per/100,000 people), even 1% is a large amount of hospitalization. It is thus not surprising that the primary cause of mortality related to foodborne pathogenic microorganisms are viral agents (112).

Other enteric viruses are not as widespread as noroviruses, but cause more serious disease or complications. Enteroviruses do not generally cause foodborne disease outbreaks, but probably infect as many or more persons as noroviruses. Whereas noroviruses propagate themselves in rapid outbreaks with a high incidence of disease, enteroviruses are disseminated themselves without causing an overt outbreak, a small subset of persons infected (1-2%) exhibit serious clinical symptoms (88, 121, 126). A 1-2% rate of severe clinical symptoms would not warrant serious scrutiny, but would be an important source of sporadic disease. Enteroviruses are the principal cause of non-bacterial meningitis, and the most significant factor of infectious infant mortality (62). Enteroviruses are also responsible for myocarditis, conjunctivitis, hemorrhagic disease of newborns and the second leading cause of the common cold (88, 126). Because the enterovirus infectious route is fecal-oral there is some concern for infections due to water, though this is mostly because enteroviruses are routinely isolated from water sources (54). Because enteroviruses have been found in water, contamination of shellfish are the primary cause of concern for foodborne illness due to enteroviruses (55, 92, 174). Though fresh fruit and vegetables are irrigated with water sources that cause concern because of the presence of enteroviruses, fresh fruit and vegetables do not seem to be a major area of focus in enterovirus associated foodborne disease.

Enteric Virus Contamination Routes of Fruits and Vegetables

Fruits and vegetables are of concern to food safety because many vegetables, like salad crops, are consumed with minimal preparation, thereby posing an increased risk of foodborne illness, if contaminated. Contamination of fruits and vegetables may arise from many routes like irrigation water, animal defecation, insects and food handling during harvest and processing (12). Furthermore, because of large scale farming or distribution, a fruit and vegetable outbreak may involve hundreds of people over various states or countries (See Impact of Enteric Viruses on Foodborne Illness). The year round demand of fresh fruits and vegetable has lead to an increased risk of foodborne illness due to fruit and vegetables irrigated with contaminated water in countries without adequate sewage treatment. Calicivirus and HAV outbreaks have been associated with imported and domestically grown produce irrigated with contaminated irrigation water (52, 77, 130).

Enteric viruses have also been isolated from carrots prior to distribution (44). This indicates a potential for not only fecal contamination of fresh carrots, but also the possible presence of human enteric viruses. The use of viral fecal indicators for identifying fecal contamination, has come into question. A large survey study in which 448 groundwater sites were assayed did not show a correlation between indicators, bacterial or viral, with the presence of either positive cell culture or RT-PCR results (1). However a study for identifying correlations between bacteriophages and human enteric viruses on fresh fruits

and vegetables has not been attempted. Previous research on the presence of enteric viruses on both surface and ground water is of concern. A study by Borchardt *et al.* attempted to determine if groundwater in La Crosse, Wisconsin contained human enteric viruses, and if the infiltration of surface water had a correlation with the presence of human enteric viruses (17). Borchardt *et al.* found 13 types of enteroviruses, hepatitis A virus and norovirus in both the groundwater and surface water. The surface water contained the highest incidence of human enteric viruses, but in the groundwater all but one of the wells tested was positive for at least one human enteric virus using RT-PCR. The use of RT-PCR gave superior sensitivity but could only detect viral nucleic acid and could not make a distinction between infectious and non-infectious virus. In order to overcome this problem, the authors also used cell culturing. Hepatitis A virus was found by cell culturing in three wells, none were positive for enterovirus. These results are difficult to interpret because the sensitivity of cell-culturing is much less than RT-PCR. Either enteroviruses are present in low concentrations or the enteroviruses are inactivated. Additionally, there was no correlation between surface water infiltration of ground water and positive RT-PCR results. The authors concluded that groundwater must have been contaminated with an urban source, not related to surface water.

Surface water has been recognized as having high levels of fecal bacteria and viruses mostly from agricultural, wildlife and septic system sources (145). The large presence of human enteric pathogens in surface water shows that

surface water contamination is heavily impacted by human waste. A study in the Netherlands found 1-2 pfu and 4-4,900 of enteroviruses and norovirus per liter of river water, respectively (101). As a means to identify the source of the human pathogenic viruses, Lodder *et al.* sampled sewage from the treatment plants and identified that treated sewage decreased the viral load from raw sewage 0.7-1.8 logs/liter for enterovirus and 0.9-2.1 logs/liter for norovirus. The treatment was largely ineffective for adequately reducing the viral load in sewage water which mostly likely contributed to the viral load in river water. These results are corroborated by another study in Germany, where treatment plant effluent and associated downstream waters were sampled for assorted enteric viruses (127). In this study, enteroviruses were found in 29-89% of the samples. Norovirus ranged from 15-44% positive. Infectious enterovirus, measured by cell culture, was found in 33% of the positive RT-PCR samples. Overall, these studies indicate that both surface waters and ground waters harbor human enteric pathogens even though the commonly used criteria of fecal indicators shows the waters are of acceptable quality. Thus, fresh fruit and vegetables have the potential to be contaminated by the use of irrigation water from both ground and surface sources.

Previous studies have also indicated that surface contamination of plants is not the only possible route of contaminating fresh produce. A study by Ward *et al.* used bacteriophage f2, a virus similar in size and structure to caliciviruses and picornaviruses, to determine if viruses can be absorbed into plants (166).

Ward *et al.* used 3 week corn (*Zea may L.*) and bean plants (*Phaseolus vulgaris L.*) grown using hydroponics as his model. Bean plants were able to absorb f2 phage through undamaged roots up to a maximum concentration of 10^3 pfu/g. If the roots were damaged, the viral concentration reached up to 10^6 pfu/g. Corn plants were able to absorb f2 phage through intact roots at a maximum concentration of 10^1 pfu/g. Damaged corn roots absorbed 10^4 pfu/g. Though the amount of f2 virus was low in certain cases, the amount absorbed was, at a minimum, able to absorb enough viruses to infect one person per gram of plant, if the f2 virus was a human enteric pathogen. Furthermore, the ability of plants to absorb virus was not dependent on the concentration of virus spiked into the growth solution. Even though Ward *et al.* used three different concentrations of f2 virus, 10^{10} , 10^7 , and 10^5 virions, the absorption of f2 phage was not affected. All the bean plants tested contained 10^5 pfu/g, irrespective of the concentration of viruses in the hydroponic solution. The study by Ward *et al.* indicates that plants are able to absorb viruses within the infectious dose, 10-100 virions, of human viral pathogens. Enteric viruses are stable infectious organisms able to survive for prolonged periods of time in soil, fresh produce, and are able to be absorbed into plant material.

Survival of Enteric Viruses on Fruits and Vegetables

The stability of enteric viruses indicates that enteric viruses are able to survive on salad crops for extended periods of time. Dawson *et al.* determined the survival of MS2 bacteriophage, as a surrogate for norovirus, on tomato, cabbage,

carrot, lettuce, parsley, peppers and strawberries (37). At 4 and 8° C, MS2 was able to survive for 87 days or until sampling wasn't feasible, due to spoilage. At these two test conditions, a reduction of no more than two logs was shown for all fresh commodities tested. Other studies have also determined that poliovirus can be isolated from lettuce and radishes after 23 days of the initial virus spike (153). A study by Croci *et al.* showed that at 4° C HAV is able to survive on lettuce, fennel and carrot. Survival of HAV was 4 days on carrots, 7 days on fennel and decreased two logs after 9 days on lettuce (35). Studies on enteric virus persistence by Allwood *et al.* investigated the persistence of MS2 and FCV on lettuce and cabbage (4). MS2 was inoculated onto the cabbage and lettuce at a titer of 13 - 14 log pfu. Feline calicivirus was inoculated at a titer of 6.0-6.5 log pfu. At 4° C, MS2 was able to persist for greater than 1 log pfu for 12 days, whereas FCV persisted for greater than 1 log pfu for 5 days. At 25° C, MS2 was able to persist for 12 days, and FCV survived for 5 days. At 37° C, FCV and MS2 survived for 3 and 12 days respectively. Even though FCV is a commonly used surrogate for norovirus, FCV is a respiratory virus, thus the physiochemical characteristics may not be comparable to other enteric viruses. Kurdziel *et al.* inoculated 10⁴-10⁵ poliovirus virions onto a variety of fruits and vegetables (romaine lettuce, green onions, white cabbage, fresh raspberries, and frozen strawberries) (90). The vegetables and fresh strawberries were held at 4° C, while the frozen strawberries were held at -20° C for 2 weeks. Poliovirus exhibited a 90% decrease after 11.6 days on lettuce, 14.2 days on white cabbage,

8.4 days on frozen strawberries, and did not decline for fresh raspberries and green onions. Differences in survival rates were attributed to desiccation, though because of the experimental design of this experiment the effect of desiccation could not be determined. Stine *et al.* used a controlled environmental chamber to determine the effect of humidity on viral and bacterial agents (146). The viruses that were tested included PRD1 bacteriophage, hepatitis A virus and feline calicivirus. On cantaloupe, survival of all three viruses was greater in dry conditions than in humid conditions. On lettuce, both PRD1 and HAV exhibited greater survival in dry conditions than in humid conditions. The animal virus, FCV, experienced a higher deactivation rate in dry conditions than in humid condition; but the difference between dry and humid, deactivation rate of 1.13 and 1.06, respectively, was minimal. On bell peppers, HAV and FCV had higher survival in dry conditions than in humid conditions. Bacteriophage PRD1 had a lower survival on dry conditions, but again the difference in deactivation rates was minimal, 0.14 and 0.08 dry and humid, respectively. The work by Stine *et al.* is important because it disputes the previously held belief that desiccation stress is important in virus survival on the surface of fresh produce. The results indicate that enteric viruses are able to survive for extended periods of time on fruits and vegetables, and therefore salad crops are able to provide a stable vector for transmission of enteric viruses.

Survival of enteric viruses at the pre-harvest stage, may be dependent not only on the physiochemical stability of the viral capsid, but also on the ability of

the epiphytic community to utilize viruses as carbon or nitrogen sources. Previous studies have identified the important role that microbial predation has on enteric viruses survival (160, 166). Some evidence on the importance that the epiphytic community has on virus survival was provided by Bidawid *et al.* in a study utilizing a modified atmosphere packaging (MAP) (13). The main objective of their study was to determine the effect of temperature and a MAP on the survival of HAV on lettuce. Modified air packaging has been extensively used in the fresh produce industry for extending the shelf-life of fresh produce by inhibiting the growth of spoilage microorganisms. At 4° C, there was no difference between using atmospheric air in an enclosed bag, MAP or unpackaged lettuce. At room temperature, the changes were significant. Using MAP, virus decreased from 0.5-1 log over the course of 12 days. The virus, HAV, decreased 1.5 logs in a bag filled with atmospheric air. Decrease of HAV on unpackaged lettuce was much greater than all other treatments, 4 logs. The authors attributed this decrease to compounds released by the lettuce during the normal cellular death of the lettuce, even though none of the experimental conditions tested for this outcome. These results could only be a result of microbial predation or viral inactivation by desiccation. The study by Stine *et al.* showed that desiccation does not effect viral survival on fresh produce, therefore the decrease of virus could only come from microbial predation. Thus, the use of carbon sources by epiphytes on leaves may be important for virus persistence.

Even though easily utilizable sugars like glucose, fructose, and sucrose are in abundance on the surface of a leaf, the uneven distribution of sugars on leaves did not allow for continual growth of epiphytic communities (95, 114). Mercier *et al.* found that even in leaves with a high microbial load, a substantial amount of sugars remained (114). Additionally, the initial concentration of sugars allowed for a rapid short-term proliferation of *Pseudomonas fluorescens* sprayed onto the leaves, but after available sugar was consumed, growth of *Pseudomonas fluorescens* stagnated. The results by Mercier *et al.* are important because it indicates that epiphytes are able to utilize carbon sources on plant surfaces. Furthermore, the rapid short term proliferation of *Pseudomonas fluorescens* indicates that R-strategist bacteria are important for initial epiphytic growth, but further growth will have to arise from K-strategist bacteria or bacteria that will utilize more complex carbon sources (5, 85). *Pseudomonas* spp. do not neatly fit into the R-strategist and K-strategist paradigm. Even though they are rapid growers, they are also able to utilize a wide range of carbon and nitrogen sources. Many of the *Pseudomonas* spp. isolated from plants are able to utilize complex carbon and nitrogen sources like most amino acids and have a variety of hydrolases that are able to utilize sucrose, maltose, trehalose and xylans (123). *Pseudomonas* are also able to utilize long-chain hydrocarbons and aromatic derivatives and has all the necessary transporters for amino acid uptake (123). The wide variety of carbon and nitrogen sources available to *Pseudomonas* spp. has been attributed to its niche where it grows and colonizes plants. Plant

exudates have been found to have sugars, like glucose, but also a wide variety of amino acids (164). Interestingly, *Pseudomonas* spp. has been found to grow well on amino acids as their sole carbon and nitrogen source (144, 164, 171).

Moreover, a study by Wilson *et al.* indicated that *Pseudomonas syringae* when inoculated onto plants with proline was able to grow better than the control (inoculation only) within the field. The addition of ammonia did not improve bacterial growth compared to the control. In the greenhouse, the effect was much pronounced. *Pseudomonas* sp. was able to utilize and grow better when inoculated in the presence of proline or serine than with fructose or glucose. The addition of proline and ammonia caused greater growth than any of the other treatments, whereas ammonia by itself was no different than the control or glucose by itself. These results are supported by a study by Sonawane *et al.* in which all four *Pseudomonas* spp. tested were able to grow well in variety of amino acids as their sole carbon/nitrogen source. Some of the *Pseudomonas* spp. grew better with an amino acid as their sole carbon/nitrogen source, than a combination of amino acid and glucose.

The ability of *Pseudomonas* spp. to utilize amino acids and proteins as a nitrogen/carbon source is important because it is the principle colonizer on the plant phyllosphere. Studies to identify the community on rye has shown that *Pseudomonas* spp. accounts for 20.1% of the resident normal flora, on olive leaves it accounts for 51% (75). Another study on strawberry plants indicated that *Pseudomonas* spp. accounted for 46.2% of the resident microflora (89). The

ability of the resident microflora to utilize the viruses as a source as carbon/nitrogen source could have important implications for the survival of viruses, which are able to use leaves as a vector for human infection. The use of food preservation technologies that either inactivate or decrease microbial activity to prolong produce shelf life may inadvertently increase the likelihood of viral transmission. The extreme conditions (oligotrophic, temperature and humidity fluctuations) have selected for microorganisms that are able to survive by utilizing all available carbon, nitrogen and other micronutrient sources for growth.

Attachment Mechanisms of Enteric Viruses onto Surfaces: Aquifer Material, Membranes and Plant Surfaces

Non-specific interactions are not directly involved in the viral infectious cycle, but are nevertheless important. Cucumber mosaic virus (CMV) is similar to many enteric viruses in terms of shape and size (122). Cucumber mosaic viruses are plant viruses, but use an insect vector to transmit the virus from plant to plant. During feeding of an infected plant, aphids retain viruses on their stylus and when the aphid feeds on another plant the stylus mechanically transmits the virus to a new plant. Unlike other insect vectors, CMVs do not reproduce inside insects nor are they ingested. Cucumber mosaic virus has a negatively charged loop on its surface capsid, which is highly conserved, and uses the negative charge to attach to the stylus of aphids (100). Mutations in the negatively charged loop do not effect the replication of virus, but mutations in this area of

the capsid alter the transmissibility of virus through aphids. Enteric viruses, unlike CMV, do not rely on an insect vector to efficiently carry the virus to another host, though the importance of non-specific binding cannot be underestimated. Because enteric viruses consist of a nucleic acid surrounded by a protein coat, an enteric virus can be thought of as a protein with a defined charge.

The charge of a virus will be dependent on the residues on the surface of the virus. As in CMV, enteric viruses also have capsid properties that allow for non-specific adsorption to a surface. The surface charge of a virus can be characterized as the isoelectric point (pI) or the point in which a virus has a net zero charge at a certain pH. The pH of a solution will determine the surface charge of a virus. A low pH solution, below the virus pI, will result in a positive surface charge, and a high pH solution, above the virus pI, will result in a negative surface charge (61).

Various studies have tried to explain adsorption of viruses to aquifer material. A commonly cited theory of non-specific viral attachment is the DLVO theory. The DLVO theory states that a layer of counter-ions develops around the adsorbent and adsorbate (61). The greater the layer of counter-ions around the adsorbent, the less adsorption occurs. The layer of counter-ions prevents adsorption by preventing the van der Waals forces to come into effect. The momentary dipole created in the van der Waal force can only occur over very short distances, thus a large counter-ion layer will prevent attraction between

the adsorbent and the adsorbate. The DLVO theory excludes all other forces except van der Waals and the double layer forces, as previously described. The DLVO theory explains viral adsorption when both virus and solid are negatively charged and is believed to be the primary forces dictating non-specific interactions (61). The DLVO theory has been used to explain the strong adsorption that clays have with viruses (28).

Multivalent ions have also been proposed to effect adsorption of viruses to solids. The multivalent ions are proposed to act as a bridge connecting two charged surfaces. These types of cations are termed salt bridges. Salt bridges have been identified as important for certain virus-adsorbent interactions (142). Other types of interactions are electrostatic interactions. A predictor of an electrostatic interaction is the pI of a virus. Dowd *et al.* determined that a viral isoelectric point is one of the main factors responsible for viral adsorption within aquifers (39). Dowd *et al.* accomplished this by looking at five different viruses with isoelectric points ranging from 3.9 to 7.3. Dowd *et al.* determined that the viral pI is the overriding factor in controlling viral adsorption in aquifer material. Redman *et al.* also studied the effect of viral pI on viral adsorption, and also concluded that viral adsorption differs due to the electrostatic charge of a virus, hence viruses with different pIs will have different adsorptive behavior (129). Huade *et al.* also determined that virus pI had an effect on virus adsorption, but concluded that the critical pH, ± 0.5 pH units of the highest pI in a system, was the most important range in virus binding to aquifer material (71).

Research involving enteric virus adsorption to membranes has identified other forces other than the pI that is important in viral adsorption. Filters that concentrate viruses from water have provided information on non-specific virus interactions. Farrah *et al.* (1981) investigated the effect of chaotropic (salts which weaken hydrophobic interactions) and antichaotropic salts (salts which strengthen hydrophobic interactions) (49). Using both chaotropic and antichaotropic salts, Farrah *et al.* (1981) determined that poliovirus bound to filters at low pH (4.0) mainly through electrostatic forces, and at high pH (9.5) using hydrophobic forces. Farrah *et al.* (1982) also examined the binding of MS2 phage to membrane filters (48). By using an antichaotropic salt (MgSO_4), Farrah *et al.* determined MS2 phage was able to bind to a nitrocellulose filter at pH 6.0. Furthermore, Tween[®] 80, a detergent, was able to reverse the adsorption of MS2 phage to the filter. The results indicate that MS2 is able to bind to a nitrocellulose filter at pH 6.0 using primarily hydrophobic interactions. Shields *et al.* also examined the effect of poliovirus on membrane binding (139). Poliovirus was able to bind to membrane filters at low pH (4.0), but only a Tween[®] 80 and 1.0 M NaCl solution was able to elute viruses from the membrane. The detergent, Tween[®] 80, or the 1.0 M NaCl solution by itself was not able to elute virus efficiently from the nitrocellulose filter membrane at pH 4.0. At pH 7.0, Tween[®] 80 was able to elute 86% of poliovirus, while 1.0 M NaCl was not able to elute virus efficiently. At pH 9.0, neither Tween[®] 80 nor NaCl eluted virus anymore efficiently than the buffer. In all cases, poliovirus elution

was most efficient with both Tween[®] 80 and NaCl. The results from Shields *et al.* indicate that poliovirus uses both electrostatic and hydrophobic interactions in binding to nitrocellulose filters. Lukasik *et al.* also examined the effect of salts on viral adsorption to filters (105). Unlike other studies, Lukasik *et al.* looked at 3-4 viruses, 4 different filters and 3 different salts including Urea and Tween[®] 80. The results obtained by Lukasik *et al.* were variable. The 1 MDS filter with a slightly positive charge at pH 7.0 bound MS2 and PRD-1 at over 95%. Poliovirus was also adsorbed by 1MDS at a lower efficiency (79%). The lowest binding efficiency was the bacteriophage ϕ X174 at 29%. The addition of 0.1 M NaCl greatly reduced the binding efficiency of all viruses (\approx 13%) at pH 7.0. At pH 3.5 all filters with the exception of a Whatman cellulose filter were able to bind virus. The addition of salt did not reduce the binding efficiency for 2 out of 4 filters, but did reduce it somewhat for the 1MDS filter. The 1MDS filters binding efficiency depended on the type of salt used in the experiment, likewise the filters not affected by the addition of salt, were affected by the addition of Urea or Tween[®] 80. Furthermore, Urea by itself did not decrease the adsorption of virus to the filters at pH 3.5. The results by Lukasik *et al.* and other studies thus indicate that 1) viruses have different binding abilities, 2) pH is important for electrostatic and hydrophobic interactions, 3) salts are able to reverse electrostatic interactions, and 4) if both electrostatic and hydrophobic interactions are present, the disruption of the electrostatic force will strengthen the hydrophobic force and vice versa. Adsorption studies with membrane filters

and aquifer material have elucidated the binding forces that may be important in plant-viral interactions.

Non-specific virus-plant interactions on plant surfaces have not been studied. Even though attachment of plant viruses to their insect vectors has been documented, as previously described, a study identifying the attachment of viruses to the surfaces of plants has not been investigated. The current state of knowledge in plant virology has not identified a virus that is transmitted through the surface of plants without injury to the plant cell. Therefore, there is an impetus to study non-specific viral attachment to plant surfaces.

Decontamination Strategies for Removing Enteric Viruses from Surfaces

The current recommended treatment (50-200 ppm chlorine) of washing fruit and vegetables to decrease microflora are ineffective against viruses (3, 4, 12, 21, 72). A consequence of not having a lipid membrane is the ability to withstand disinfectants commonly used for bacteria. Commonly used disinfectants like bleach, hydrogen peroxide, Tsunami[®] 100, and sodium bicarbonate have not been effective in neutralizing enteric virus infectivity (4). In another study by Malik *et al.*, the effect of sodium bicarbonate was found to significantly reduce viable FCV, a norovirus surrogate, on environmental surfaces (108). Though, the effect on foods and how closely FCV mimics norovirus is currently not known. Furthermore, newer sanitizing methods, like electrochemical inactivation, do not reduce viral numbers in significant amounts (40). Other

methods, like ozone, are effective in decreasing total microbial counts on fresh produce (83). Thurston-Enriquez *et al.* tested the virucidal activity of ozone on FCV and Adenovirus (152). Ozone was found to be effective in reducing FCV and adenovirus in water, though the efficacy of ozone on decreasing viral pathogens on the surface of fruits or vegetables has yet to be shown. Gulati *et al.* tested 9% n-quaternary ammonium compound (QAC), 10% QAC, 5% QAC and 2% sodium bicarbonate, 5.25% sodium hypochlorite, 15% peroxyacetic acid and 11% hydrogen peroxide, 4.75% *o*-benzyl *p*-chlorophenol and 4.75% *o*-phenylphenol, and 5% *o*-benzyl *p*-chlorophenol and 10.5% *o*-phenylphenol for their effectiveness against calicivirus at their recommended concentrations (72). Gulati *et al.* determined that none of the sanitizers were effective against calicivirus. Most sanitizers are developed and tested based of their bactericidal not virucidal activity. Because bacteria have a lipid membrane, an effective bactericidal sanitizer will have limited success against a non-enveloped virus. Enteric viruses are also more difficult to disinfect because they are not actively interacting with their environment. Unlike bacteria and eukaryotes, viruses do not need to maintain a basal metabolic level to survive. Enteric viruses are in stasis until activated by a host cell. As a result, viral survival is solely a factor of structural and genetic stability. An effective method to decontaminate viruses from surfaces is a solution of $\approx 1,000$ ppm of chlorine (25). At a minimum 1,000 ppm is 5X-20X the FDA recommended treatment for fruits and vegetables (21). Therefore, there is no current treatment for fruits and vegetables to rinse salad

crops to deactivate enteric viruses without significantly reducing the quality of salad crops.

CHAPTER III

THE EFFECT OF CRITICAL pH ON THE ADSORPTION OF VIRUS TO BUTTERHEAD LETTUCE*

Overview

Enteric viruses account for the majority of foodborne illness in the United States. The objective was to determine whether the isoelectric point (pI) of viruses such as feline calicivirus (FCV), echovirus 11, and bacteriophages ϕ X174 and MS2 had any effect on their attachment to butterhead lettuce. Viral adsorption to lettuce was found to be variable. Bacteriophage MS2 was the only virus showing attachment at its critical pH and was the only virus fitting the current DLVO model. Echovirus 11 had the highest affinity to lettuce surface. Echovirus 11 exhibited reversible attachment above the virus pI, whereas below the viral pI a strong adsorption was observed. Adsorption of FCV was at its maximum above the viral pI. Bacteriophage ϕ X174 exhibited the most complex adsorption pattern with attachment occurring only at the pH extremes (pH 3.0 and pH 8.0). The results suggest the current model for virus adsorption to sediment does not adequately explain the variability in viral attachment patterns that was observed. Importantly, the results suggest that current sample processing methods can select for only certain viral types.

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¹Texas A&M University, ²Dynamac Corp. ³USDA-ARS

Introduction

The CDC has recognized that among the food-borne disease outbreaks between 1993 and 1997, over half of the cases of “unknown etiology” exhibited characteristics of viral illnesses (120). In 2000, viral agents accounted for 28% of all documented food borne illness cases, whereas bacterial agents accounted for 25% of all food-borne disease outbreaks (22). Mead *et al.* (113) have suggested that enteric viruses may actually account for as much as 67% of all food-borne disease related gastroenteritis in the United States. The large number of enteric virus infections can be attributed in part to their low infectious doses (≤ 100 PFU), their stability in the environment, and our relatively limited knowledge about their presence in foods (4, 8, 148).

Although the incidence of food-borne illnesses associated with fresh produce was believed to be relatively low, the number of outbreaks associated with fresh produce has recently doubled (8). In actuality, salads and fresh produce account for the top two vectors in norovirus foodborne disease transmission (170). Studies have also reported that carrots, cilantro and parsley can harbor fecal indicator viruses (43, 44). Lettuce is of particular concern among salad crops because it is consumed in relatively large quantities with minimal preparation, has a large surface area (hence, greater pathogen attachment sites), and is grown in close proximity to the soil. Lettuce was implicated in a large viral disease outbreak. In 1988, a hepatitis A virus outbreak

involving over 200 people was attributed to lettuce being contaminated via irrigation water (132).

The initial attachment of enteric viruses to salad crops and herbs is a key step in the contamination chain of events. Understanding the factors (physical, chemical and biological) that control the attachment process can provide insight into appropriate intervention methods that can be used to either prevent attachment or remove the attached viral pathogens. There is a significant amount of information related to factors that control the attachment of enteric viruses to soil and aquifer sediments. Factors such as virus type, pH, ionic concentration, presence of multivalent cations, and organic matter, are thought to be involved (31, 39, 67, 129, 136, 150). The factors controlling enteric virus attachment to salad crops have, however, not been adequately studied. The attachment of enteric virus particles to lettuce leaf surfaces can be envisioned as involving both kinetic adsorption and equilibrium attachment processes (67). Since the isoelectric point of a virus can influence the net surface charge of a virus at a particular pH, we hypothesized that the isoelectric point of the virus is a controlling factor dictating the attachment of enteric virus particles to butterhead lettuce surfaces. Because viruses consist of nucleic acid surrounded by a protein coat, a virus particle can be thought of as a protein with a defined surface charge. The ultimate charge of a virus would, therefore depend on the amino acid residues on the virus surface and the pH of the surrounding medium (39, 71, 129, 150).

In order to delineate the factors controlling the non-specific attachment of enteric viruses to produce surfaces, the attachment of viruses to lettuce surfaces was studied using batch experiments. The primary objective of this study was to determine if the isoelectric point of viruses controlled their attachment to the lettuce surface. The viruses that were studied included model viruses (bacteriophages and FCV) as well as a known enteric virus (echovirus 11). Only if the factors controlling virus attachment to lettuce are identified, can effective and scientifically-based washing or virus recovery protocols be developed.

TABLE 3.1. Physicochemical Properties of Viruses Used in the Study. The table lists the physicochemical attributes important when selecting the viruses used for the experiments.

Virus	pI	Structure	Shape	Envelope	Transmission route	Host
MS2	3.9 ^a	T=3	Icosahedral	No	Fecal-oral	<i>E. coli</i>
FCV	4.9 ^b	T=3	Icosahedral	No	Respiratory	Feline
Echovirus 11	5.9 ^b	Pseudo T=3	Icosahedral	No	Fecal-oral	Human
φX174	6.6 ^a	T=1	Icosahedral	No	Fecal-oral	<i>E. coli</i>

^a The pI of viruses MS2 and φX174 was obtained from the manuscript by Dowd *et al* (39).

^b The pI of echovirus 11 and FCV was obtained by inserting the amino acid sequence of the capsid into the space provided at the website <http://www.embl-heidelberg.de/cgi/pi-wrapper.pl>.

Materials and Methods

Viruses and Cells. In order to study the influence of the isoelectric point on virus attachment patterns we chose viruses of varying isoelectric points that included bacteriophages and known enteric and mammalian viruses (Table 3.1). Bacteriophage MS2 (ATCC # 15597-B1) was propagated in *E. coli* HS(pFamp)R (ATCC # 700891). Bacteriophage ϕ X174 (ATCC # 13706- B1) was grown in *E. coli* host CN13 (ATCC # 700609). The MS2 and ϕ X174 phages were enumerated using the double agar layer (DAL) method using their respective hosts (160). Echovirus 11 (ATCC # VR-1052) was propagated in buffalo green monkey cells (BGMK). Feline calicivirus strain F9 (FCV) was grown in Crandell-Reese feline kidney cells (CRFK). (The virus and host cells were a generous gift from Dr. Sagar Goyal at the University of Minnesota). The mammalian viruses were enumerated using a soft agar overlay method (156). Propagation and maintenance media was identical to Gulati et al., with the exception of lactalbumin hydrolysate (72). Lactalbumin hydrolysate was not used for CRFK cells. Buffalo green monkey cells were grown and maintained in media similar to CRFK cells with the exception of 25 mM of HEPES and 10% fetal bovine serum.

Attachment Studies. Butterhead lettuce was purchased locally from a farmers' market that sold fresh farm products. The lettuce leaves were severed from the base and cut into 25 cm² pieces using appropriate sterile techniques. The pieces were placed in a 20 oz Whirl-Pak bag[®] (Fort Atkinson, WI). Twenty

milliliters of citric phosphate buffer (0.1M) (pH range 3-5) were added to appropriately labeled bags. Twenty milliliters of sodium phosphate buffer (0.1M) (pH range 6-8) were similarly added to labeled bags. The buffers were titrated to their respective pH using HCl or NaOH, as necessary with an acceptable pH buffer variation of ± 0.01 pH units. (The pH meter was calibrated with pH 4, 7, and 10 calibration buffers prior to each use). The pH range was specifically chosen so as to include the pI of the viruses being studied. A virus suspension (0.1 mL of a 10^5 PFU per ml) was added to each bag to achieve a total of 10^4 PFU per bag. The control treatments consisted of the bag containing 20 mL of buffer inoculated with 0.1 ml of a 10^5 PFU/ml lysate diluted in 0.1% peptone but without the piece of lettuce. The virus suspensions and the buffers used in the experimental and control treatments were prepared from the same stock. After the virus was inoculated into the control and experimental units, virus adsorption was allowed to proceed for 30 minutes at room temperature on a rocking platform.

The experimental and control units were sampled in a staggered fashion. After 30 minutes, the control and experimental units were cut open using flame-disinfected scissors and 0.1 ml of buffer was aspirated from the bags. The bacteriophage assays were carried out immediately after each bag was cut open since their assay was rather straightforward. The 0.1 ml aliquots were assayed for MS2 and ϕ X174 bacteriophages without any further dilution. The aliquots for echovirus and FCV analysis were first diluted into Eagles minimal essential

media (cat. # M0643) (Sigma-Aldrich, St. Louis, MO). From the diluted buffer, 0.2 ml was inoculated into each well for the plaque assay. The animal virus samples were collected and then assayed all together once the adsorption phase of the experiment was complete. To avoid experimental variability, buffer volumes, dimensions of the lettuce squares, sequence of addition of viruses, lettuce squares and viral stocks were all standardized to the maximum extent possible.

Data Analysis. Multiple, independent experimental trials, with each trial consisting of three experimental replicates and three control replicates were performed. The samples from the control and experimental treatments were assayed at the same time. The “percent attachment” for each virus was calculated as follows: $[(\text{Mean Control} - \text{Mean Experimental}) / \text{Mean Control}] \times 100$. We measured attachment as a function of a “difference” because relying on virus titers based directly on extraction or removal from the leaf surface was prone to significant errors since it was impossible to choose a buffer that guaranteed high recovery efficiency for the different viruses. Potential virus aggregation/de-aggregation and attachment to the bag was controlled by analyzing the data in terms of the experimental bag (with lettuce) to the control bag (without the lettuce). The data is presented as “percent attachment” with the trend line (based on the median value) along with the sample interquartile range (25th and 75th percentiles) (131). Significant differences ($p \leq 0.05$), if any,

in the percent attachment across the pH range for a given virus was calculated based on the Wilcoxon Sign Rank Test using SPSS 11.0.1 (Chicago, IL).

Results

The MS2 phage exhibited the most dramatic change in adsorption as a function of pH compared to the other three viruses (Fig. 3.1).

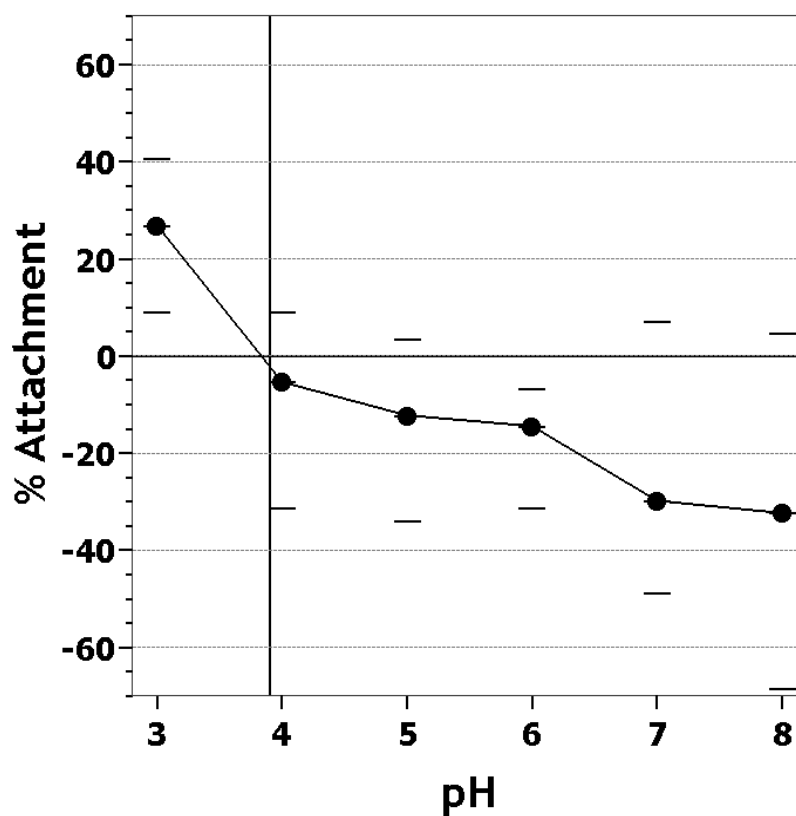


FIG. 3.1. MS2 Adsorption to Lettuce. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 6 independent replicates with each experiment consisting of 3 replicates.

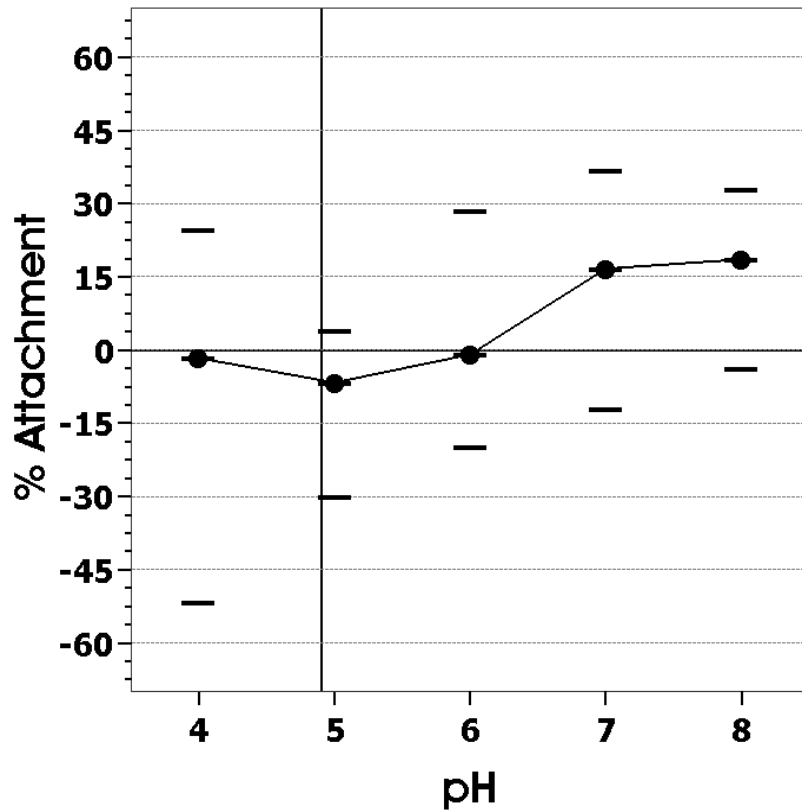


FIG. 3.2. Feline Calicivirus Adsorption to Lettuce. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 10 independent replicates for pH 4, 6 and 8, and 9 independent replicates at pH 5 and 7 with each experiment consisting of 3 replicates.

At pH 3.0, MS2 phage showed the maximum adsorption (28%) which corresponded to 2,690 PFU/25cm²). At pH 8.0, the majority of the viruses were unattached (-32%).

Feline calicivirus showed maximal attachment at pH 8.0 (19% corresponding to 1860 PFU/25 cm²) and minimal attachment at pH 5.0 (Fig. 3.2). The FCV were rapidly inactivated at pH 3.0 and hence only data from pH 4.0 and above are presented. Though the FCV results indicate that there was significant adsorption and desorption of viruses at all of the pH ranges that were tested, it is apparent from the trend line that as the pH increased from pH 5.0 to 8.0, the % of viruses that were attached to the lettuce surface increased significantly (Table 3.2). An equal proportion of viruses appeared to be attached and unattached at pH 6.0 (Fig. 3.2). It must be noted that the difference in attachment of FCV at pH 5.0 as compared to pH 4.0 was only 3.4% (which is equivalent to 340 PFU/25 cm²).

TABLE 3.2. Obtained *P*-values Between Virus Attachment at Each pH. *P*-values obtained when comparing percent attachment of four different viruses in lettuce to five different pH buffers.

pH	Virus	Values at pH ^a :				
		4.0	5.0	6.0	7.0	8.0
3.0 ^b	MS2	0.03*	0.03*	0.03*	0.03*	0.12
	φX174	0.04*	0.08	0.08	0.25	0.75
	Echovirus 11	0.25	0.75	0.04*	0.05*	0.04*
4.0	MS2		0.75	0.60	0.46	0.46
	φX174		0.60	0.75	0.75	0.17
	FCV		0.68	0.24	0.01*	0.01*
	Echovirus 11		0.75	0.04	0.12	0.08
5.0	MS2			0.92	0.75	0.46
	φX174			0.35	0.92	0.17
	FCV			0.01*	0.01	0.01*
	Echovirus 11			0.08	0.12	0.04*
6.0	MS2				0.46	0.60
	φX174				0.60	0.05*
	FCV				0.03*	0.01*
	Echovirus 11				0.50	0.08
7.0	MS2					0.75
	φX174					0.08
	FCV					0.95
	Echovirus 11					0.22

^a Numbers with asterisks represent significant differences based on the Wilcoxon sign rank test ($P < 0.05$)

^bThe FCV was nonviable at pH 3.0

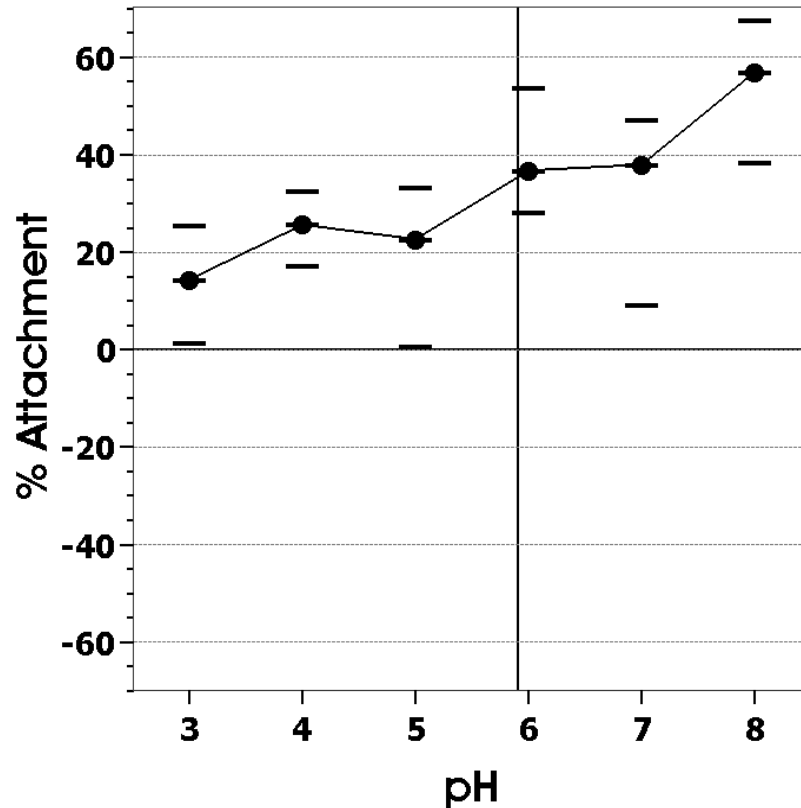


FIG. 3.3. Echovirus 11 Adsorption to Lettuce. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 6 independent replicates for pH 3 thru 5, 5 independent replicates for pH 6 and 8 and 7 independent replicates for pH 7 with each experiment consisting of 3 replicates.

Echovirus 11 exhibited the greatest attachment to butterhead lettuce among the four different viruses that were employed in this study (Fig. 3.3). The virus appeared to be strongly bound to the lettuce surface at pH 3.0 (14.4% corresponding to 1440 PFU/25 cm²) and tended to increase with increasing pH. Maximal attachment was observed at pH 8.0 57% or 5700 PFU/cm²).

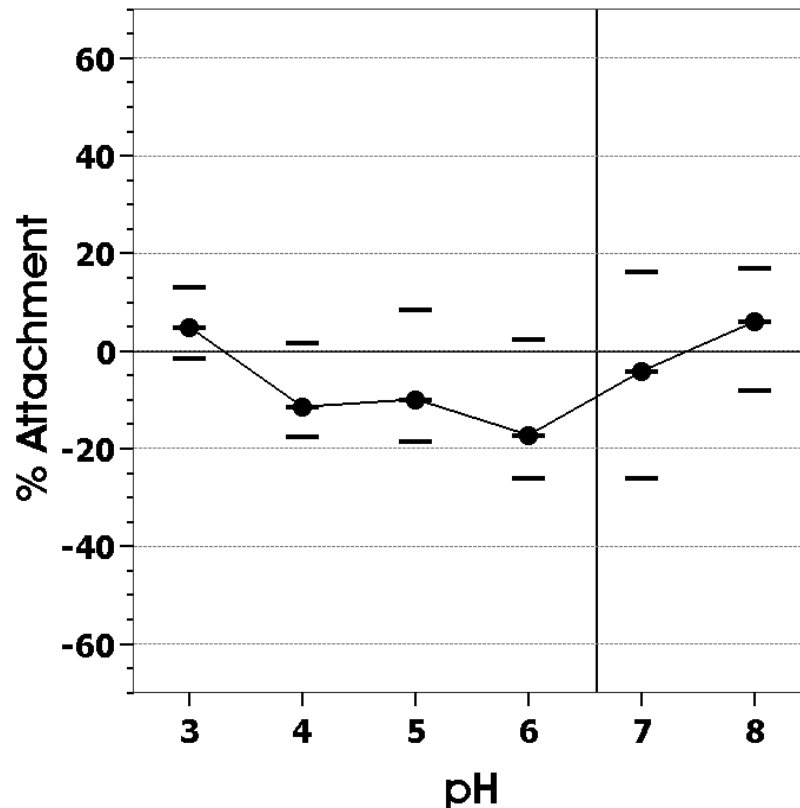


FIG. 3.4. ϕ X174 Adsorption to Lettuce. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 6 independent replicates for pH 3-8 with each experiment consisting of 3 replicates.

The phage ϕ X174 exhibited the most complex attachment and detachment pattern of the four viruses that were studied (Fig. 3.4). At the extreme ends of the pH range tested, (pH 3.0 and pH 8.0), ϕ X174 phage exhibited only 4.9% and 6% (490 and 600 PFU/25 cm² respectively) attachment, respectively. There appeared to be no attachment of virus particles to the leaf surfaces between these two pH values.

Discussion

The physicochemical forces that control the interaction between enteric viruses and plant surfaces have not been adequately studied in the past. A better understanding of the controlling factors can lead to improved washing or other intervention strategies. The use of positively charged filters is a common strategy used to concentrate enteric viruses from large volumes of water and sharp drops in pH are exploited as a strategy to concentrate viruses in suspension. The apparent reversible and irreversible attachment that was observed in these studies (based on the data spread) was expected because these viruses do not employ specific cell-surface receptors to attach to the lettuce surface.

In batch experiments using sediment material, virus concentrations (in the buffer) normally decline in time (due to adsorption onto the solid surface) after which they remain constant. When the numbers remain constant, the viruses are said to be at equilibrium adsorption which is achieved due to reversible adsorption (8, 131). It has been postulated that two processes are involved in the formation of the adsorption equilibrium with a solid surface namely, mass transport of viruses close to the surface and secondly the immobilization of the viruses to the surfaces by physical and chemical interactions. Electrostatic interactions, van der Waals forces, and hydrophobic effects are three major forces that are thought to be responsible for the interactions between virus particles and solid substrates (136). The Derjaguin-Landau-Verwey-Overbeek

(DLVO) theory serves as a conceptual framework to understand non-specific interaction of virus particles to solid surfaces under different conditions such as pH, and ionic strength. The DLVO theory states that only the van der Waals and electrostatic forces are of any consequence in colloidal particle adhesion to surfaces. The van der Waals force is always attractive and the electrostatic force is always repulsive. Factors that decrease or increase the electrostatic component will invariably reduce or enhance the influence of van der Waals force, thereby directly impacting adsorption. Even though hydrophobic interactions are thought to be involved in virus adsorption to solid surfaces, hydrophobic interactions are not considered in the DLVO theory. Previous studies have shown that virus attachment to solid surfaces decreases with increasing pH (67, 136). The DLVO theory attempts to explain this phenomenon by suggesting increased repulsion by negatively charged virus particles and solid surfaces. The overall negative charge on the virus surfaces increases above the pI of the virus particle, whereas below the viral pI the overall net charge becomes increasingly positive. Even though the surface charge of the lettuce is relatively unknown (to the best of our knowledge), the experimental design normalized the impact of the lettuce charges across all experiments. Among the four viruses that were studied, only the MS2 phage behaved per the DLVO theory (Fig. 3.1). As the pH increased from pH 3.0 to pH 8.0, the percentage of virus attachment decreased. The enteric virus, echovirus 11, exhibited a pattern of increasing attachment (Fig. 3.3). Below the pI of the virus (i.e. 5.9), the viruses were

“tightly” bound to the leaf as compared to “reversible” binding above the pI. (The reversible binding is deduced by the spread of the data points at pH levels greater than the pI). Guan et al. have recently reported the existence of a “critical pH” range at which the virus behavior changes abruptly (71). The critical pH was reported to be 0.5 pH units below the highest isoelectric point of the virus and the solid substrate. If the pH of the suspending medium is below the critical pH, they suggest that the virus has an opposite charge to at least one component of the solid substrate and thus becomes irreversibly adsorbed to the substrate. The critical pH concept, however, based on this study, does not appear to be valid for any virus other than MS2 phage. The phage ϕ X174 exhibited a completely different adsorption pattern compared to the other 3 viruses with attachment occurring only at the extreme pH levels

Previous studies involving hepatitis A virus (HAV) and poliovirus recovery from fruits and vegetables showed variable efficiencies, suggesting that the detachment process is a net result of complex virus particle-surface interactions (94, 165). Studies by Legitt et al. and Ward et al. employed a high pH (9.0) buffer wash to recover viruses from lettuce (94, 165). Legitt et al. recovered only 16% of inoculated poliovirus and HAV from lettuce. Ward et al. recovered 58% and 49% of poliovirus from 2.9 kg and 3.3 kg of lettuce, respectively, suggesting poliovirus adsorption to lettuce. The majority of the virus particles were, therefore, being “lost” after the first pH 9.0 wash. Furthermore, the results seem to suggest that the greater the amount of lettuce used, the greater was the “loss”

of virus particles. Though our study did not employ poliovirus or HAV, it employed echovirus 11, a member of the picornavirus family (similar to poliovirus and HAV). We hypothesize that many enteric picornaviruses share similar adsorption potential to fruits and vegetables. The low recoveries of echovirus in the present study similar to the low recoveries observed by Legitt et al. support this hypothesis. The increased attachments of echovirus 11 to lettuce as a function of increasing pH suggest the involvement of electrostatic forces. However, other investigators have suggested that hydrophobic interactions may also be playing a key role in virus attachment (106, 139).

The FCV is routinely used as a surrogate for norovirus since they are genetically and structurally related (3, 72, 148). The FCV infect the respiratory tract and, therefore, there is little evolutionary pressure to maintain capsid integrity at low pH. The virus is sensitive to pH 3.0 as observed in this study. Duizer et al. also reported that <0.005% of FCV-F9 virus survived at pH 3.0 after 30 minutes (42). The results from this present study suggest that FCV is not a suitable surrogate for low pH studies. Nevertheless, in this study FCV was able to bind to lettuce at pH 7.0 and 8.0. The FCV was able to bind to butterhead lettuce at around 18.6% or 1860 PFU/25 cm² at neutral to basic pH. The ramifications of these results, however, have to be considered carefully since the transmission route of FCV is different from that of the typical enteric norovirus. It is plausible that the attachment patterns of norovirus may be actually closer to that of echovirus 11 rather than FCV, since noroviruses and echovirus 11 are

enteric viruses.

Overall, these results suggest that different viruses can exhibit differing attachment patterns to lettuce. Importantly, unlike the situation with aquifer sediments, the isoelectric point of the virus is not the controlling factor governing their attachment to lettuce. This study is significant in that it demonstrates that the attachment of enteric virus particles to lettuce cannot be generalized from the pattern of a single commonly used enteric virus surrogate such as the MS2 or ϕ X174. These results suggest that current fruit and vegetable washing/rinsing protocols to rinse or recover viruses need be re-examined because pH interactions between the virus particle, the surface, and the buffer can alter the attachment or detachment of the viruses to the surfaces. The study also suggests that using a particular pH buffer to recover virus particles could artificially select for the recovery of a particular type or group of viruses. This may be the reason why in an earlier study, we recovered only DNA-containing phages from cilantro and parsley samples (43).

CHAPTER IV
IDENTIFICATION OF FORCES CRITICAL FOR VIRUS
ADSORPTION TO LETTUCE

Overview

Enteric viruses are estimated to be responsible for the majority of foodborne gastroenteritis in the United States. Though, recent years have seen an increase in viral foodborne disease caused by viruses, there is a distinct lack of data involving mechanisms of viral attachment to the surfaces of fruits and vegetables. The objective of this study was to determine the forces responsible for non-specific virus attachment on the surface of butterhead lettuce. We have previously determined that viruses attach to the surface of butterhead lettuce at varying amounts. As a follow up to our previous work, echovirus 11, FCV, MS2 bacteriophage and ϕ X174 bacteriophage were independently assayed to determine the forces affecting viral attachment to butterhead lettuce. Three different conditions 1) 1% Tween 80, 2) 1 M NaCl and 3) 1% Tween 80 with 1 M NaCl were compared to determine attachment forces responsible for non-specific viruses attachment on the surface of lettuce. Only 1 M NaCl exhibited a consistent significant decrease in viral attachment at pH 7 and 8. These results indicate that viruses are able to attach to lettuce using electrostatic forces and that a phosphate buffer with 1M NaCl is effective in removing virus attachment to butterhead lettuce. These results will provide an effective method to wash lettuce to remove enteric viruses from the surface.

Introduction

Recent years have seen a dramatic increase in food borne disease cases caused by enteric viruses. Between the years 1993-2004, the number of cases where enteric viruses have been implicated in U.S. food borne diseases have increased from 4.7% in 1997 to as much as 52% in 2004 (26, 120). These numbers are probably higher in developing or under-developed nations of the world. Foods especially fresh fruits and vegetables can get contaminated with viral pathogens either at pre-harvest or during post-harvest processing. Though many viral food-borne disease cases are associated with food handler contamination at food service establishments, there have been many instances where foods, most notably fresh or frozen produce, have been contaminated on the farm (57, 70, 77, 132). A large outbreak in Pennsylvania associated with HAV contamination of green onions was traced back to contamination at the field in Mexico (169). Viral properties such as low infectious dose, resistance to chlorination, and resistance to commonly used produce-washing regimes have made enteric viruses on fresh produce rather difficult to control (4, 87).

The initial attachment of enteric viruses to salad crops and herbs is a key step in the chain of contamination events. Understanding the factors (physical, chemical and biological) that control the attachment process can provide insight into appropriate intervention methods that can be used to either prevent attachment, or remove the attached viral pathogens. We have previously shown that viruses exhibit differing levels of attachment to lettuce surfaces, and that

electrostatic forces were involved in the attachment of four different viruses (ϕ X174, MS2, FCV and echovirus 11) to butterhead lettuce (161). However, given the variability in the attachment patterns that could be explained solely by electrostatic interactions, we were interested in identifying the role that hydrophobic interactions have in the attachment of enteric viruses to lettuce. Compared to the amount of information that is available related to the attachment of viruses to aquifer sediments, information explaining the non-specific attachment of enteric viruses to fresh produce, such as lettuce, is relatively scant. Developing a fundamental understanding of the forces responsible for virus adsorption or attachment of lettuce can lead to the formulation of appropriate rinse solutions that can be used during post-harvest processing, in food service establishments or even in homes to remove attached virus particles. Experimentally, an appropriate lettuce wash solution can also help in eluting viruses off lettuce during sample processing to obtain accurate estimations of the viral loads on produce. The primary objective of this study was to compare the relative contribution of electrostatic and hydrophobic forces controlling virus attachment to butter-head lettuce. Four different viruses (MS2, ϕ X174, feline calicivirus and echovirus 11) were employed in these studies.

Materials and Methods

Cells and Viruses. The four viruses used in this study included ϕ X174, Ms2, feline calicivirus and echovirus 11 (4, 37). These four viruses were chosen because they have been used in the past as indicators of fecal viruses, as well as pathogenic viruses (57, 61, 90). We had previously shown that electrostatic forces appeared to be involved in their attachment to butterhead lettuce (Table 4.1). The feline calicivirus (FCV) was chosen because it is used as a norovirus surrogate. The echovirus 11 is a human pathogen belonging to the enterovirus group. The echovirus 11 has been implicated as the principle cause of non-bacterial meningitis and a variety of other human diseases (104). More importantly, these four viruses have similar physicochemical attributes, like structure, size, and varying isoelectric points (161).

TABLE 4.1. Medians of Control Virus Adsorption to Lettuce. The table lists the median % attachment of selected viruses in 0.1 M NaPO₄ buffer.

	Medians (% Attachment at pH)^a					
	3	4	5	6	7	8
Echovirus 11	25.4	25.7	22.7	36.8	38.0	57.0
FCV	-	-1.7	-6.7	-0.9	16.7	18.6
MS2	26.9	-5.4	-12.2	-14.4	-29.8	-32.3
ϕX174	4.9	-11.4	-9.9	-17.2	-5.0	3.0

^aData is summarized from Vega *et al.* (161).

Echovirus 11 (ATCC VR-1052) was grown with buffalo green monkey cells (BGMK), FCV (F9) was grown in Crandell Reese feline kidney cells (CRFK), bacteriophage MS2 (ATCC 15597-B1) was propagated with *Escherichia coli* HS(pFamp)R (ATCC 700891) and bacteriophage ϕ X174 (ATCC 13706-B1) was propagated in *E. coli* host CN13 (ATCC 700609). Feline calicivirus and CRFK cells were a generous gift from Dr. Sagar Goyal at the University of Minnesota. The bacteriophages were assayed using the double agar layer method and the animal viruses using the agar overlay method, respectively (156, 160). The growth and maintenance media were identical to previously described protocols (161).

Attachment Studies. Butterhead lettuce was purchased locally from a farmers' market that sold fresh farm products. The lettuce leaves were severed from the base and cut into 25 cm² pieces using appropriate sterile techniques. The pieces were placed in a 20 oz Whirl-Pak bag[®] (Fort Atkinson, WI). Twenty milliliters of citric phosphate buffer (0.1M) (pH range 3-5) was added to appropriately labeled bags. Twenty milliliters of sodium phosphate buffer (0.1M) (pH range 6-8) was similarly added to labeled bags. The buffers were titrated to their respective pH using HCl or NaOH, as necessary with an acceptable pH buffer variation of $\pm .01$ pH units. (The pH meter was calibrated with pH 4, 7, and 10 calibration buffers prior to each use). In order to determine whether electrostatic or hydrophobic forces were involved in the attachment, it was necessary to sequentially remove these forces and observe the viral

attachment patterns. Three different treatments (namely removal of hydrophobic interactions, removal of electrostatic interactions and removal of hydrophobic and electrostatic interactions) were evaluated in this attachment study. The detergent Tween[®] 80 (Sigma-Aldrich, St. Louis, MO) (catalog no. P1754) (1% v/v) was added to the buffers (pH 3-8) to remove hydrophobic interactions. A high concentration of salt, 1M NaCl (Sigma-Aldrich) (catalog no. S7653), was added to the buffers (pH 3-8) to remove electrostatic interactions. Both Tween[®] 80 (1% v/v) and NaCl (1 M) were added to the buffers (pH 3-8) to remove both electrostatic and hydrophobic interactions. The experimental design for determining attachment was identical to Vega *et al.* with the exception of the suspending medium (161). Briefly, a 25 cm² piece of lettuce was placed inside a 20 oz Whirl-Pak bag (Fort Atkinson, WI). Twenty milliliters of buffer followed by virus (total concentration of 10⁴ pfu/20 ml) was added, a total of three replicates per experiment were carried out. The lettuce in the virus and the buffer solution was the experimental set. The control set consisted of the 20 oz Whirl-Pak bag with the buffer and virus solution at identical concentrations of the experimental but without the lettuce piece. The control set had three replicates.

The virus suspensions and the buffers used in the experimental and control treatments were prepared from the same stock. Both the control and experimental sets were prepared and assayed concurrently.

Bacteriophages were assayed without dilution, whereas the animal viruses were diluted in Eagle's minimal essential media (catalog no. MO643) (Sigma-Aldrich, St. Louis, MO.). For the NaCl experiments, samples were diluted to at least 1 to 3 to avoid cell culture toxicity. For experiments with both Tween[®] 80 and NaCl, the samples were diluted to at least 1 to 8 to avoid cell culture toxicity. Aliquots (0.2 ml) were removed and were inoculated onto the cell monolayer for the agar overlay assays. All experiments were repeated 5 times.

Data Analysis. Multiple, independent experimental trials with each trial consisting of 3 independent replicates and 3 control replicates were performed. Each experiment was repeated five times. Attachment was determined by the equation: $[(\text{mean control}) - (\text{mean experimental})]/(\text{mean control}) = \%$ attachment, where "mean experimental" is the lettuce submerged in the virus and buffer solution and the "mean control" is virus and solution without the presence of lettuce.

We measured attachment as a function of a “difference” (between the control and the experiment) because relying on virus titers based directly on extraction or removal from the leaf surface was prone to significant errors since it was impossible to choose a buffer that guaranteed high recovery efficiency for the different viruses. Potential virus aggregation/de-aggregation and attachment to the bag was controlled by analyzing the data in terms of the experimental bag (with lettuce) to the control bag (without the lettuce). The data is presented as “percent attachment” with the trend line (based on the median value) along with the sample interquartile range (25th and 75th percentiles) and connecting the median of the five experimental replicates at a specific pH (131). The horizontal lines above and below the median represent the 25th and 75th percentiles, respectively. The Wilcoxon sign rank test was used calculate significant differences (P less than or equal to 0.05) between treatments for a given pH. The statistical software SPSS version 11.0.1 (SPSS Inc., Chicago, Ill.) was used for statistical analysis. The “no-treatment” data employed for identifying the significant differences is from our recently published work (161).

TABLE 4.2. Obtained *P*-values Comparing Treatments. The table lists the *P*-values comparing % attachment of the viruses onto lettuce at each pH for each treatment. *P*-values represent positive or negative differences.

Virus	Treatment	pH (P-values)					
		3	4	5	6	7	8
Echovirus 11	No Treatment vs 1M NaCl	0.04	0.08	0.50	0.04	0.04	0.04
	No Treatment vs 1% Tween 80	0.35	0.75	0.89	0.69	0.23	0.08
	No Treatment vs 1% Tween 80 and 1M NaCl	0.14	0.04	0.04	0.04	0.04	0.04
	1M NaCl vs 1% Tween 80	0.04	0.23	0.89	0.04	0.04	0.04
	1M NaCl vs 1% Tween 80 and 1M NaCl	0.23	0.50	0.35	0.04	0.50	0.89
	1M NaCl and 1% Tween 80 vs 1% Tween 80	0.04	0.08	0.23	0.14	0.04	0.04
Feline Calicivirus	No Treatment vs 1M NaCl	-	0.35	0.23	0.50	0.08	0.08
	No Treatment vs 1% Tween 80	-	0.89	0.04	0.08	0.50	0.50
	No Treatment vs 1% Tween 80 and 1M NaCl	-	0.50	0.50	0.89	0.50	0.04
	1M NaCl vs 1% Tween 80	-	0.04	0.08	0.04	0.23	0.50
	1M NaCl vs 1% Tween 80 and 1M NaCl	-	0.89	0.23	0.50	0.14	0.50
	1M NaCl and 1% Tween 80 vs 1% Tween 80	-	0.04	0.14	0.04	0.50	0.89
φX174	No Treatment vs 1M NaCl	0.23	0.14	0.04	0.89	0.89	0.89
	No Treatment vs 1% Tween 80	0.23	0.08	0.14	0.89	0.89	0.89
	No Treatment vs 1% Tween 80 and 1M NaCl	0.35	0.04	0.04	0.35	0.89	0.89
	1M NaCl vs 1% Tween 80	0.69	0.35	0.89	0.35	0.69	0.89
	1M NaCl vs 1% Tween 80 and 1M NaCl	0.08	0.50	0.08	0.23	0.50	0.50
	1M NaCl and 1% Tween 80 vs 1% Tween 80	0.23	0.35	0.04	0.35	0.35	0.89
MS2	No Treatment vs 1M NaCl	0.04	0.50	0.23	0.23	0.69	0.50
	No Treatment vs 1% Tween 80	0.04	0.14	0.04	0.14	0.23	0.50
	No Treatment vs 1% Tween 80 and 1M NaCl	0.08	0.35	0.23	0.04	0.14	0.35
	1M NaCl vs 1% Tween 80	0.35	0.23	0.23	0.89	0.69	0.04
	1M NaCl vs 1% Tween 80 and 1M NaCl	0.23	0.69	0.35	0.35	0.04	0.14
	1M NaCl and 1% Tween 80 vs 1% Tween 80	0.69	0.23	0.89	0.08	0.08	0.35

The *P*-values were calculated using the Wilcoxon-rank sum test.

RESULTS

The figures represent the attachment of the 4 different virus particles to lettuce under the different experimental conditions while Table 4.2 summarizes the statistical analysis of the results.

Echovirus 11. In the presence of 1 M NaCl, echovirus 11 showed increasing attachment (from -25% to + 17%) with increasing pH up to pH 5.0 (Fig 4.1). Beyond pH 5, adsorption decreased and remained below 0% (indicating zero adsorption). The addition of 1 M NaCl was effective in removing virus adsorption to lettuce at all pH levels except pH 5.0, though at pH 4.0 the reduction was not significant ($P = 0.08$) (Table 4.2). The decrease in attachment was significantly different ($P < 0.05$) compared to the control (Table 4.1). There was no significant difference in the adsorption at pH 4.0 and 5.0 between the treatment and the control samples (Table 4.2).

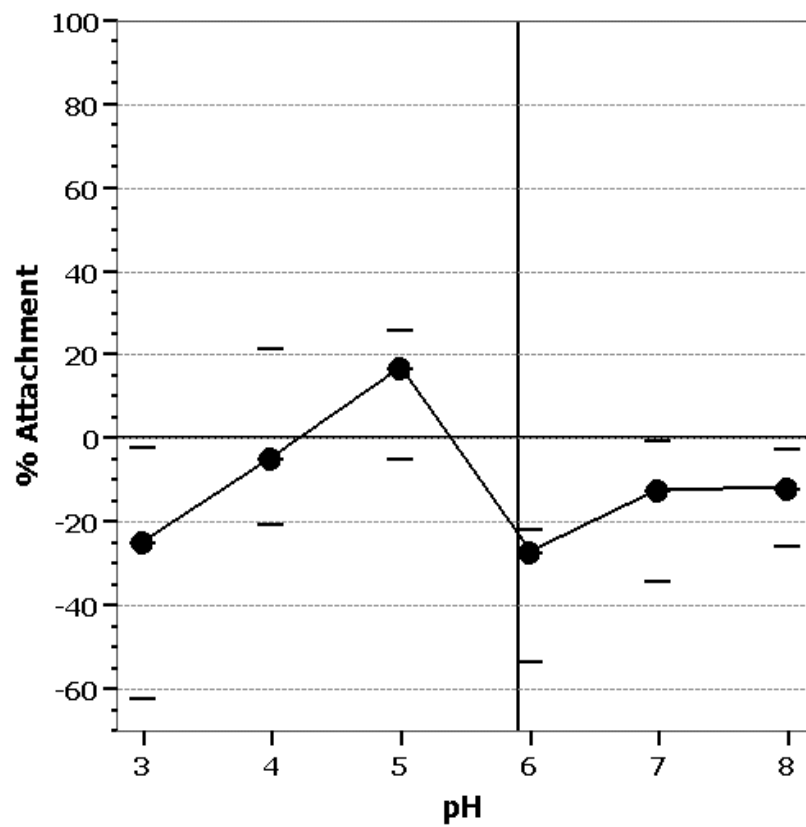


FIG. 4.1. Echovirus 11 Adsorption to Lettuce with 1M NaCl. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 5 independent experiments.

The addition of 1% Tween[®] 80 (which was used to remove hydrophobic interactions) indicated a demonstrable virus attachment at all pH levels. Attachment increased from 27% at pH 3.0 up to 84% at pH 8.0 (Fig 4.2). The increased attachment was not sufficiently greater than the control to obtain a significant difference.

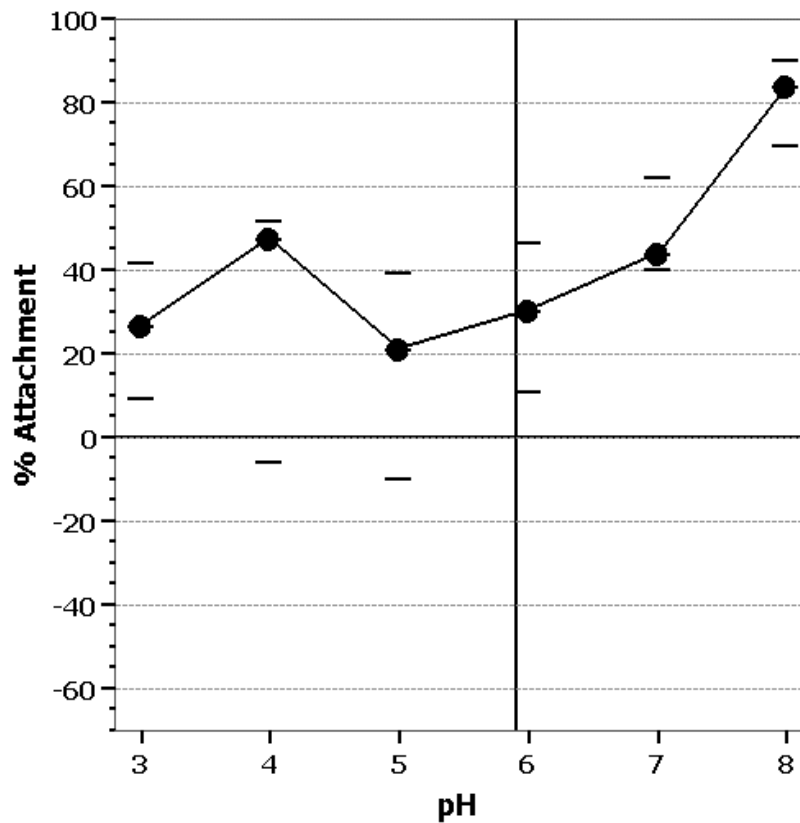


FIG. 4.2. Echovirus 11 Adsorption to Lettuce with 1% Tween[®] 80. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 5 independent experiments.

In the presence of both 1M NaCl and 1% Tween[®] 80, echovirus 11 adsorbed to the lettuce only at pH 5 and 6 albeit at low levels of 11% and 12% respectively (Fig 4.3), nevertheless attachment was statistically different ($P < 0.05$) at all ranges but pH 3.0 ($P = 0.14$).

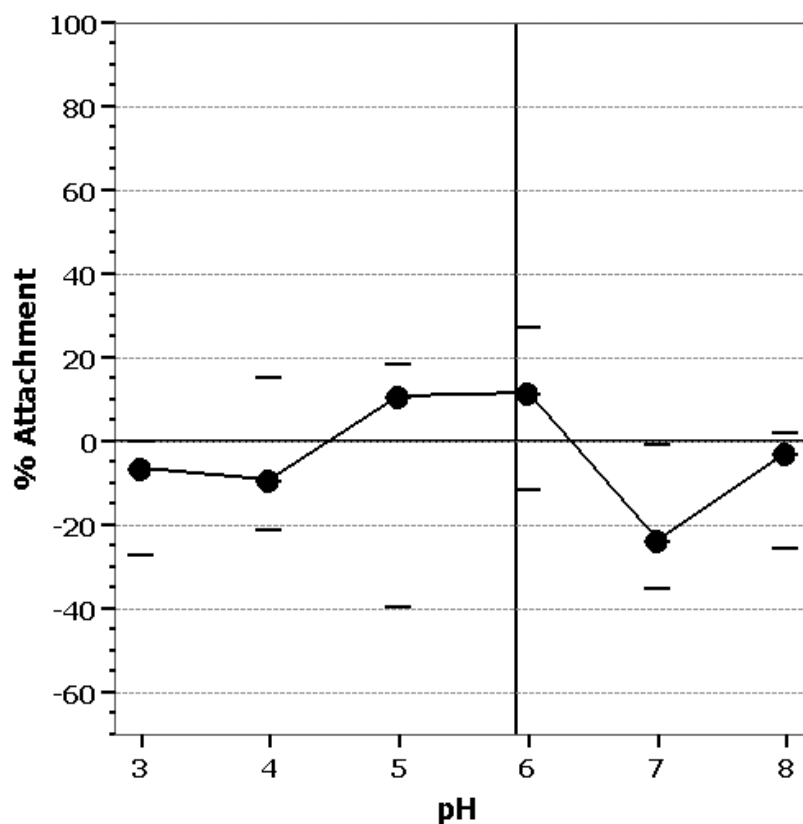


FIG. 4.3. Echovirus 11 Adsorption to lettuce with 1M NaCl and 1% Tween[®] 80. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 5 independent experiments.

Feline Calicivirus. In the presence of 1M NaCl, FCV adsorption was less than 0% at all pH levels (Fig 4.4).

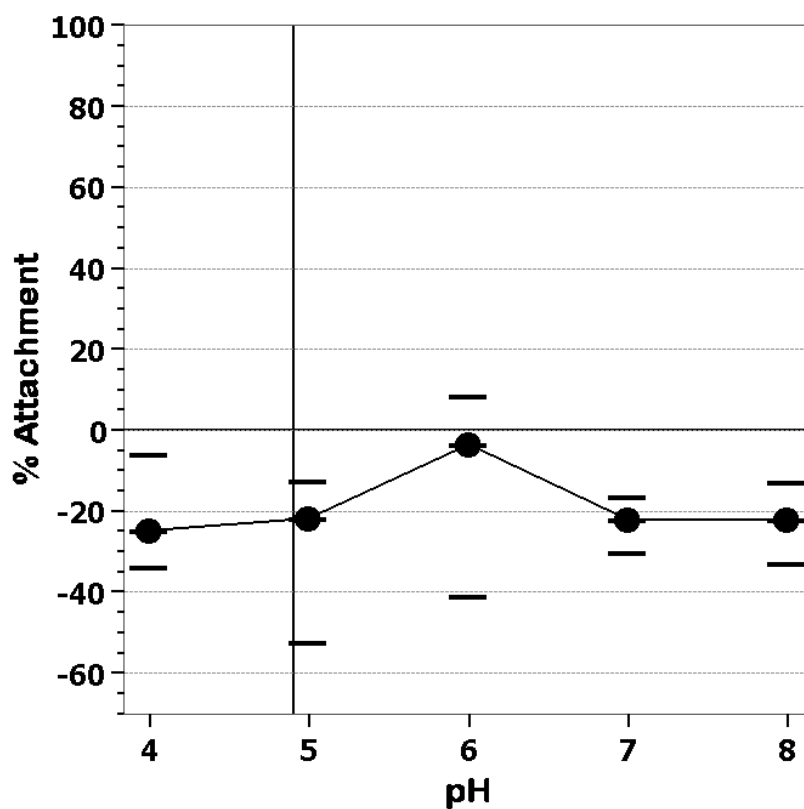


FIG. 4.4. Feline Calicivirus Adsorption to Lettuce with 1M NaCl. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 5 independent experiments.

There was no adsorption at any of the other pH levels beyond its isoelectric point. In the presence of 1% Tween[®] 80, there was minimal adsorption at pH 4 (5%), pH 5 (7%), pH 6 (11%) and pH 8 (14%) (Fig 4.5).

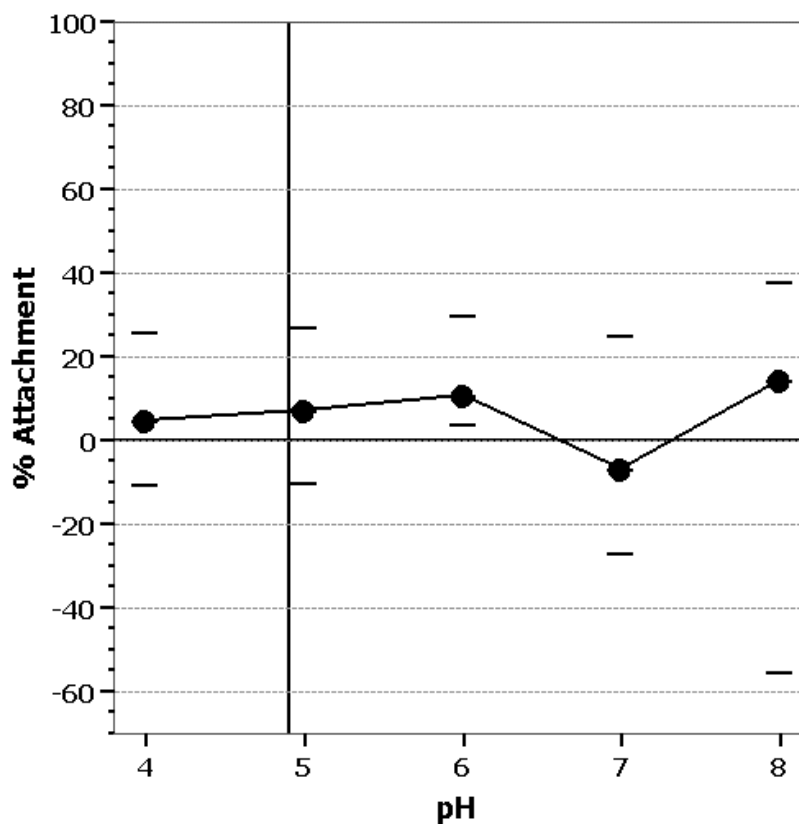


FIG. 4.5. Feline Calicivirus Adsorption to Lettuce with 1% Tween[®] 80. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 5 independent experiments.

In the presence of both Tween[®] 80 and 1M NaCl, there was no adsorption at any of the pH treatments (Fig 4.6).

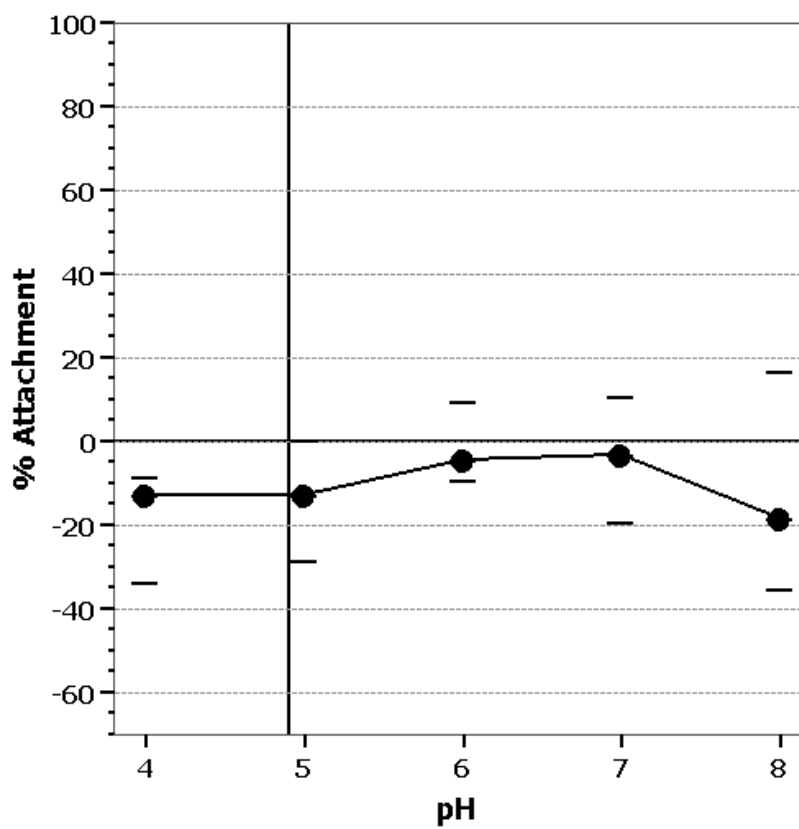


FIG. 4.6. Feline Calicivirus Adsorption to Lettuce with 1M NaCl and 1% Tween[®] 80. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 5 independent experiments.

MS2 Bacteriophage. The addition of 1M NaCl caused increasing adsorption to occur from pH 3 to pH 5 (Fig 4.7). From pH 3, (-12%) adsorption increased to 2% at pH 4 then continued to increase up to pH 5.0 at 14%. Thereafter, MS2 bacteriophage attachment decreased rapidly down to -10% at pH 6 and remained unattached up to pH 8.

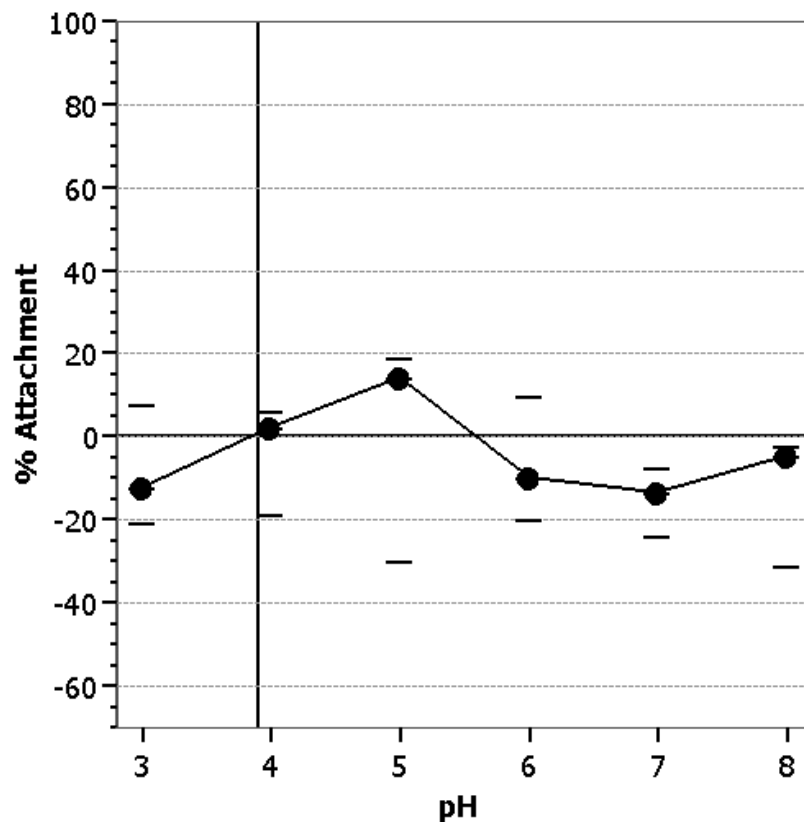


FIG. 4.7. MS2 Adsorption to Lettuce with 1M NaCl. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 5 independent experiments.

The addition of 1% Tween[®] 80 caused a decreasing adsorption of MS2 bacteriophage from a high at pH 3 (9%) to a steadily decreasing low at pH 7 corresponding to -10% (Fig 4.8).

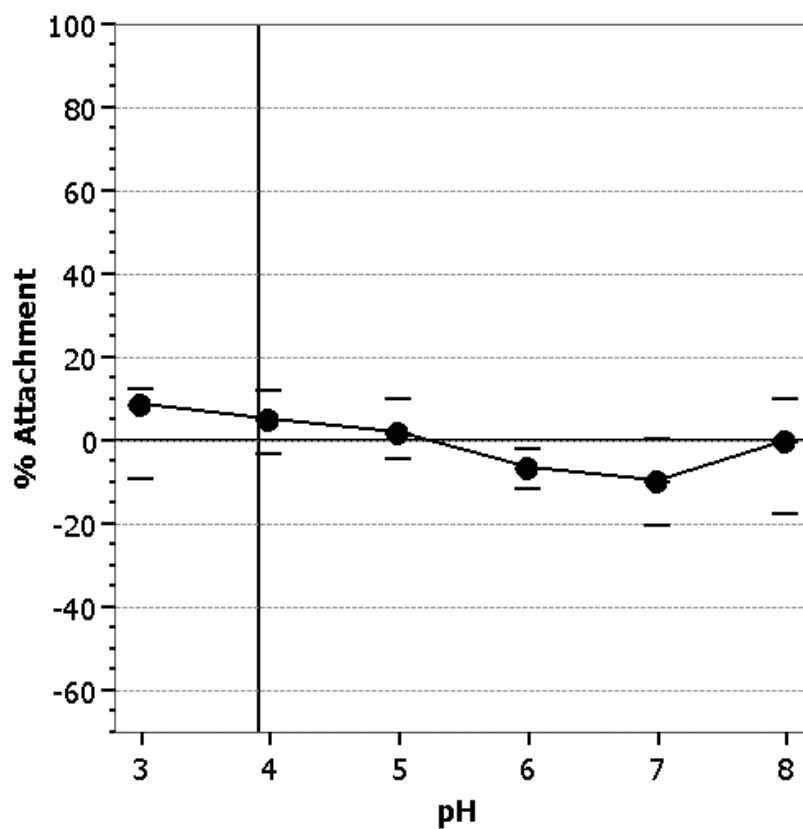


FIG. 4.8. MS2 Adsorption to Lettuce with 1% Tween[®] 80. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 5 independent experiments.

The bacteriophage MS2 did not exhibit any substantive change in adsorption between pH 3 to pH 8 with 1M NaCl and 1% Tween[®] 80. In the presence of 1% Tween[®] 80 and 1M NaCl, there was minimal adsorption (6% from pH 3 to pH 8) (Fig 4.9).

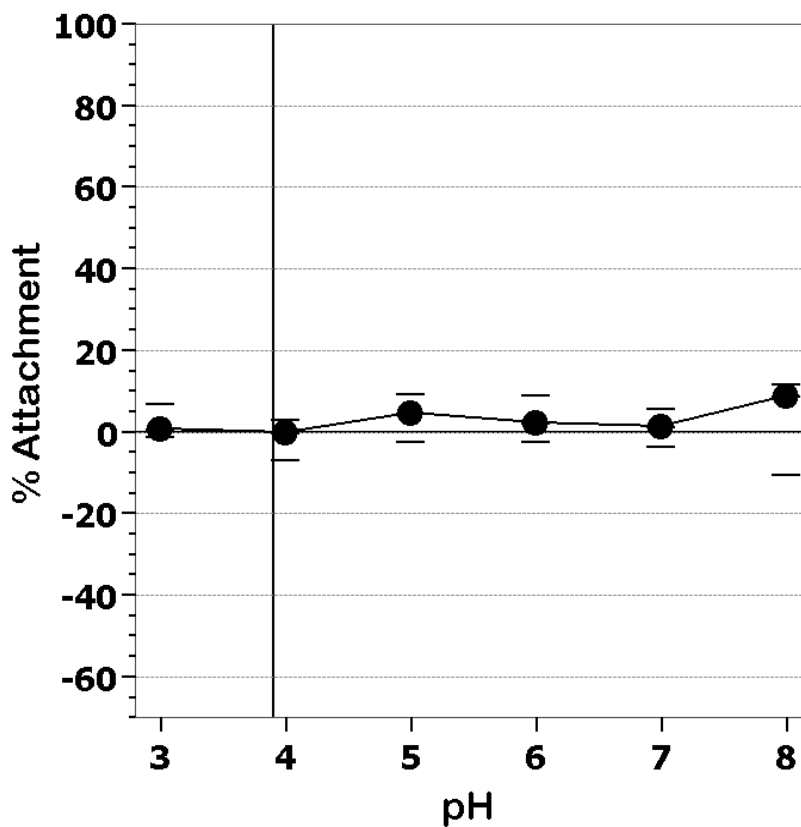


FIG. 4.9. MS2 Adsorption to Lettuce with 1M NaCl and 1% Tween[®] 80. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 5 independent experiments.

ϕ X174 Bacteriophage. In the presence of 1M NaCl, adsorption increased rapidly from pH 3 (-5%) to pH 4 (12%), thereafter decreased steadily to pH 7 at -15% (Fig 4.10).

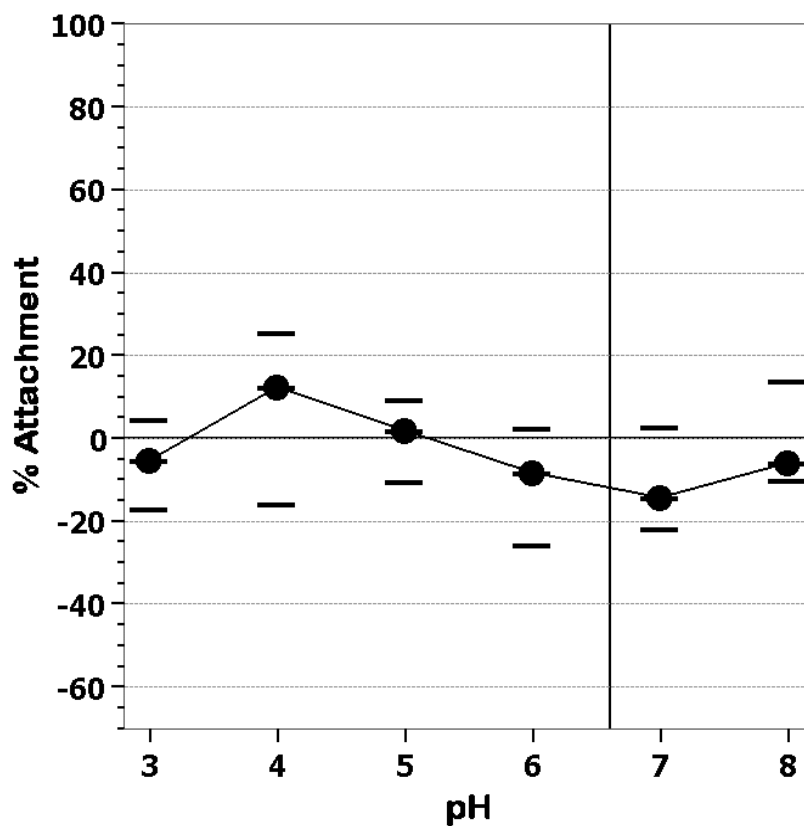


FIG. 4.10. ϕ X174 Adsorption to Lettuce with 1M NaCl. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 5 independent experiments.

The addition 1% Tween[®] 80, caused an increase of adsorption from pH 3 (-9%) to pH 4 (12%) (Fig 4.11). A rapid decline then occurred from pH 4 to pH 6 (-25%). Thereafter, adsorption increased at pH 7 and pH 8, -4% and 0%, respectively.

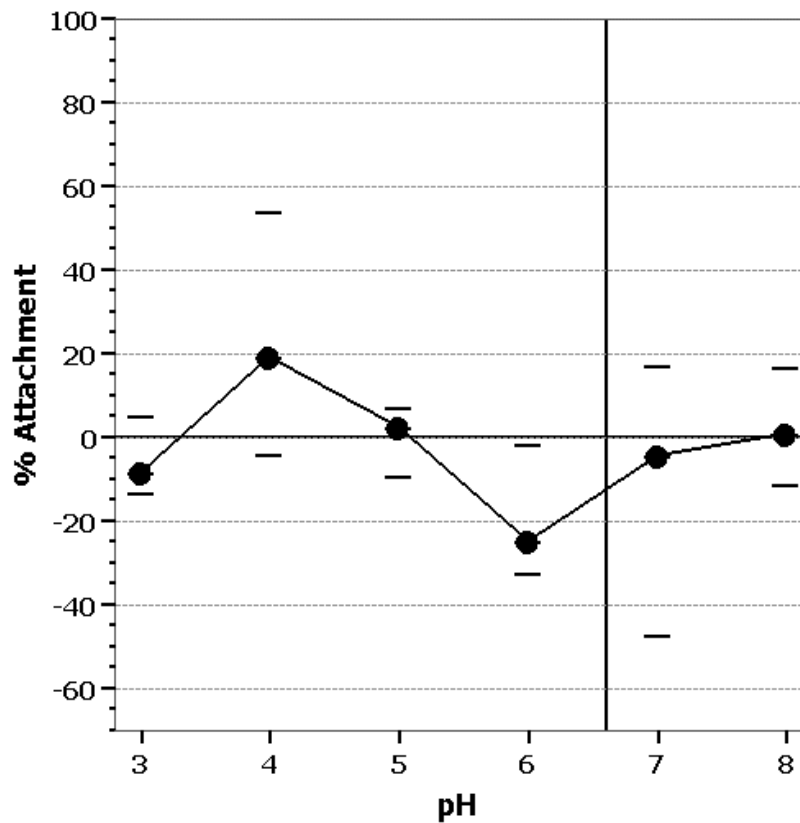


FIG. 4.11. ϕ X174 Adsorption to Lettuce with 1% Tween[®] 80. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 5 independent experiments.

In the presence of both 1M NaCl and 1% Tween[®] 80, adsorption steadily declined from pH 3 at 17% to no attachment pH 7. There appeared to be a slight increase in adsorption to 12% at pH 8 (Fig 4.12).

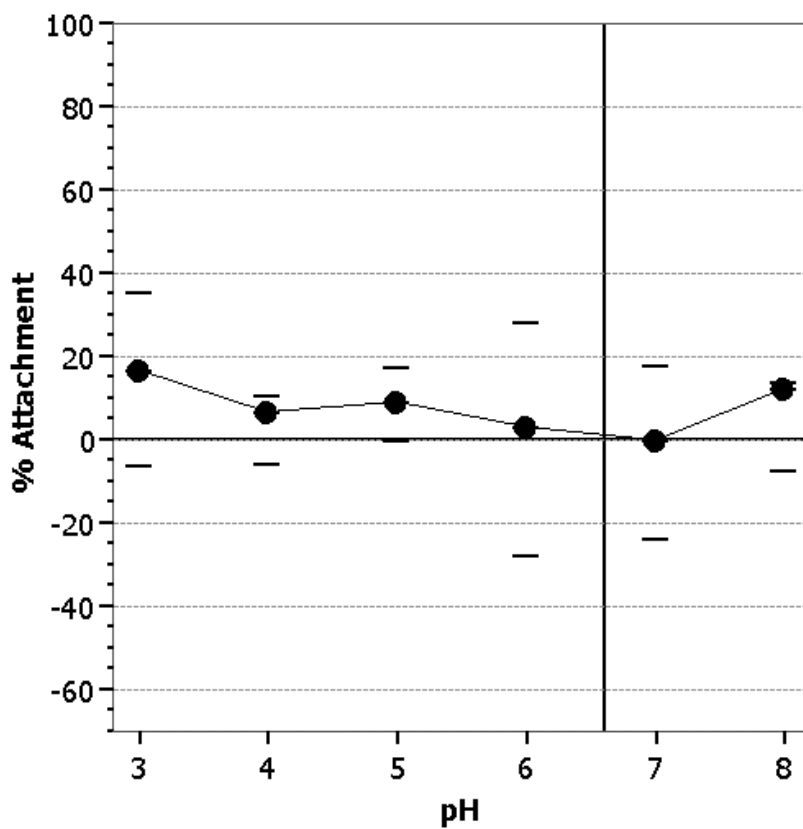


FIG. 4.12. ϕ X174 Adsorption to Lettuce with 1M NaCl and 1% Tween[®] 80. The trend line is shown connecting the median values. The horizontal bars represent the 25th and 75th percentiles. The vertical line represents the virus isoelectric point (pI). The experiment consisted of 5 independent experiments.

Discussion

Most work on enteric viruses and foods such as vegetables has focused on virus survival on the surfaces of fresh fruits or vegetables (4, 37, 90, 153). Even though there have been multiple papers on virus elution and concentration protocols (41, 43, 92, 94), virus loss due to adsorption has not been addressed. Our previous research (see Chapter III) suggested that echovirus 11 exhibited the greatest adsorption to lettuce (25.4-57% attachment) (Table 1). The addition of 1 M NaCl to the buffer solution removed all virus adsorption above the virus pI of 5.9 (Fig 4.1). Unexpectedly, adsorption at pH 5 (17.1%) and to some extent pH 4 (-4.8% median attachment, percentile range of -20.1% to 21.6%) was not inhibited (Fig 4.1). Only with the addition of Tween[®] 80 and NaCl to the milieu did the adsorption of echovirus 11 decrease at pH 5 (10.5%) and pH 4 (-9.4%). However, attachment was not completely inhibited (Fig 4.3 and Table 4.1). This leads us to believe that adsorption at pH 5 is due to van der Waals forces. A high concentration of NaCl produces highly charged, highly mobile ions in solution, which compress the Gouy layer (a layer of oppositely charged ions extending into the surrounding medium) so that electrostatic interactions or repulsions are not a factor in surface to surface interactions (61). Additionally, the detergent Tween[®] 80 removes hydrophobic interactions. Because neither NaCl nor Tween[®] 80 had any discernible effect, we can only conclude that van der Waal forces are responsible for the interaction at pH 5.

At the pH range of 7 (-13.5%) and 8 (-4.5%), FCV adsorption was also inhibited, much like echovirus 11 (Fig 4.4). Though FCV adsorption was reduced to below zero, the difference was not statistically different due to the large variation in FCV adsorption without any treatment (Table 4.2). At higher pH ranges, the addition of 1M NaCl removed all interaction between the lettuce and animal viruses.

The bacteriophage data was not as conclusive as the animal virus data. Our previous work indicated that adsorption of MS2 bacteriophage was only present at pH 3 (26.9%) (Table 4.1). The addition of NaCl completely removed adsorption at this pH (-12.3%), but also increased adsorption at the other pH ranges (Table 4.1 and Fig 4.7). Though, there may not be any significance because the increase in adsorption occurred in the negative range with the exception of pH 5 (14% with NaCl and -12.2% without treatment).

Bacteriophage ϕ X174 adsorption also decreased at pH 3 (-5.4% with NaCl and 4.9% untreated) and pH 8 (-5.9% with NaCl and 3% untreated), though the initial adsorption was low and further decreased with the addition of NaCl nevertheless, the difference was not significant (Table 4.2). Additionally, ϕ X174 proved to be recalcitrant to any treatment that was used. This result is not surprising, since our previous work did not show any discernable trend in ϕ X174 adsorption to lettuce, and other research indicates that ϕ X174 is a relatively inert virus, which lacks any adsorption pattern (136, 161).

Overall, the detergent Tween[®] 80 did not remove viruses from lettuce, with the exception of MS2 phage at pH 3 (8.7% with Tween[®] 80 and 26.9% without treatment). Though NaCl reduced MS2 bacteriophage adsorption to a greater extent (-12.3%). In other cases, Tween[®] 80 had either no difference compared to a non-treated solution or increased adsorption to lettuce, this was most noticeable with echovirus 11 (adsorption range of 21.1%-83.8% with Tween[®] 80 and 22.7%-57% without treatment). These results indicate that the waxy cuticle of the lettuce surface does not affect the adsorptive capacity of the lettuce. Additionally, the lack of any reduction in viral adsorption by Tween[®] 80 indicates that viruses must not be adsorbing on the waxy cuticle. Adsorption must be occurring on some other, as of yet unidentified, surface structure.

The virus adsorption trends shows that most of the viruses were not completely inhibited within the pH range of 5-6 in the presence of NaCl, and that within this range a complete reversal of adsorption occurred, i.e. adsorption changed from positive to negative. This is most pronounced with echovirus and MS2 bacteriophage. Even FCV, which exhibited a negative adsorption in the presence of 1M NaCl, also trended upward at pH 5.0 before decreasing at pH 6.0. A secondary pI, that of the lettuce surface or a specific part of the lettuce, must be accounting for the adsorption phenomenon within this range. The only exception was ϕ X174, which shows adsorption at pH 4, but still shows an adsorption reversal between pH 5 and 6. Because van der Waals forces are weak and only interact at very close distances, the strength of the repulsion of the

electrostatic force is the main factor responsible for driving van der Waals interactions (61). In the absence of electrostatic repulsion, like in the presence of high salts or at the pI, either hydrophobic interactions or van der Waals can only be a factor in interactions. Because we determined that hydrophobic interactions are not involved in virus to lettuce interactions, only van der Waals forces must be responsible for any attachment in the presence of both NaCl and Tween[®] 80.

The identification of forces critical for virus adsorption is important for vegetable and fruit processing for viruses or as a preventive measure to avoid foodborne disease caused by viral agents. Additionally, other authors have found high levels of enteric viruses in discharged water from wastewater treatment plants (101, 127, 157). The ability of human pathogenic viruses to adsorb to lettuce is of great concern given the amount of virus in waters that may not be treated before irrigation. Though bacterial indicators are commonly used to identify poor water quality, previous research has shown that bacteria does not serve as reliable indicators for enteric virus contamination (44, 146, 154). Hence, the non-specific adsorption of enteric viruses to minimally processed fresh produce may result in either large outbreaks or sporadic outbreaks which would be difficult to trace, given the current science of viral elution protocols.

This research has identified the forces that are responsible for virus adsorption to the surface of lettuce. Electrostatic forces are the principle force, and the addition of 1 M NaCl at pH 7 and 8 will inhibit all interactions between

the lettuce and virus. In light of this research many elution protocols, those using a high pH to elute viruses, may need to be re-evaluated. Because we did not test the effect of beef extract at a concentration or pH range, pH > 8, commonly used for elution protocols, we cannot make a final recommendation on the proper elution procedures. The fact though, is that these procedures commonly have virus losses equivalent to the amount of virus adsorbed in this study and our previous study shows that these protocols need to be reevaluated to determine adequate elution protocols. This study shows that increasing the pH without greatly compressing the gouy layer will result in enhanced adsorption of virus to lettuce.

CHAPTER V

**THE SURVIVAL OF VIRUSES ON LETTUCE AND THE
IMPACT OF THE MICROBIAL FLORA ON VIRAL
PERSISTENCE**

Overview

The stability of enteric viruses on fresh produce is an important virulence trait that has an impact on the infectivity of a virus. It has been shown that viruses are able to adsorb onto the surface of lettuce, but the long-term persistence on the surface of the lettuce has not been fully investigated. The objective of this study was to determine the effect of the microbial flora on the surface of lettuce on the survival of viruses. Four viruses were assayed for their ability to persist in the presence of the normal flora. The human pathogen, echovirus 11, and FCV exhibited prolonged survival when incubated with the microbial flora of lettuce compared to a filtered rinsate or buffer only. The bacteriophages, MS2 and ϕ X174, were not affected in the presence of the microorganism obtained from the lettuce, filtered rinsate or buffer. On the lettuce surface, echovirus 11 and feline calicivirus, survived at an equivalent or greater rate than the bacteriophages at 4° C, whereas at room temperature the animal viruses were inactivated at a greater rate than the bacteriophages. The microbial flora was able to utilize the amino acids, phenylalanine and alanine, preferentially over complex carbohydrates, xylose and xylan, and simple sugars, fructose and sucrose, but was not able to utilize the viruses as energy sources.

Overall, these results suggest that viral persistence on the surface of lettuce may be temperature dependent and may not be affected by the resident microorganisms on the surface of the lettuce.

Introduction

The surface of leaves has a large microbial community, which is made up of transient and specialized microbial inhabitants. The specialized microbial community survives in a hostile environment with alternating conditions of high and low humidity and temperature fluctuations (98). The epiphytic community, microorganisms that comprise the community on the aerial portion of plants, must be able to utilize a wide range of nutrient sources when available. To this extent it is no surprise that common epiphytes are those bacteria that are able to utilize a wide variety of carbon sources, like *Pseudomonas spp.* (123). When transient microorganisms are placed within this community, they can either compete for resources or be out competed. This type of competitive exclusion has been previously shown for the inhibition of ice nucleating bacteria and the colonization of plant pathogens (97, 98, 171). The inhibitory effect of the epiphytic bacteria has also been shown against human bacterial pathogens *E. coli* O157:H7, *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* Montevideo (138). The effect of the normal flora on the survival of viruses has not been previously studied. Unlike bacteria or fungi, viruses cannot compete with microorganisms for survival or are they able to react to environmental stimuli. Even though this contributes to viral stability in the environment,

because viruses do not have to maintain basal metabolic activity and hence are not limited by nutrient availability or growth conditions, it can also cause viruses to become predation targets in a nutrient limited environment. The objective of this study was to determine if the presence of the microbial community of lettuce is able to utilize viruses as a nutrient source. Four viruses, the bacteriophage ϕ X174 and MS2 and animal viruses, FCV, a commonly used calicivirus surrogate, and the human pathogen echovirus 11, were studied for their ability to survive in the presence of the microbial community. A series of microcosm, lettuce surface and aerobic respiration studies were assayed to determine the amount of bacterial predation on viruses.

Materials and Methods

Cells and Viruses. Echovirus 11 (ATCC VR-1052) was grown in buffalo green monkey cells (BGMK), feline calicivirus (F9) was grown in Crandell Reese feline kidney cells (CRFK), bacteriophage MS2 (ATCC 15597-B1) was propagated with *E. coli* HS(pFamp)R (ATCC 700891) and bacteriophage ϕ X174 (ATCC 13706-B1) was propagated in *E. coli* host CN13 (ATCC 700609). Feline calicivirus and CRFK cells were a generous gift from Dr. Sagar Goyal at the University of Minnesota. Both bacteriophages were assayed using the double agar layer method (160). Animal viruses were assayed by the agar overlay method (161). Growth and maintenance media was identical to previously described protocols (161).

Virus Purification. Viruses were purified from their respective raw lysates to remove all nutrients that could be used for bacterial growth. The purification of viruses was similar to the method used by Zhou *et al.* for FCV (175). Briefly, the CRFK cell line was grown to 90-100% confluency then infected with a multiplicity of infection of 1-10 virus particles. After 24-48 hours, the infected CRFK cells were freeze-thawed twice. The raw lysate was then centrifuged at $3,313 \times g$ for 30 minutes at 4°C . Solid NaCl (1M) was added to the raw lysate and incubated in ice water for 1 hour. After incubation, PEG MW 3350 was added to the lysate (10% w/v) and incubated at 4°C overnight. The lysate was centrifuged at $11,000 \times g$ for 10 minutes at 4°C then the supernatant was discarded. The precipitate was resuspended in 1/10 - 1/100 of the total volume in boric acid buffer, pH 7.4 (0.2 M boric acid and 0.5 M NaCl). The concentrated lysate was then mixed in a boric acid CsCl solution to obtain a final concentration of 1.31 g/ml of CsCl. The sample was centrifuged at $193,911 \times g$ for 20 hours at 4°C . To remove the CsCl salt, samples were dialyzed using the Slide-A-Lyzer[®] dialysis cassette (10,000 mw) using sodium phosphate buffer. Briefly, the samples were dialyzed using 1 L of 0.1 M NaPO_4 (pH 7.0) buffer at room temperature for 2 hours. After 2 hours, spent buffer was discarded and replaced with 1 L of 0.1 M NaPO_4 buffer. Dialysis was allowed to continue for another 2 hours at room temperature. After the second incubation, spent buffer was discarded and 1 L of 0.01 M NaPO_4 (pH 7.0) was added and incubated overnight

at 4° C. Viruses were then aliquoted into 0.2 ml portions and stored at -80°C until further use.

Washing Microorganisms from Lettuce. Lettuce leaves were severed at the base and placed in 42-oz Whirl-Pak bags (Fort Atkinson, WI).

Approximately 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) was added to the bag for every gram of lettuce. Bags were heat sealed after removing as much air from the bag as possible to allow maximum buffer contact on the surface of the lettuce. The bags with lettuce were sonicated for 1 minute on each side and then vortexed for 30 seconds at maximum speed. The resulting lettuce wash was removed and placed in a 40 ml conical tube until further use. Half of volume was filtered through a 0.22 µm filter to remove microorganisms and used immediately for the microcosm studies.

Microcosm Study. The total volume needed for the microcosm studies was calculated and prepared accordingly. Equal volumes of 0.1 M NaPO₄ buffer (pH 7.0) filtered lettuce wash and lettuce wash was aliquoted into conical tubes. Purified virus was thawed and equal amounts were added to each of the three tubes. A total volume of 1.2 ml was aliquoted into a microcentrifuge tube. All tubes were placed in cryogenic storage boxes and stored out of direct sunlight at room temperature. Three tubes for each condition was removed from the box and sampled for virus titers starting at day 0.

Lettuce Surface Survival Study. To identify the effect of the microbial normal flora on the lettuce survival, viruses were aliquoted onto the surface of a

25 cm² lettuce piece and assayed for their presence. Briefly, 25 cm² of lettuce was cut and placed in petri plates, 100 µl of purified virus was aliquoted onto the surface of lettuce and incubated for 30 minutes at room temperature. Half of the lettuce samples were placed at room temperature and the other half was placed at 4° C. Because any treatment to remove the microbial normal flora will result in a change in the surface characteristics of the lettuce, the only method to decrease the metabolic activity of the normal flora was to decrease the temperature to 4° C. Any difference on the survival of virus can only be attributable to desiccation or the decreased respiration of the normal flora. Because all the viruses used are non-enveloped, desiccation stress is minimal. Furthermore, a study by Stine *et al.* indicated that desiccation is not a major factor for virus inactivation on fresh produce (146). Viruses were eluted from the surface of the lettuce using 0.1 M NaPO₄ buffer (pH 7.0) amended with 1M NaCl. Previous research has shown that this buffer will remove 100% of viruses from the surface of the lettuce.

Microbial Nutrient Utilization Study. To fully investigate the ability of the microbial flora to utilize complex carbon sources, two different assays were conducted using the BD Oxygen Biosensor System (BD Biosciences, Bedford, Mass.). The first assay consisted of analyzing the usage of carbon sources from lettuce grown using a hydroponic system, retail bibb lettuce and retail iceberg lettuce. Finally, the ability of the lettuce epiphytes to use viruses as energy sources was also investigated. The normal flora was eluted as previously

described in the microcosm survival study protocol. A total of 150 μl consisting of 50 μl of phosphate-buffered mineral salts, 50 μl of the microbial flora and 50 μl of a carbon source was aspirated into the BD Oxygen Biosensor microplate (Garland *et al.* 2003) The amount of fluorescence was read every 15 minutes for 48 hours. The microplate was maintained at 30° C for the duration of the experiment. An increase of fluorescence is a direct indication of oxygen consumption and thus active metabolic activity.

Data Analysis. All statistical analysis was performed using SPSS statistical software, version 11.0.1 (SPSS Inc., Chicago, Ill.). Both the microcosm and lettuce surface studies were analyzed with the same criteria. All data was graphed using the C/C_0 , where C is the viral concentration at the time the sample was taken and C_0 is the viral concentration at day 0. Two methods were used for the data analysis of survival data. If initial virus concentrations were not similar than a pair wised sign-test was used for data analysis after the C/C_0 transformation. (NRF). In determining the utilization of carbon sources, the maximum NRF for each carbon source was used in determining the median of each data set.

Differences were considered significant if the p-value was less than 0.05 using the two-tailed test (i.e. the differences are greater or less than 0) sign test. For samples that had a similar day 0, the data was first transformed using the box-cox transformation, then significant differences were tested using a two-tailed pair-wise t-test. Differences between treatments were considered significant if the p-value was less than 0.05. In the microbial respiration studies, the data was normalized by dividing all the data points from a well by its fluorescence at 1 hour. This is expressed as normalized relative fluorescence

Results

The results for the microcosm and lettuce surface study represent viable virus over a period of 14 days for bacteriophages and 15 days for animal viruses. Each data point is the mean of three experimental replicate.

Microcosm Survival Study. The survival of bacteriophage ϕ X174 was assayed in the presence of the lettuce wash, filtered lettuce wash and in 0.1 M phosphate buffer (Fig 5.1). There was no significant decrease in bacteriophage ϕ X174 over the course of 14 days in any of the three conditions. There was a rapid increase of ϕ X174 in lettuce wash and a slower increase of ϕ X174 for both buffer and filtered lettuce wash, but overall there were no significant differences.

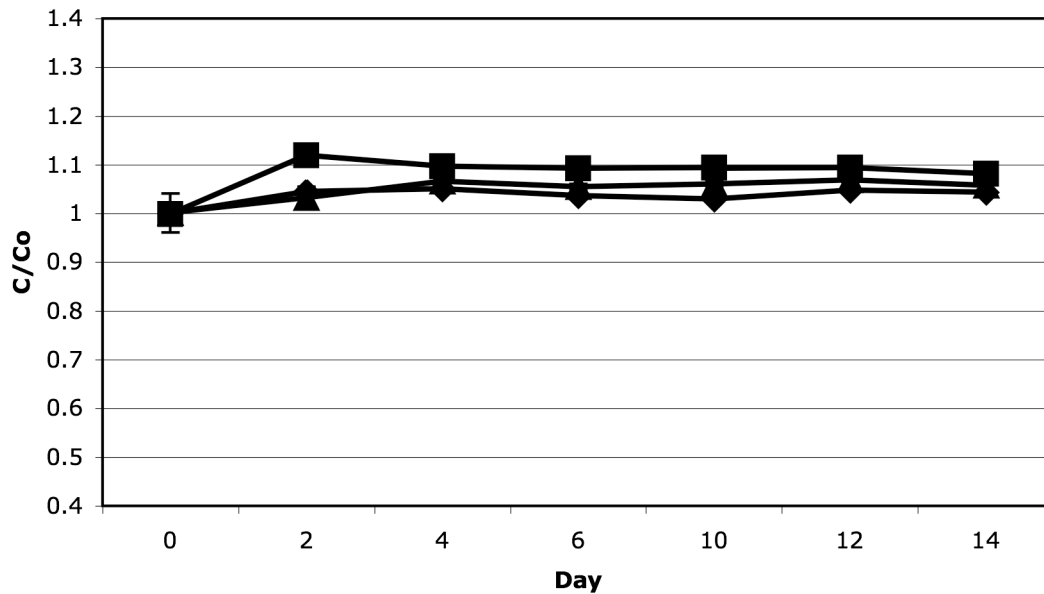


FIG. 5.1. ϕ X174 Microcosm Survival. The graph represents the survival of ϕ X174 in microcosms over a period of 14 days. The square points represents the survival of ϕ X174 in the presence of the lettuce microbial flora, the triangle points represents the survival of ϕ X174 in the presence of filtered lettuce wash and the diamonds represent the survival of ϕ X174 in buffer. C/C_0 was obtained by dividing the mean virus pfu of day n by the mean pfu at day 0. The error bars represent ± 1 SE.

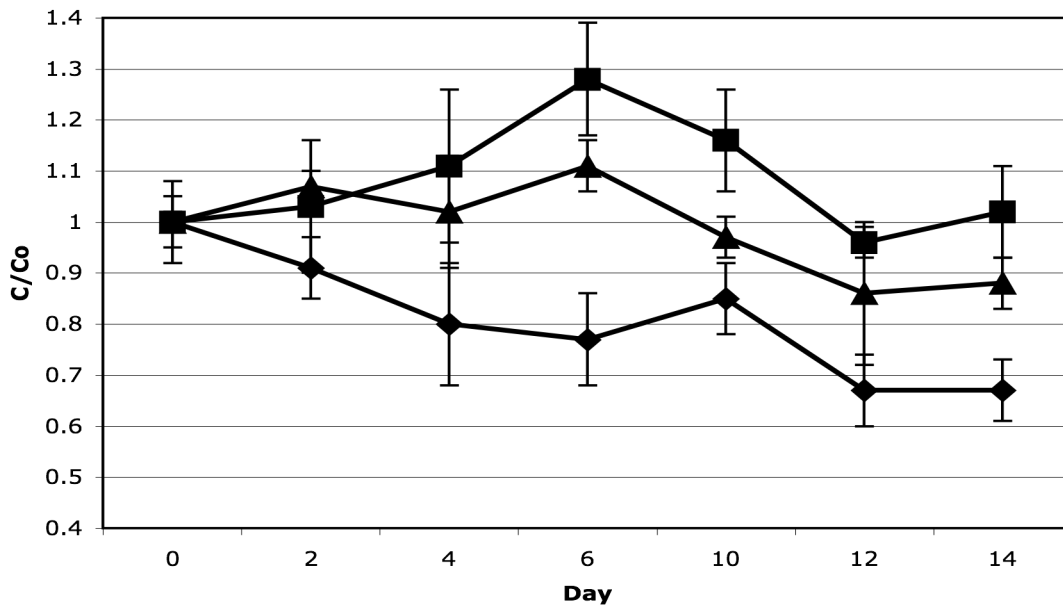


FIG. 5.2. MS2 Microcosm Survival. The graph represents the survival of MS2 in microcosms over a period of 14 days. The square points represents the survival of MS2 in the presence of the lettuce microbial flora, the triangle points represents the survival of MS2 in the presence of filtered lettuce wash and the diamonds represent the survival of MS2 in buffer. C/C_0 was obtained by dividing the mean virus pfu of day n by the mean pfu at day 0. The error bars represent ± 1 SE.

In the presence of the lettuce wash, MS2 bacteriophage exhibited an increase in observed virus titers, probably due to de-aggregation, which continued up to day 6 (Fig 5.2). This trend was also similar to MS2 in buffer, but de-aggregation was not as pronounced. The survival of MS2 in the presence of the filtered lettuce wash had the greatest effect on MS2 survival. The bacteriophage MS2 decreased steadily in filtered lettuce up to 30%, whereas MS2 bacteriophage in buffer decreased to 20%, whereas the MS2 phage had the lowest decrease of 0% by the end of the two weeks. The MS2 bacteriophage in filtered lettuce wash was significantly different ($P < 0.05$) than lettuce wash and buffer.

The animal virus, FCV, decreased steadily under all conditions during the course of the experiment (Fig 5.3). Both filtered lettuce wash and buffer inoculated virus were similar in deactivation, whereas FCV in the presence of lettuce wash exhibited the slowest deactivation trend during the course of the experiment. Both the buffer and filtered lettuce wash exhibited a decrease of FCV greater than 40% by day 15, whereas FCV in the presence of lettuce wash also decreased but at a reduced rate. The differences were most pronounced at day 9, but by day 15 the decrease for all three conditions were similar. Nevertheless, the increased survival of FCV in lettuce wash was significantly different than both buffer and filtered lettuce wash ($P < 0.05$).

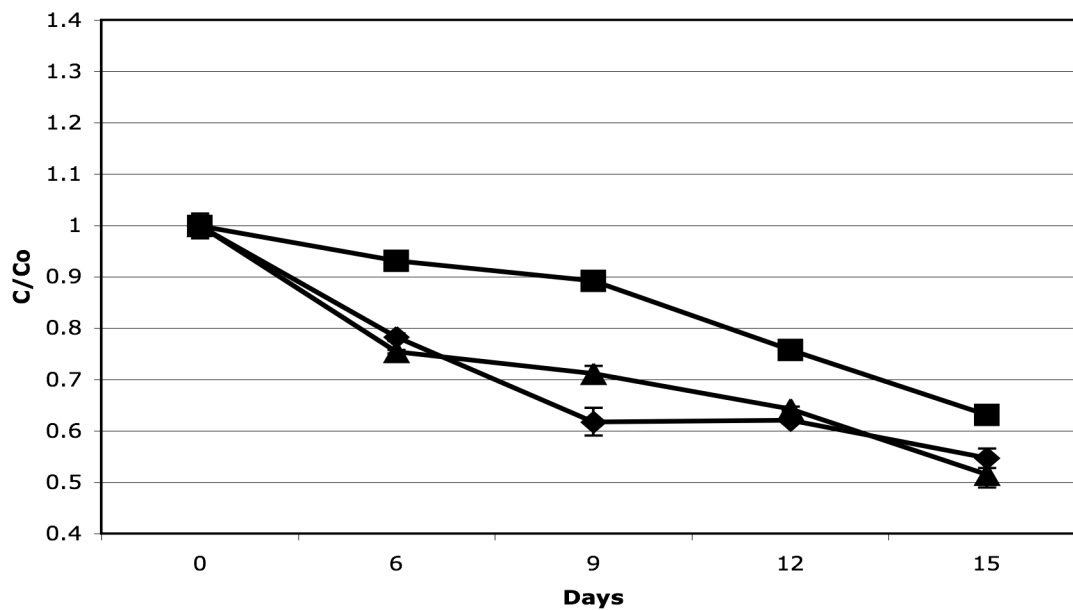


FIG. 5.3. Feline Calicivirus Microcosm Survival. The graph represents the survival of FCV in microcosms over a period of 15 days. The square points represents the survival of FCV in the presence of the lettuce microbial flora, the triangle points represents the survival of FCV in the presence of filtered lettuce wash and the diamonds represent the survival of FCV in buffer. C/C_0 was obtained by dividing the mean virus pfu of day n by the mean pfu at day 0. The error bars represent ± 1 SE.

Survival of echovirus 11 was not significantly different during the survival experiment (Fig 5.4). All treatments decreased at approximately the same rate at 40-50% by day 15. The decrease was rapid throughout all treatment conditions with no significant differences between any of the treatments. Surprisingly, the enterovirus exhibited a faster decline than FCV, which is a respiratory virus. Both animal viruses declined at a faster rate than the bacteriophages. The bacteriophages exhibited a small decrease, if any. Overall the presence of the microbial normal flora of lettuce had little effect on the persistence of virus, and if a difference existed, it was protective in nature.

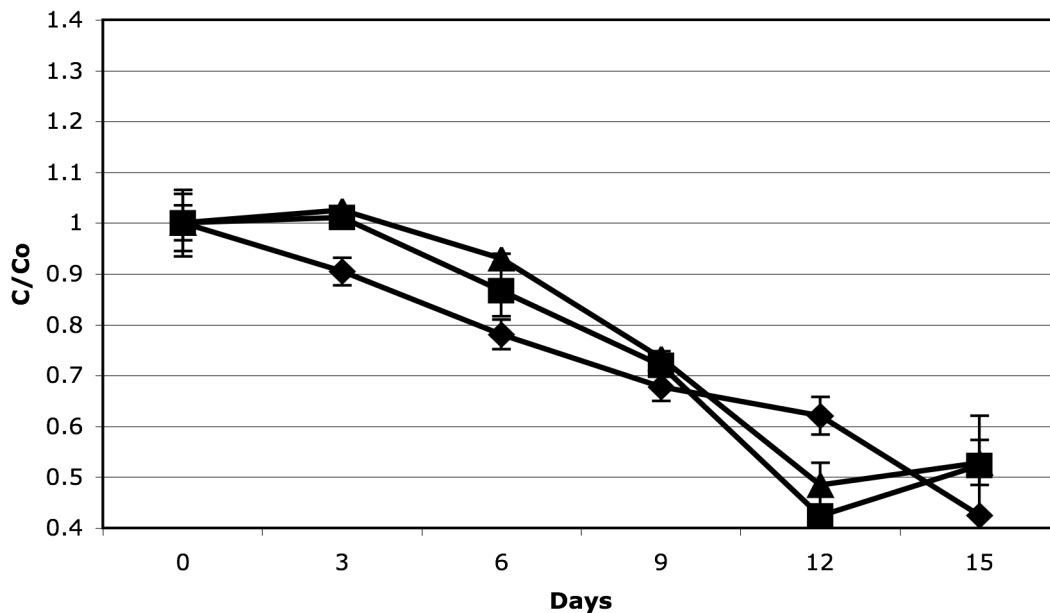


FIG. 5.4. Echovirus 11 Microcosm Survival. The graph represents the survival of echovirus 11 in microcosms over a period of 15 days. The square points represents the survival of echovirus 11 in the presence of the lettuce microbial flora, the triangle points represents the survival of echovirus 11 in the presence of filtered lettuce wash and the diamonds represent the survival of FCV in buffer. C/C_0 was obtained by dividing the mean virus pfu of day n by the mean pfu at day 0. The error bars represent ± 1 SE.

Lettuce Surface Survival Study. Bacteriophage ϕ X174 exhibited two different survival characteristics on the surface of lettuce (Fig 5.5). At 4° C, ϕ X174 survival did not decrease until after day 4. Thereafter, ϕ X174 decreased 25% by day 14. At room temperature, ϕ X174 exhibited a steady decline decreasing 60% by day 10 -14. The survival of ϕ X174 at room temperature and at 4° C was significantly different ($P < 0.05$).

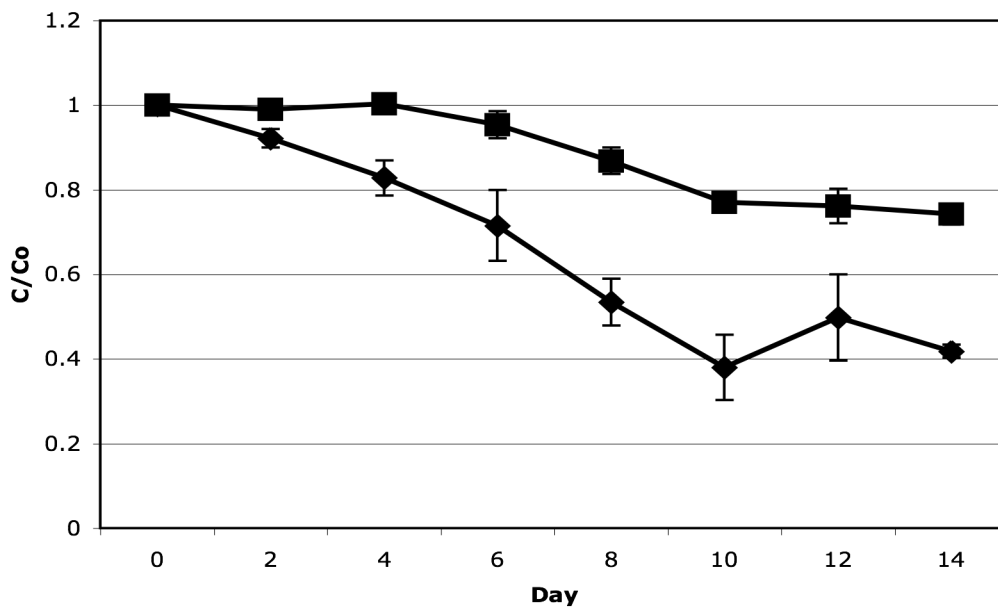


FIG. 5.5. ϕ X174 Lettuce Surface Survival. The figure represents survival of ϕ X174 on the surface of lettuce. The square data points represent ϕ X174 survival at 4° C and the diamond data points represent ϕ X174 survival at room temperature. C/C_0 was obtained by dividing the mean virus pfu of day n by the mean pfu at day 0. The error bars represent ± 1 SE.

The bacteriophage MS2, declined started at day 2 at 4° C (Fig 5.6). By the end of day 14, MS2 had a 40% decrease. At room temperature, MS2 survival rapidly decreased. Approximately, 90% of the initial MS2 bacteriophage on the lettuce had become deactivated starting at day 8 and remained relatively constant up to day 14. The differences in MS2 survival were significantly different between room temperature and at 4° C.

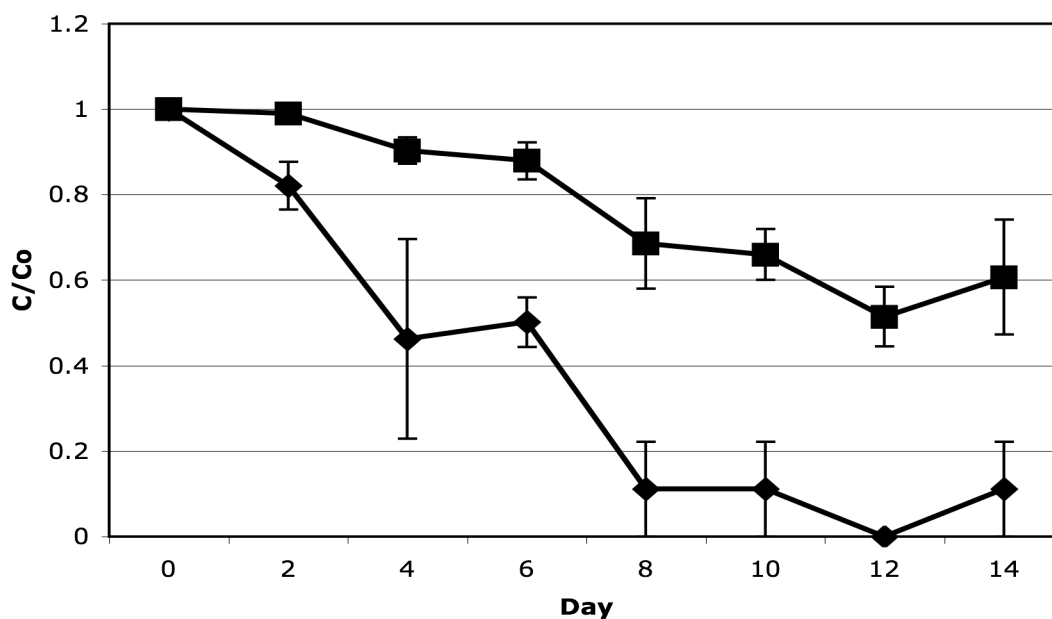


FIG. 5.6. MS2 Lettuce Surface Survival. The figure represents survival of MS2 on the surface of lettuce. The square data points represents MS2 survival at 4° C and the diamond data points represent MS2 survival at room temperature. C/C_0 was obtained by dividing the mean virus pfu of day n by the mean pfu at day 0. The error bars represent ± 1 SE.

Differences in FCV survival were the greatest of all the viruses assayed. At 4°C, FCV had decreased 25% by day 9 (Fig 5.7). By day 15, FCV survival had decreased another 15% to 60%. At room temperature, no viruses were detectable by day 6. The survival differences were significantly different between room temperature and 4°C ($P<0.05$).

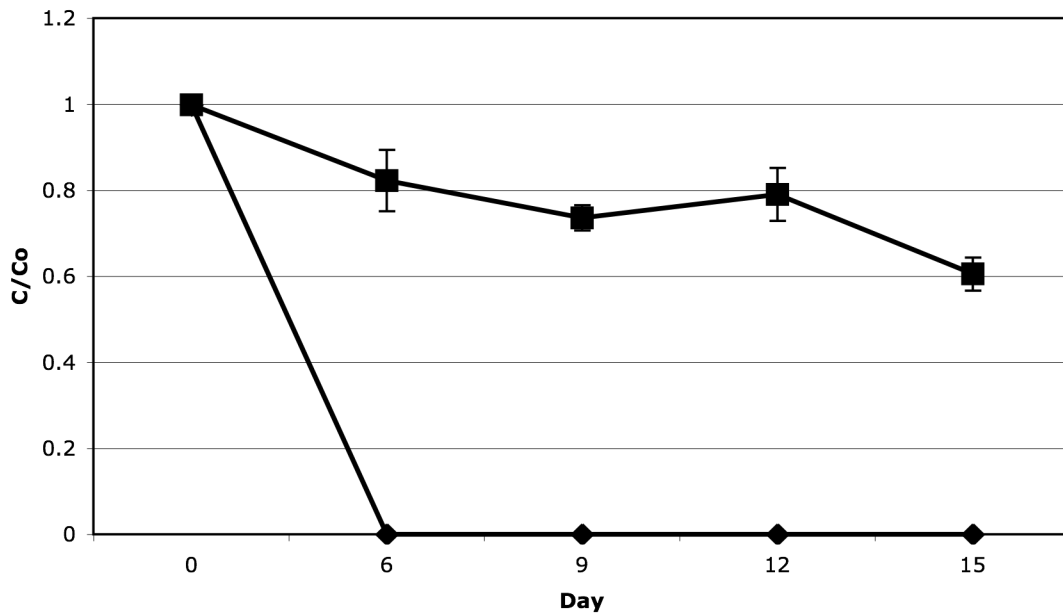


FIG. 5.7. Feline Calicivirus Lettuce Surface Survival. The figure represents survival of FCV on the surface of lettuce. The square data points represents FCV survival at 4°C and the diamond data points represent FCV survival at room temperature. C/C_0 was obtained by dividing the mean virus pfu of day n by the mean pfu at day 0. The error bars represent ± 1 SE.

The human pathogen, enterovirus 11, exhibited the greatest survival of all the viruses at 4° C (Fig 5.8). Up to day 6, there was no detectable decrease of echovirus 11 on the surface of lettuce. At day 9, echovirus 11 decreased 10%, but did not decrease any further until day 15 with 80% of the virus remaining viable. The survival at room temperature was significantly different than at 4° C ($P < 0.05$). By day 6, some samples contained no detectable viruses, and by day 12 virus was not detectable on any of the samples at room temperature.

Microbial Utilization of Viruses. The ability of the lettuce microbial community to utilize viruses was investigated. The ability of the microbial flora to utilize complex carbon sources as well as amino acid was investigated. After the initial carbon utilization studies, the ability the microbial flora to metabolize the viruses and use them as carbon sources was assayed. Utilization of the viruses was measured by a decrease of oxygen concentration in each well. This method has been previously used to monitor microbial carbon utilization (58). The consumption of solubilized oxygen was measured to determine microbial growth in the presence of carbon sources and viruses. Because the viruses were purified from any contaminating carbon sources, oxygen utilization is a direct indication of aerobic growth with viruses as the only available carbon source.

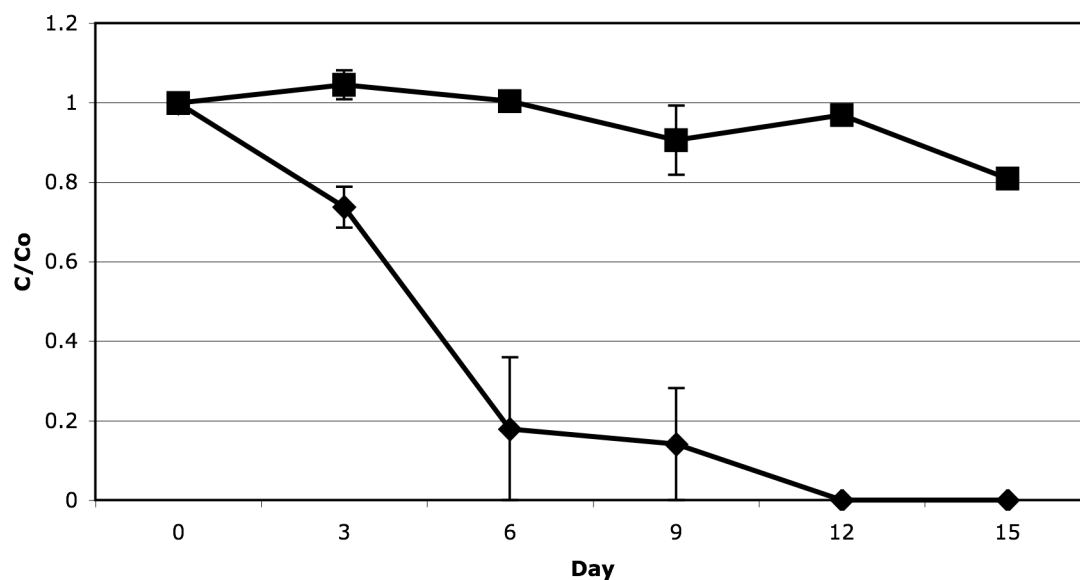


FIG. 5.8. Echovirus 11 Lettuce Surface Survival. The figure represents survival of echovirus 11 on the surface of lettuce. The square data points represents echovirus11 survival at 4° C and the diamond data points represent echovirus 11 survival at room temperature. C/C_0 was obtained by dividing the mean virus pfu of day n by the mean pfu at day 0. The error bars represent ± 1 SE.

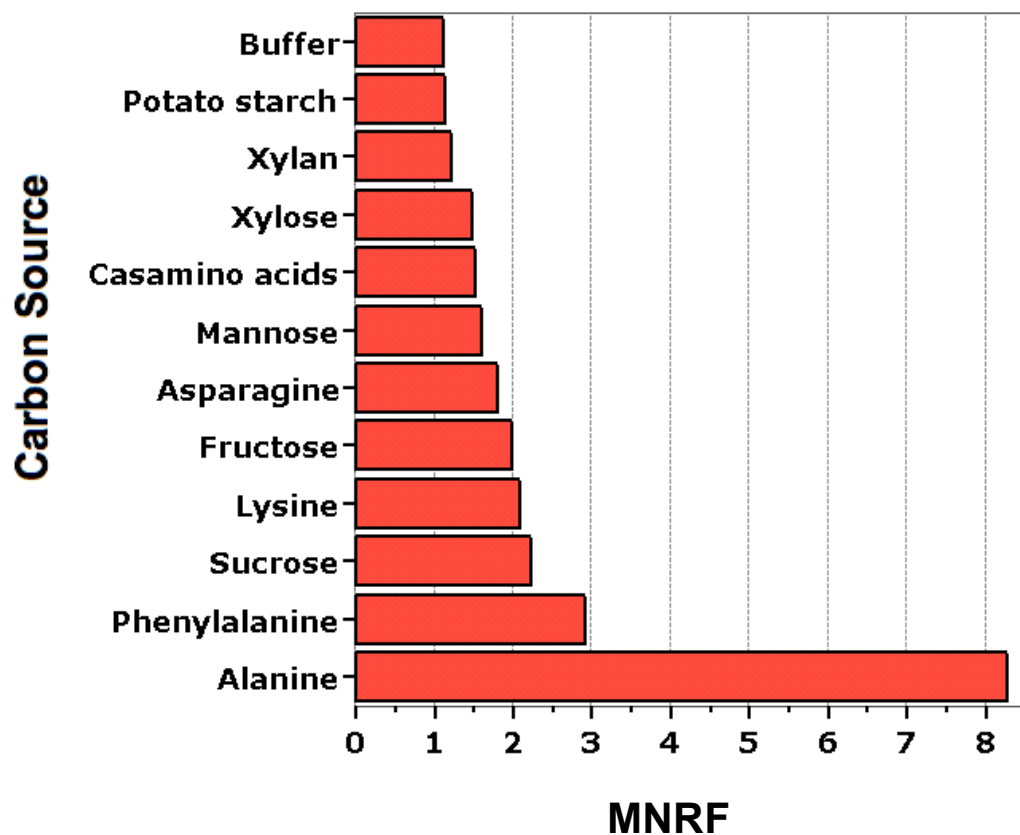


FIG. 5.9. Bibb Lettuce Microbial Flora Carbon Utilization. The bar graph represents the carbon utilization of retail bibb lettuce normal flora during a 2 day experiment. The x-axis lists the carbon sources used for this study. The y-axis is the median of the maximum normalized relative fluorescence (MNRF). The data was normalized by dividing all the data obtained from a single well by the fluorescence value obtained at 1 hour. The MNRF was obtained by calculating the mean of the maximum normalized value of each well. The median is the result of fluorescence signal from 8 wells.

The retail bibb lettuce microbial flora was able to utilize alanine at the greatest extent of all the carbon sources assayed, 8.5 fold, in retail bibb lettuce (Fig 5.9). The next highest carbon source was phenylalanine exhibiting a 2.9 fold maximum relative fluorescence in bibb lettuce. Not surprisingly, the simpler carbon sources were utilized more than the complex carbon sources (Fig 5.9). Though, only select amino acids (phenylalanine and alanine) were utilized and other amino acids and casamino acids were either not utilized or utilized at a reduced extent.

The iceberg lettuce exhibited a carbon utilization response similar to bibb lettuce (Fig 5.10). Like bibb lettuce, phenylalanine and alanine had the greatest response, 8 fold and 5.5 fold, respectively. Though, the iceberg lettuce response was inverted for alanine and phenylalanine, alanine > phenylalanine, compared to the bibb lettuce response. Additionally, the amino acids provided the strongest response but like bibb lettuce, casamino acids had the lowest response of all the amino acids. After the amino acids, the simple sugars provided a reduced response whereas the complex carbon sources provided a response no greater than buffer only.

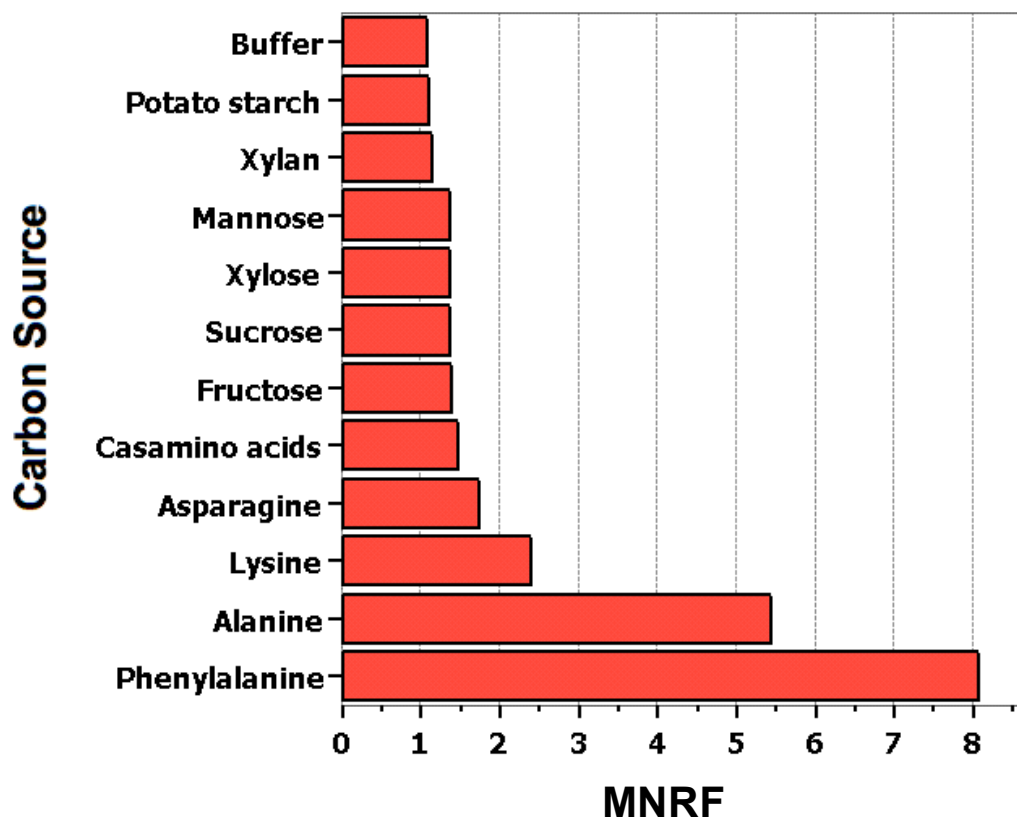


FIG. 5.10. Iceberg Lettuce Microbial Flora Carbon Utilization. The bar graph represents the carbons utilization of retail iceberg lettuce normal flora during a 2 day experiment. The x-axis lists the carbons sources used for this study. The y-axis is the median of the maximum normalized relative fluorescence (MNRF). The data was normalized by dividing all the data obtained from a single well by the fluorescence value obtained at 1 hour. The MNRF was obtained by calculating the mean of the maximum normalized value of each well. The median is the result of fluorescence signal from 8 wells.

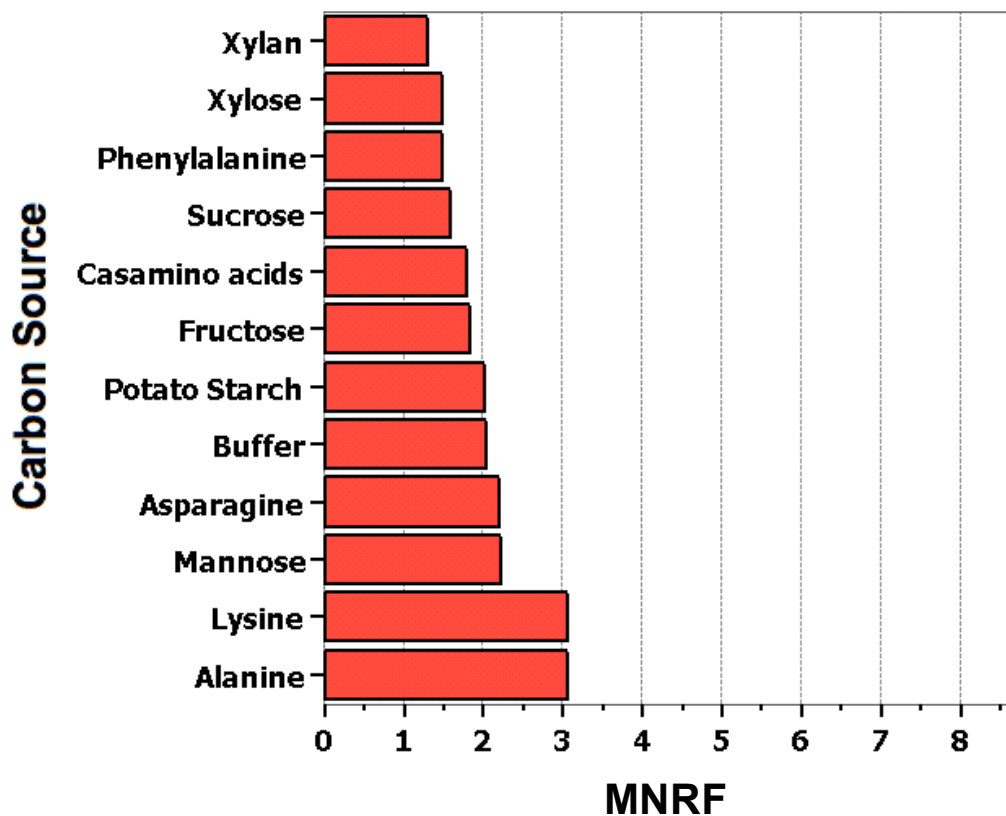


FIG. 5.11. Hydroponic Bibb Lettuce Microbial Flora Carbon Utilization. The bar graph represents the carbons utilization of hydroponic bibb lettuce normal flora during a 2 day experiment. The x-axis lists the carbons sources used for this study. The y-axis is the median of the maximum normalized relative fluorescence (MNRF). The data was normalized by dividing all the data obtained from a single well by the fluorescence value obtained at 1 hour. The MNRF was obtained by calculating the mean of the maximum normalized value of each well. The median is the result of fluorescence signal from 8 wells.

The microbial community in the hydroponic grown bibb lettuce was dissimilar from the bibb and iceberg lettuce community response (Fig 5.11). The amino acids provided the top two responses, but unlike the retail samples, lysine and alanine provided the greatest response. Only asparagines and mannose responded greater than the buffer, mostly because the wash buffer was 0.1% peptone where in iceberg and bibb lettuce utilized a 0.1 M sodium phosphate buffer.

The high utilization of amino acids indicated that the viral protein coat had the potential to be utilized. As controls for the next study, phenylalanine and alanine were utilized to assure that the functional community remained the similar to previous results (Fig 5.12). The assay exhibited greater than a 10-fold response on phenylalanine and alanine indicating an identical functional response with phenylalanine and alanine by the lettuce microbial flora.

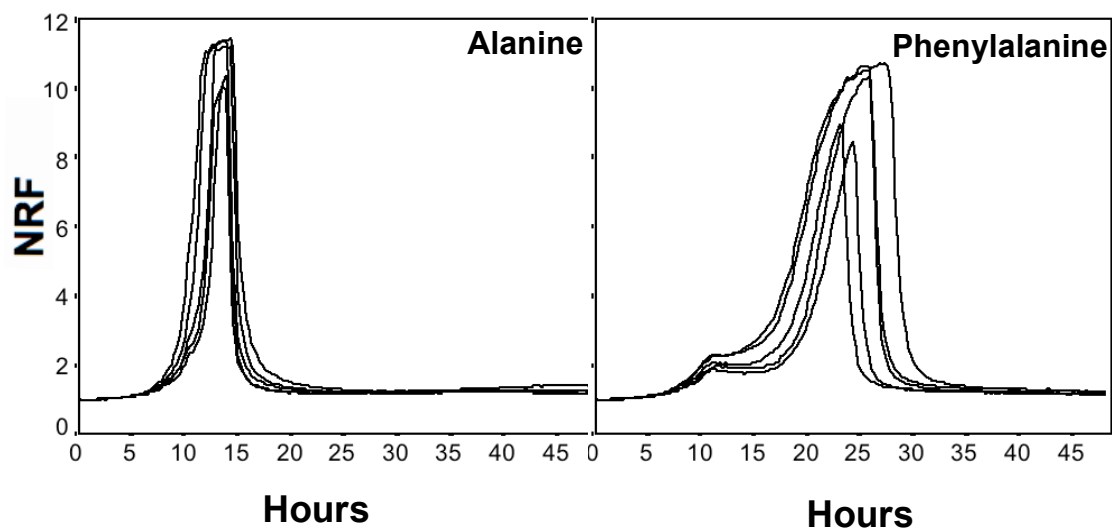


FIG. 5.12. Amino Acid Control Microbial Flora Utilization. Graphs represent the carbon utilization of 300 ppm of phenylalanine and 300 ppm of alanine. The y-axis represents the normalized relative fluorescence (NRF). The data was normalized by the data point at 1 hour. The x-axis is in time in hours and represents the signal at a given time point. The data was normalized by dividing all the data obtained from a single well by the fluorescence value obtained at 1 hour.

The response in viral degradation for all 4 viruses was low, approximately 1.2-1.6 maximum relative fold response. In general, the unwashed cells with virus exhibited a greater response than washed cells with virus (Fig 5.13 & Fig 5.14).

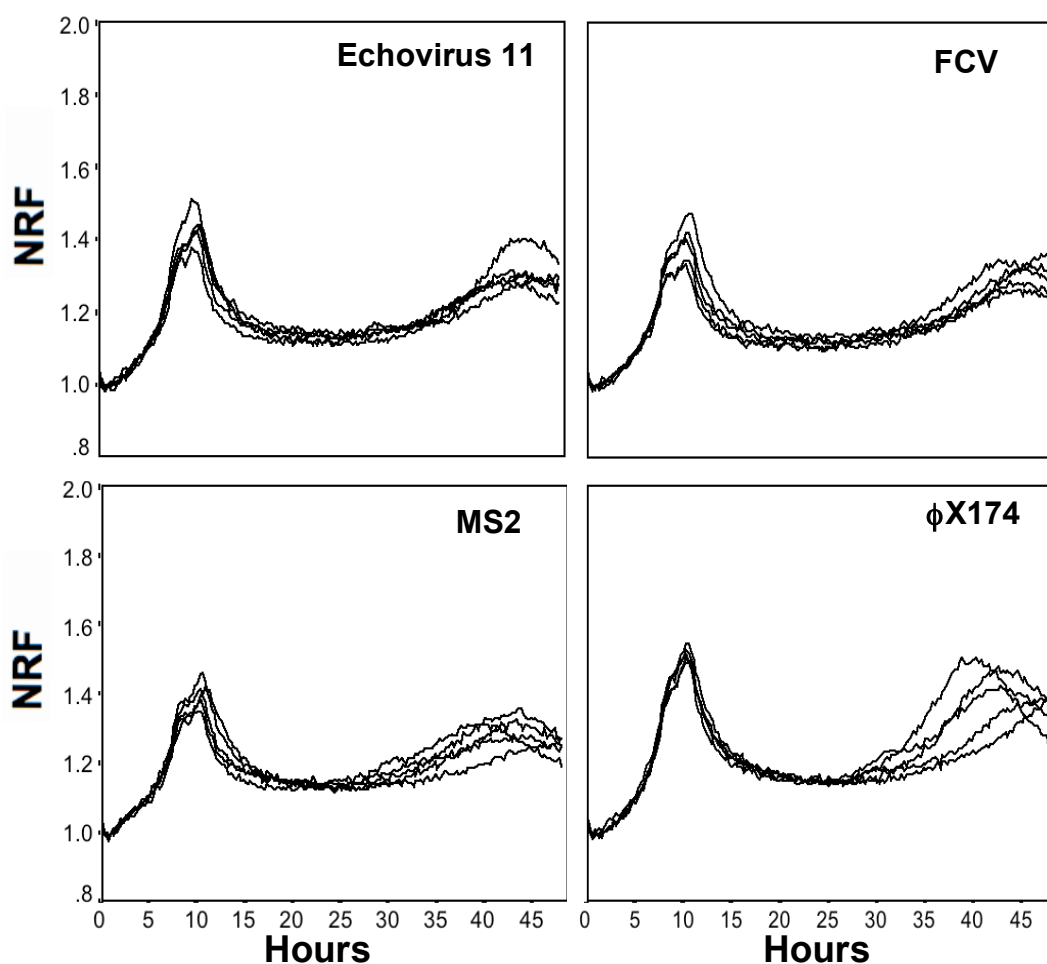


FIG. 5.13. Microbial Flora Virus Utilization. Graphs represent the carbon utilization of the unwashed microbial flora in the presence of each virus listed in the top right corner of each panel. The y-axis represents the normalized relative fluorescence (NRF). The data was normalized by the data point at 1 hour. The x-axis is in time in hours and represents the signal at a given time point. The data was normalized by dividing all the data obtained from a single well by the fluorescence value obtained at 1 hour.

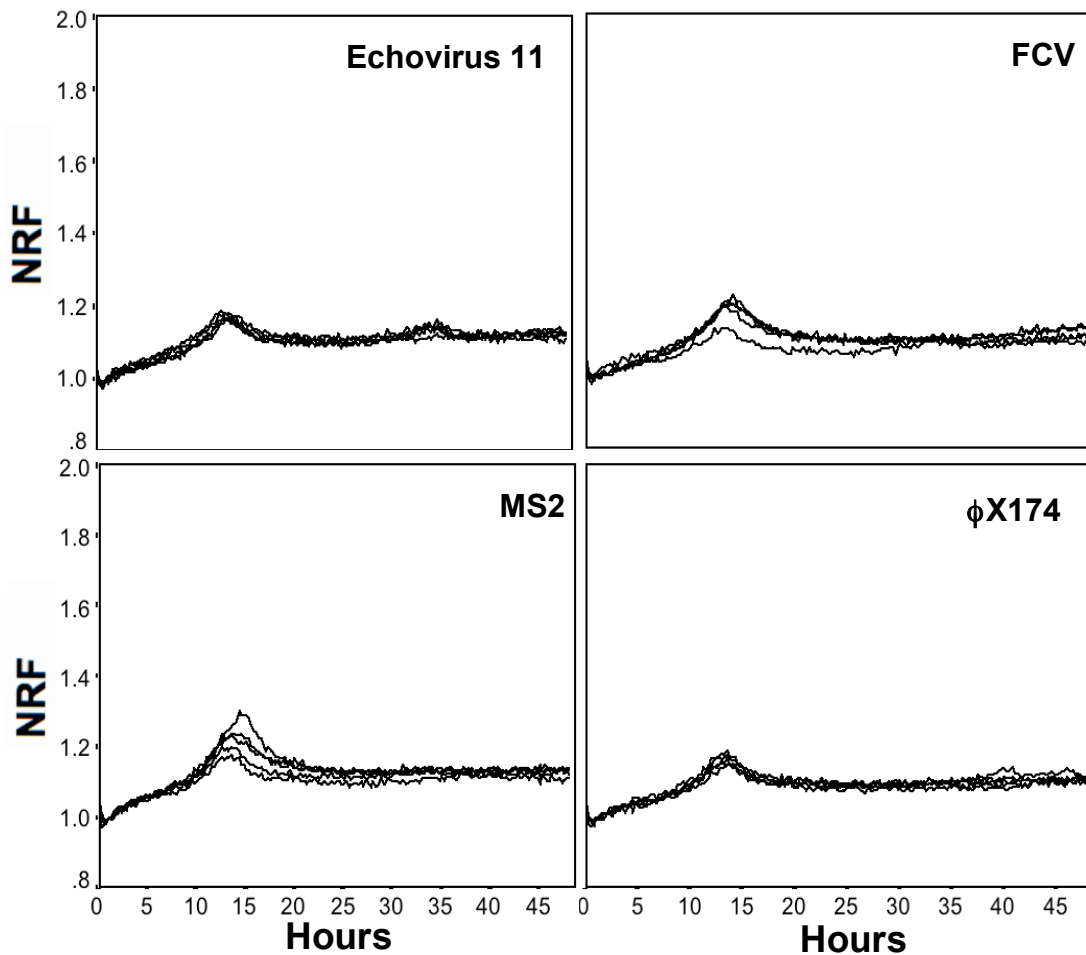


FIG. 5.14. Washed Microbial Flora Virus Utilization. Graphs represent the carbon utilization of the washed microbial flora in the presence of each virus listed in the top right corner of each panel. The y-axis represents the normalized relative fluorescence (NRF). The data was normalized by the data point at 1 hour. The x-axis is in time in hours and represents the signal at a given time point. The data was normalized by dividing all the data obtained from a single well by the fluorescence value obtained at 1 hour.

This was initially interpreted as low-level viral utilization. A comparison of the washed cells and unwashed cells to the virus samples indicated that the low level respiration is for the most part background microbial respiration (Fig 5.15).

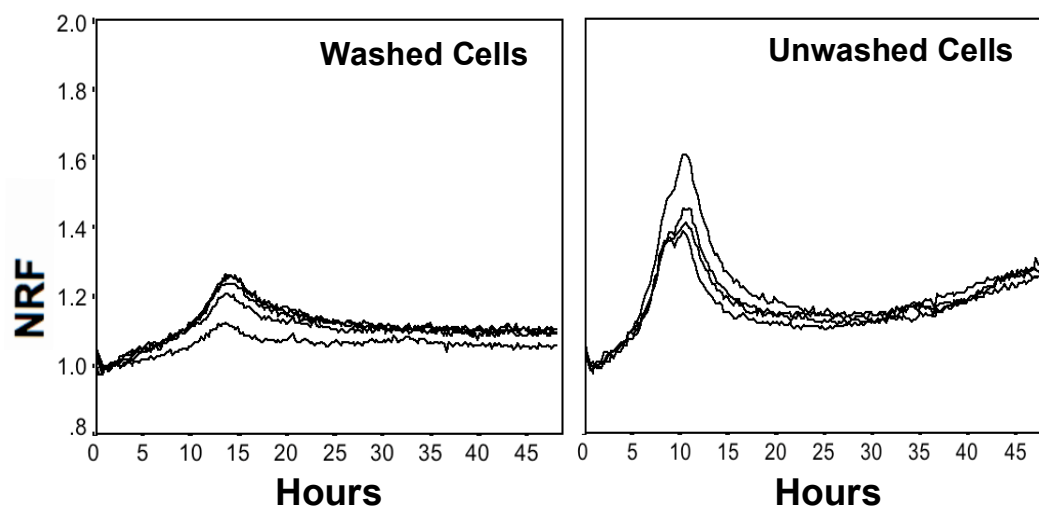


FIG. 5.15. Background Microbial Respiration. Graphs represent the background respiration of the washed and unwashed microbial flora. The y-axis represents the normalized relative fluorescence (NRF). The data was normalized by the data point at 1 hour. The x-axis is in time in hours and represents the signal at a given time point. The data was normalized by dividing all the data obtained from a single well by the fluorescence value obtained at 1 hour.

The initial response or first peak is not significantly different from the background respiration. A secondary response occurs late in the incubation, but only in non-washed samples. Though, a secondary response also occurred in the unwashed cells, it was not as great as the virus and unwashed cells. The difference though is minimal. The bacteriophage ϕ X174 had the greatest secondary response similar to the primary response, 1.4 fold.

Discussion

The results of the microcosm studies was not consistent with the results from the lettuce survival studies. The bacteriophage ϕ X174 had a 60% decrease in survival on the surface of lettuce whereas ϕ X174 survival in the microcosm did not show any decrease in viral numbers for the duration of the experiment. The bacteriophages in the microcosm studies exhibited a greater survival rate than the bacteriophages on the lettuce surface. This could indicate that microcosm studies have different factors that could affect viral persistence. A study by Stine *et al.* did not show a consistent difference in virus survival on the surface of produce in low or high humidity, but desiccation of lettuce at room temperature was much more rapid than at 4° C (146). The significant differences between room temperature and 4° C could be attributable to temperature sensitivity of the viruses.

In a study by Allwood *et al.* virus survival on the surface of lettuce and cabbage was investigated (4). At 4° C. MS2 and FCV exhibited a 90% decrease in 5 and 1.5 days, respectively. In this study, none of the 4 viruses exhibited a 90%

decrease at 4° C during the duration of the experiment. At 25° C, MS2 and FCV exhibited a 90% decrease at 3.5 and 1 day, respectively. Other than FCV, the persistence was greater for the current study than with the study by Allwood *et al.* The bacteriophage ϕ X174 did not decrease to 90% during the duration of the experiment, and MS2 and echovirus 11 did not reach 90% deactivation until day 8 and 9, respectively.

Dawson et al. also assayed the survival of bacteriophage MS2 in a buffer and on a variety of vegetables (37). Bacteriophage MS2 decrease 90% (1 log) in buffer at 4° C until day 50. On the surface of fresh produce a 90% decrease did not occur until after day 20. These results for MS2 bacteriophage are comparable to the results obtained in this study for MS2 bacteriophage. The results for MS2 bacteriophage, though cannot be extrapolated to the other viruses. Other studies by Croci *et al.* determined that HAV, a picornavirus like echovirus, had a 90% decrease after 4 days on lettuce (35). The picornavirus, echovirus 11, more than doubled the survival of HAV on lettuce at 9 days. The study by Kurdziel *et al.* had the most similar survival pattern for echovirus 11, though Kurdziel *et al.* measured poliovirus survival (90). Polioviruses, echoviruses and coxsackie viruses are all enteroviruses and are considered different strains of the same virus species, thus the similarity in survival 90% decrease in 9 days for poliovirus and 90% decrease in echovirus 11 in 8 days at 4° C is not surprising.

There are many factors that could have caused the varying results in all these studies. The primary reason is that none of the previous studies purified virus to the extent of the current study. This could have varying effects on virus survival. Additionally, previous studies have shown that MS2, FCV and echovirus 11 have varying adsorption properties to lettuce (161). Whereas MS2 does not adsorb to lettuce above neutral pH in a phosphate buffer, FCV and to a much greater extent echovirus 11 adsorb to lettuce. This alone would cause a significant bias in survival of MS2 compared to FCV and echovirus. Additionally, the elution buffer used for this study has been shown to elute 100% of the viruses used for this study providing confidence in comparing the survival rates between viruses.

In general, the presence of the microbial flora did not have a negative impact on virus survival, moreover the presence of the microbial flora had a protective effect on virus survival. Though the observed effect could have been masked by filtered exudates greater than 0.22 μm . A study by Konowalchuk *et al.* showed that viruses were able to be concentrated from water using a lettuce floc (86). The lettuce floc coated the viruses and caused the viruses to become large flocs so that a low speed centrifugation (1,000 x g) was able to pellet the viruses from solution. The survival differences between filtered lettuce wash and lettuce wash may be due to a filterable colloid protecting the viruses in the microcosm studies.

The persistence of viruses on the surface of the lettuce was different from the microcosm studies. Whereas in the microcosm studies the bacteriophages had enhanced survival compared to the animal viruses, on the lettuce surfaces the bacteriophages were either equal to or worse than the animal viruses. The difference though was temperature dependent. At room temperature the bacteriophages were superior to the animal viruses, but at 4° C the animal viruses, echovirus 11, did not decrease significantly. These studies show that at refrigeration temperatures, bacteriophages are not conservative surrogates for virus survival. In addition, results from our recent work indicating the varying attachment efficiencies of viruses along with the lack of being a conservative surrogate, strongly suggests that bacteriophages and other viral surrogates are poor virus surrogates; therefore results obtained using surrogates should be interpreted with caution in fresh produce experiments.

The microbial flora was able to utilize amino acids suggesting a predation effect on the survival of viruses on lettuce. On further investigation, the microbial flora was selective on the type of amino acids that were metabolizable. Additionally, there are factors that may explain the lack of utilization of the viruses. Though the viral capsid is a protein coat, the stability and the size of capsids may cause the bacteria to either not sense the presence of the amino acid source or bacteria may not be able to hydrolyze the proteins in an intact capsid. Additionally, the carbon utilization experiments were carried out over a period of two days. The microcosm and surface experiments did not show a significant

viral decrease if any until after two days. The amount of microbial respiration in unwashed samples indicated there was sufficient utilizable carbon sources on the surface of the lettuce. The study by Lindow *et al.* showed that there are sufficient carbon sources on the surface of plants, but the availability may be constrained by accessibility (95).

The survival of viruses on the surface of lettuce was not comparable to the survival of viruses in the microcosm studies. The microcosm studies overestimated the survival of bacteriophages and underestimated the survival of animal viruses. Furthermore, refrigeration temperatures enhanced the survival of the animal viruses compared to the bacteriophages. The carbon utilization studies did not detect predation of viruses though the microbial flora responded to select amino acids over simple sugars. The use of viral surrogates should be carefully interpreted since they are not similar to neither the attachment nor the survival of the human pathogenic virus, echovirus 11.

CHAPTER VI

SUMMARY

Virus adsorption and the subsequent survival of viruses on fresh produce is of concern because of the increasing amount of fresh produce consumed in the United States. Because fresh produce is not cooked to the same extent as meat products, salads and salad crops provide an ideal vector for virus transmission. Viruses are difficult to work with, especially when compared to bacteria, and previous research has attempted to circumvent this problem by using surrogate viruses, especially the bacteriophages. On the surface the justification is reasonable. The bacteriophages and the animal viruses are similar in size and shape. Unfortunately, these similarities are not sufficient. On careful consideration, the bacteriophages and animal viruses infect completely different organisms, have different replication strategies and in the case of animal viruses must be able to withstand or evade the immune response. The differences between bacteriophages and animal viruses are vast. If the infectious route and type of host or host cell is considered, then true similarities can be used to select appropriate surrogate viruses. Similarities must go beyond size and shape of a virion.

Previous research with membrane filters and sediments have elucidated the mechanisms for virus adsorption and desorption within those systems. The results presented indicate that these forces and elution procedures are not transferable to lettuce. Moreover, each virus had an independent adsorption

pattern further casting doubt on the rationale on using surrogates and protocols designed for all viruses, but only optimized with a few viruses, especially the bacteriophages. These results are significant for both public health and industry to develop a method to either remove viruses from fresh produce at home or at the processing level.

These studies were conducted to determine the varying adsorption efficiencies of four different viruses echovirus 11, FCV, MS2 and ϕ X174. These four viruses are important for their pathogenicity to humans and their use as surrogates. Additionally, their survival characteristics were also assayed to identify their adsorption characteristics and their subsequent persistence in the presence of the microbial flora.

The Effect of Critical pH on the Adsorption of Virus to Butterhead

Lettuce

The adsorption efficiency was measured for echovirus 11, FCV, MS2 and ϕ X174. Batch studies were conducted and repeated three times for each experiment and each experiment was repeated at least 5 times. The results indicated that each virus had a different adsorption pattern. The human pathogenic virus was not similar to any other virus. Moreover, the bacteriophages do not adsorb at pH 7 and 8, whereas the animal viruses had the greatest adsorption at this pH range. The enterovirus, echovirus 11, adsorbed the greatest amount of all the viruses tested. Both animal viruses had increasing adsorption with increasing pH indicating a potential electrostatic interaction. The MS2 bacteriophage behaved as per the DLVO theory and was greatly affected by its pI. The bacteriophage ϕ X174 did not have any discernable pattern.

Identification of Forces Critical for Viral Adsorption to Butterhead

Lettuce

The main objective was to identify the forces responsible for non-specific virus adsorption to lettuce. A high concentration of salt, 1M NaCl, detergent 1% Tween[®] 80 and a combination treatment of 1M NaCl and 1% Tween[®] 80 was used to identify the force responsible for viral adsorption. The detergent was the least effective treatment in preventing non-specific virus adsorption to lettuce. The virus, echovirus 11, exhibited an enhanced adsorption capacity in the presence of the detergent, and had either no effect of increased adsorption albeit

at low levels. At pH 7 and 8, 1 M NaCl removed all viral adsorption for all viruses and at pH 3 removed MS2 bacteriophage adsorption to lettuce. The combination treatment was effective, but was equivalent to 1 M NaCl by itself. Adsorption at pH 5 proved to be difficult to remove for all viruses. This may indicate a van der Waals force adsorption occurring with an unidentified surface structure at or near pH 5. The results indicate that non-specific viral adsorption to lettuce occurs primarily through electrostatic interactions.

The Survival of Viruses on Lettuce and the Impact of the Microbial Flora on Viral Persistence

The effect of the normal flora on virus survival was investigated using microcosm, lettuce surface and microbial oxygen utilization studies. The microcosm studies indicated that the bacteriophages were very stable, whereas the animal viruses decreased during the entire length of the experiment. The microbial flora did not have a negative effect on the persistence of virus. Moreover, viruses seemed to have a slower die off in the presence of the microbial flora. The lettuce surface studies exhibited conflicted results from the microcosm studies. The animal viruses survived at equivalent or greater rates than the bacteriophages at refrigerated temperatures. At room temperature, the survival of animal viruses was reduced compared to the bacteriophages. Even though the microbial flora was able to utilize amino acids, the microbial flora was not able to utilize the viruses as carbon sources. Because viruses are small and stable, their ability to be metabolized may be greatly limited, unless a

sufficiently large concentration of viruses is present or the time needed for utilization may be greater than 2 days.

Conclusions

1. Each virus has its own adsorption efficiency.
2. The electrostatic force is mainly responsible for viral adsorption to the surface of lettuce and the addition of 1M NaCl is sufficient to prevent adsorption of viruses at pH 7 and 8.
3. Microcosm studies do not accurately model the survival characteristics of viruses on the surface of lettuce.
4. Echovirus 11 is able to survive on the surface of lettuce for the length of the lettuce shelf life without a large decrease in viral numbers.
5. At refrigeration temperatures, bacteriophages are not conservative surrogates of animal viruses.
6. The microbial flora does not utilize viruses as metabolites.

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APPENDIX A

PROTOCOLS AND PROCEDURES

Cell Culturing and Virus Assay Procedures

Bacteriophages. Bacterial host *E. coli* HS(pFamp)R was grown in Tryptic soy broth (TSB) (VWR West Chester, PA) in 10 ml tubes. Growth medium TSB was supplemented with 0.1 ml of filter sterilized (0.22 μ m filter) ampicilan and streptomycin solution at a concentration of 0.15 g/100 ml ampicilin and 0.15 g/100 ml of streptomycin sulfate. The bacterial host *E. coli* CN13 was grown in TSB supplemented with 0.1 ml of 1 g/100 ml of nalidixic acid filter sterilized (0.22 μ m filter). Both *E. coli* hosts were incubated at 37° C in a shaking water bath incubator for 12-16 hours.

The double agar overlay assay consisted of TSA plates supplemented with nalidixic acid (1 g/100 ml) or streptomycin sulfate and ampicilin (0.15 g/100 ml) for *E. coli* host CN13 and *E. coli* HS(pFamp)R, repectively. The soft agar overlay consisted of TSB with the addition of 0.7% bacteriological agar. Briefly, the soft agar was brought to a boil then allowed to cool, then 5 ml was aliquoted into test tubes. The soft agar was then autoclaved and stored until needed. Prior to use, soft agar was melted by autoclaving for 5 minutes, then the soft agar was placed in a water bath set at 50° C for at least 30 minutes to allow the soft agar to equilibrate with the water bath.

The top agar or soft agar was maintained at 50° C. The top agar was removed from the water bath and rapidly supplemented with 0.2 ml of the

appropriate bacterial culture (host *E. coli* F-amp for MS2 or *E. coli* CN13 for ϕ X174) followed by 0.1 ml of sample. The top agar was briefly vortexed and its entire contents poured over the appropriate TSA plate supplemented with antibiotics. The top agar was allowed to solidify for 15 – 60 minutes then the plates are inverted and incubated at 37° C for 12 – 16 hours.

Animal Viruses. The host used for feline calicivirus F9 was Crandell Reese feline kidney cells or CRFK. The CRFK cells were grown in Eagles minimal essential media (MEM) (Sigma # MO643) supplemented with 5.5 ml of 100X antibiotic /antimycotic solution (Sigma # A5955), 8% fetal bovine serum (FBS) and 8.1 ml of 1M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (15 mM final concentration) (HEPES) (Hyclone # SH30237.01). A substitution of 8% FBS with 10% newborn calf serum (NBCS) (Sigma # N4637) does not change the growth characteristics of the CRFK cells.

The host for echovirus 11 was buffalo green monkey cells (BGMK). The BGMK cells were grown in Eagles MEM supplemented with 5.5 ml of 100X antibiotic/antimycotic solution, 10% FBS and 12.5 ml of HEPES (25 mM final concentration). A substitution of 10% NBCS did not cause any discernable growth or morphological changes to the BGMK cells.

Maintenance of Animal Cells. Both the CRFK and BGMK cells were maintained using identical protocols. After 80% confluency was achieved, approximately 4-5 days at 37° C with 5% CO₂, the cells were washed 3X with Hanks buffered salt solution (H2387) (HBSS). The volume used for growth and

washings were identical but varied on the size of the growth flask. A T25, T75 and T150 needed 5, 15 and 30 ml, respectively. After washing the cells 3X with HBSS, the cells were removed from the flask by enzyme digestion.

After removing the wash solution 1, 3 and 5 ml of 0.25% HyQ[®] Trypsin with EDTA (Hyclone SH30042.01) was aliquoted onto the cellular monolayer of the T25, T75 and T150 flasks, respectively. After the cells become rounded, the flasks were tapped firmly on each side of the flasks to dislodge the cells.

Afterwards, a total volume of 10, 30 and 40 ml for the T25, T75 and T150 was used to deactivate the trypsin reaction, respectively. The sloughed cells were collected and centrifuged at 435 x g for 5 minutes at 4° C. After centrifugation the supernatant was removed and replaced with 10, 30 or 60 ml of fresh growth media for the T25, T75 or T150, respectively. A total volume of 1, 3 or 6 ml was used for a T25, T75 or T150, respectively. Fresh media was added to bring the volume up as previously described.

Agar Overlay Assay. The agar overlay assay was used to determine the plaque forming units (PFU) of solutions. Animal cells, BGMK or CRFK, were transferred onto cell culture treated 6 well plates. An 80% confluent monolayer was passaged as previously described but resuspended into 40 ml for a T75 or 80 ml for a T150 flask after pelleting the cells to remove trypsin. After resuspending the cells in fresh media, 2 ml of cells were aliquoted onto each well. Thereafter, the 6-well plates were incubated for 2 days for CRFK cells and 3 days for BGMK cells.

After incubation, the monolayer was washed 3X with 2 ml of HBSS. Each time the media and HBSS was removed from the monolayer by tipping the 6 well plates over a large graduated cylinder (aspiration of HBSS may damage the cells). The cells were infected by carefully aspirating 0.2 ml of solution onto the cellular monolayer. The infected plates were gently rocked back and forth and incubated at 37° C with 5% CO₂ for 1 hour. Every 15 minutes the plates were rocked back and forth to spread the virus inoculum over the wells. At 30 minutes into the incubation the agar overlay (1.6% v/v low temperature melting, low electroendosmosis agarose) was melted in a microwave and placed in a 50°C water bath until further use. After the 1 hour incubation the agarose was mixed 1:1 with 2X Dulbecco's modified MEM (DMEM) (Gibco, cat no 12800-017) supplemented with 1X antibiotic/antimycotic solution, 25 mM HEPES, 2% FBS or NBCS. After thoroughly mixing the agarose and DMEM the agar overlay was gently aspirated onto the infected monolayer. The agarose plug was allowed to set for 10-15 minutes. After the incubation the plates were placed into the incubator for 48 hours.

After the 48 hour incubation the 6 well plates were removed from the incubator and a solution of 3.7% formaldehyde was pipetted into the wells and was allowed to stand for at least 4 hours at room temperature. After fixing the cells with formaldehyde, the plugs were removed by inverting the plates and firmly hitting the plates to eject the agarose plugs. After the plugs were removed the plaques are visualized by pipetting enough 1.5% crystal violet to cover the

bottom of the well. After at least 2 minutes the wells were washed using deionized water. After washing the wells the plaques were counted.

Harvesting and Purification of Viruses

Bacteriophages. A bacteriophage high titer raw lysate was obtained by infecting approximately 20 plates using the double agar layer assay as previously described. A virus solution was diluted to obtain >300 pfu/plate. After the plates were incubated 0.1% peptone solution or 0.1 M NaPO_4 pH 7.0 buffer was aspirated onto the plate to cover the entire surface (≈ 10 ml). The plates were allowed to stand at room temperature for a minimum of 2 hours. After incubation the solution was aspirated from the plate. The raw lysate was then centrifuged at $3,313 \times g$ for 30 minutes at 4°C . No further purification was done for lysates used in the viral adsorption studies. Lysate was collected and stored at 4°C until needed. For survival and viral carbon source utilization studies the bacteriophages were further purified to remove media and other potential carbon sources.

Secondary purification involved polyethylene glycol (PEG) purification followed by a CsCl isopyknic purification. The total volume of the lysate was measured and solid NaCl was added to obtain a 1M NaCl solution. After the NaCl was dissolved the solution was incubated for 1 hour on ice. After the 1 hour incubation the lysate was centrifuged at $3,313 \times g$ for 30 minutes at 4°C . After centrifugation solid PEG 3350 was added to final concentration of 10% w/v. The PEG was dissolved on a magnetic stirrer at low speeds. After the PEG was

completely dissolved the magnetic stir bar was removed and the lysate was incubated overnight at 4°C. After the overnight incubation the lysate was centrifuged at 11,000 x g for 10 minutes at 4°C. After centrifugation the supernatant was discarded and the pelleted precipitate was resuspended in 1/10 – 1/100 of the original volume in Boric acid buffer adjusted to pH 7.4 [0.2 M boric acid and 0.5 M NaCl]. After resuspension of the precipitate the lysate was mixed with 1.5 g/ml of CsCl to obtain a final concentration of 1.31 g/ml CsCl solution. Polycarbonate tubes were chemically sterilized for 30 minutes by immersing the tubes in a 0.1% chlorine solution adjusted to pH 6-7 (156). Residual chlorine was removed by draining the chlorine solution then immersing the tubes in autoclaved water amended with 2.5 ml of a 2% sodium thiosulfate solution per liter of sterile water. The PEG precipitated virus was centrifuged at 193,911 x g for 20 hours at 4°C. Fractions were collected and the absorbance at 260 and the absorbance ratios of 260/280 were recorded.

The CsCl was removed from the viruses by dialysis using the Slide-A-Lyzer® dialysis cassette (10,000 mw) (Pierce Rockford, IL). The virus fraction was injected into the dialysis cassette and dialyzed with 1 L of 0.1 M NaPO₄ (pH 7.0) buffer at room temperature for 2 hours. After 2 hours spent buffer was discarded and replaced with 1 L of NaPO₄ buffer. Dialysis was allowed to continue for another 2 hours at room temperature. After the second incubation spent buffer was discarded and 1 L of 0.01 M NaPO₄ (pH 7.0) was added to the dialyzing beaker. The viruses were allowed to further dialyze overnight at 4° C.

Viruses were then aliquoted into 0.2 ml portions and stored at -80°C until further needed.

Animal Viruses. The general methodology for FCV and echovirus 11 was similar to the bacteriophage protocol. Approximately 5 to 8 T150 flasks were seeded with BGMK or CRFK cells. The cells were allowed to grow up to 80-100% confluency. The cells were washed 3X with HBSS before infecting the cells. A multiplicity of infection (m.o.i.) of 1-10 was used to infect the cells. The infected cells were incubated 24 hours or until 80-100% of the cells exhibited cytopathic effect (CPE). The FCV infected cells were freeze-thawed no more than two times, whereas the echovirus 11 infected BGMK cells were freeze-thawed three times to release all virions. Thereafter the cells were centrifuged at 3,313 x g for 30 minutes at 4°C. The animal virus lysate was collected, titered and diluted to 10⁵ pfu/ml. The diluted lysate was aliquoted into 5 ml portions and stored at -20°C until needed.

The viruses were purified using the same methodology as previously described for the virus survival and carbon utilization studies.

Fluorescence Microscopy Protocol

A 25 cm² piece of lettuce was cut and placed in a 20-oz Whirl-Pak bag (Fort Atkinson, WI). A total volume of 10 ml of 0.1% peptone solution was aspirated into the bag with the lettuce. The bag was heat-sealed and was either rubbed in a circular motion for 1 minute on each side (handwash) or placed in a sonicator for a total of 2 minutes, 1 minute on each side. A total volume of 1 ml

of rinsate was aspirated from the bag. The rinsate was aspirated into a microcentrifuge tube and centrifuged at 10,000 RPM for 5 minutes. The supernatant was discarded and the pellet was resuspended in 0.1% peptone. The obtained bacteria were stained according to the Live/Dead® BACLight™ manufacturers instructions. After staining of the cells the solution was syringe filtered through a cellulose plain black 13 mm², 0.22 µm filter (Osmonics Inc./GE Water Technologies, Fairfield, CT) placed on a 13 mm polyester mounting membrane (Poretics Corp., Livermore, CA). After filtration the membrane is placed on a glass slide for viewing (see figure A.1).

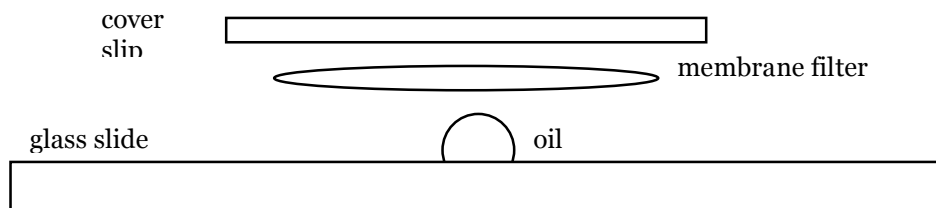


FIG. A.1. Fluorescence Microscopy Slide Assembly. The graphic depicts the order of the mounting oil and membrane on the slide for correct viewing of the bacterial cells using 100x power. After assembly of the glass slide, the cover slip is sealed along the edges with clear nail polish. The oil used for mounting the membrane is supplied with the Live/Dead® BACLight™ kit. A total 7 µl of oil was used for mounting the membrane.

PCR and Denaturing Gradient Gel Electrophoresis

PCR. The 16s rDNA gene was amplified from lettuce wash samples for use in the denaturing gradient gel (DGGE). The DGGE methodology was used to identify the diversity of the lettuce epiphytic community. Because all bacteria contain the 16s rDNA, the gene was targeted for amplification. The PCR product size for DGGE was limited by the resolution limit of the gel assay (500 bp limit). Primer pairs were chosen by their ability to anneal to the greatest amount of bacteria while discriminating against microbial members of the *Archea* and *Eucarya*. The full 16s rDNA gene is approximately 1.5 kbp long and is comprised of 16s rDNA variable regions 1-8. Variable regions 6-8 were chosen for DGGE analysis based on its specificity to the *Eubacteria* (137). The forward primer sequence named F968 is 5'-(GC clamp)-AAC GCG AAG AAC CTT AC-3'. The reverse primer sequence named R1492 is 5'-TAC GGY TAC CTT GTT ACG ACT T-3'. The GC-clamp was added to the 5' end of the forward primer to prevent rapid denaturation of the amplicon. The GC-clamp sequence used was 5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3'. The predicted product for the primer pair F968 and R1492 was 585 bp.

A reaction volume of 100 μ l was needed due to the large sample volume for DGGE analysis, 25-30 μ l of product with an equal volume of loading dye. The reaction conditions for the 16s rDNA PCR are: 1X PCR Buffer II, 100 mM of dNTP mix (25 mM each dNTP), 80 nM of primer F968, 80 nM of primer R1492, 0.025 U/ μ l of polymerase and 5% v/v Dimethyl Sulfoxide (DMSO). The

chemical compound DMSO was needed to prevent hairpin formation of the GC-clamp and provide enhanced amplification yields. In addition a hot start was incorporated into the reaction to improve amplification efficiency. The addition of wax to the reaction prevented volume loss due to evaporation and cross contamination when opening tubes during the hot start.

The touchdown-PCR (TD-PCR) methodology was used to amplify the sequence to minimize non-specific amplification. The TD-PCR condition used was:

1. Step 1: Hot Start
 - a. Initial denaturation: 5 minutes at 94°C
 - b. Hold: 80°C
2. Step 2: TD-PCR cycle (10 cycles with a decrease of 1°C every cycle at the annealing step)
 - a. Denaturation: 30 seconds at 94°C
 - b. Annealing: 1 minute at 60°C (1st cycle)
 - c. Elongation: 2 minutes at 72°C
3. Step 3: Amplification cycle (20 cycles)
 - a. Denaturation: 30 seconds at 94°C
 - b. Annealing: 1 minute at 50°C
 - c. Elongation: 2 minutes at 72°C

4. Step 4: Final Elongation Step

- a. 5 minutes at 72°C

5. Step 5: Cool down

- a. Hold at 4°C

After TD-PCR the samples were visualized on a 1% agarose gel. The agarose gel was run at 80-100 volts in 1X TBE buffer. Representative gels are in figure A.2.

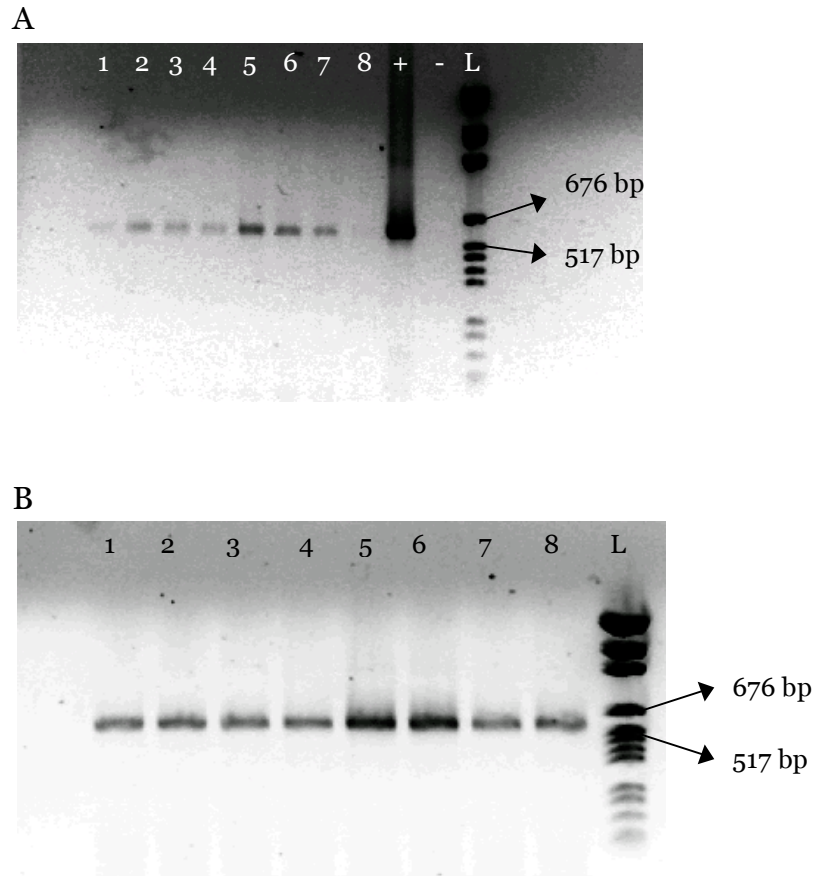


FIG. A.2. Representative Gel of 16s rDNA PCR Product. Figure A and B are representative agarose gels showing the amplicon product for the primer pairs F968 and R1492. Figure A shows the product from community DNA from Bibb lettuce obtained from a retail chain in Florida. Figure B represents the amplicon products from community DNA from Bibb lettuce obtained from Kennedy Space Center, FL. The ladder is a DNA D-15 ladder. Figure A: Lanes numbered 1-8 are the amplification products of community DNA from different leaves on the lettuce. The positive control is a pure culture environmental isolate. Figure B: Lanes numbered 1-8 are the amplification products from different lettuce leaves. Positive and negative controls for figure B are not shown.

DGGE. The DGGE apparatus was assembled based on the Bio-Rad manufacturers instructions for the gradient delivery system model # 475. To determine the optimal denaturation range, a perpendicular gel was cast according to the manufacturers instructions. A 6% denaturant, 1 mm, 16 x 10 cm² polyacrylamide gel was made with a denaturant range of 0-100%. The results are shown in figure A.3.

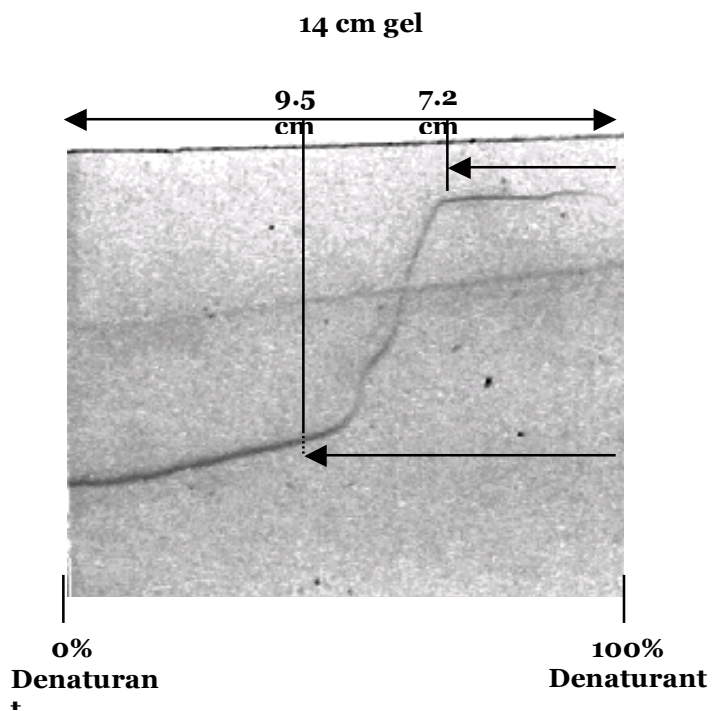


FIG. A.3. DGGE Perpendicular Gel. A perpendicular gel with a 16s rDNA product gel was run to determine the optimal denaturation range to use. To obtain the optimal denaturant, the length from the end of the plateau indicating completely denatured product would be the maximum denaturant used in future gels. The end of the decline represents products not yet denatured indicating the minimum denaturant concentration. Assuming a constant denaturant gradient, 7.2 cm and 9.5 cm is equivalent to a denaturation concentration range of 33% - 50% is optimal before further optimization with actual sample. Gel measurements not drawn to scale.

After further optimization the denaturation gradient was established at 45% -70%. Glass plates were treated with PlusOne™ Repel-Silane ES (GE Helathcare, Fairfield, CT) to prevent the polyacrylamide gel from sticking to the glass plates. A representative DGGE gel is shown in figure A.4

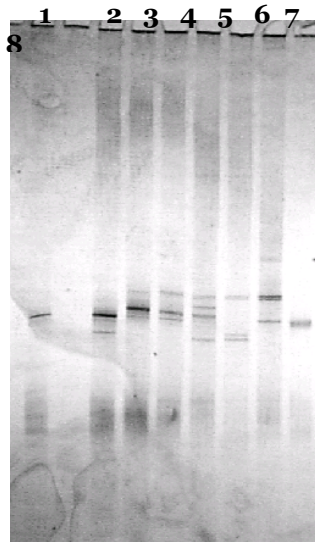


FIG. A.4. DGGE Parallel Gel. A representative DGGE polyacrylamide gel. Gel visualized by silver staining. Lanes 1-7 are a community analysis of amplicon products using degenerate primers from individual lettuce leaf washes.

Silver Staining. The DGGE gels were visualized by silver staining. Silver staining was chosen because gels can be fixed on a solid substrate and stored for later reference. Additionally, bands can be excised for cloning or sequencing without damaging the DNA. The protocol is based on the Promega silver staining protocol (20). The protocol was designed for 1 mm thick gels. The thickness of the gel will either decrease or increase incubation times. A shallow tray was used as the incubation vessel. The trays were scrubbed before the silver staining to decrease background staining. In addition a fiberglass screen (window screen) was used as a support for the gel to ease manipulations and prevent the gel from being aspirated between steps. The optimized silver staining protocol used for 1 mm DGGE gels:

1. Step 1: Fixation
 - a. The fixer solution (7.5% Acetic acid, Glacial acetic) was poured onto the gel
 - b. Gel was incubated for 20 minutes
 - c. The fixer solution was removed
2. Step 2: Wash (3x)
 - a. Deionized water (12-18 M Ω) was poured into the vessel
 - b. The gel was incubated for 3 minutes
 - c. The water was removed

3. Step 4: Silver Impregnation
 - a. The silver solution was poured onto the gel (1.5 g/L AgNO₃ and 0.056% formaldehyde was added 15 minutes before use)
 - b. Incubation for 30-40 minutes
 - c. Silver solution was removed (gel was minimally handled to decrease background)
4. Step 4: Rinse
 - a. Gel was rinsed with deionized water
 - b. Total rinse time was 10 seconds (an increase in time will decrease the sensitivity of the stain)
 - c. Water was removed (minimal handling of gel)
5. Step 5: Image Development and Quenching of Reaction
 - a. Addition of developer solution (30g/L Na₂CO₃, 0.056% formaldehyde, 400 µg/L sodium thiosulfate)
 - i. Sodium thiosulfate was made up in bulk, aliquoted and stored at -20°C. Portions were thawed just prior to use
 - ii. Formaldehyde and sodium thiosulfate were added to developer solution 5-15 minutes prior to use
 - b. Developer was used at 8-12°C (removal the developer from a 4°C refrigerator 15 minutes before use will increase the temperature of the developer to 8-12°C)

- c. The gel was carefully watched until the desired staining had occurred
 - d. Cold fixer solution was poured (4°C) onto the gel to stop reaction
 - e. The lack of new bubbles indicates the reaction has been stopped
6. Storing of gel
- a. Gel was sandwiched between an two overhead sheets (thick plastic film) and sealed along the edges with tape and stored at room temperature

A representative silver stained gel is in figure A.4

APPENDIX B

SUPPORTING EXPERIMENTS

Characterization and Identification of the Lettuce Microbial Flora

Various assays and methodology were used to characterize the lettuce normal flora. Initial studies included iceberg, butterhead and romaine lettuce. Spread plate assays, bacterial counts using fluorescence microscopy, fatty acid analysis and denaturing gradient gel electrophoresis were used to define the epiphytic community on lettuce. Different parameters like outer leaves, inner leaves, temporal and geographic changes were considered as possible factors affecting the survival and attachment of viruses on lettuce.

Elution of the Microbial Flora. The effect of lettuce material on downstream analysis and the addition of carbon sources from the lettuce was investigated. The main objective was to remove the microbial community for analysis and maintain the integrity of the lettuce leaf to minimize any soluble compounds in the rinsate. A 25 cm² piece of lettuce was used for all elution experiments. Conditions considered included massaging by hand for 2 minutes, sonication for various time periods, the addition of Tween[®] 80 and different types of media. Figure B.1 are the results of the experiment.

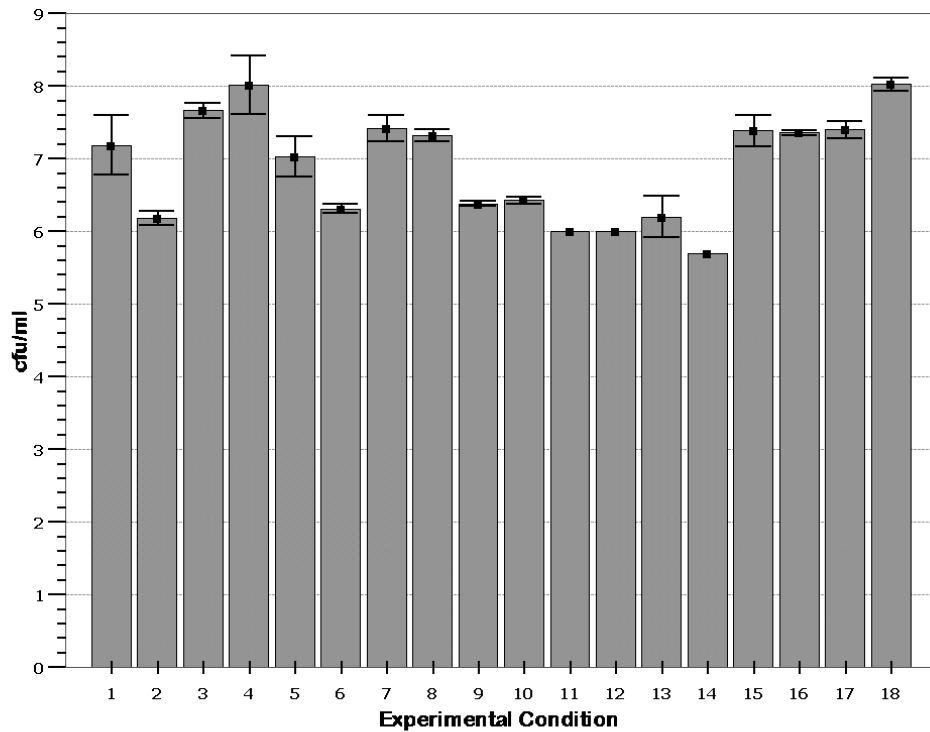


FIG. B.1. Comparison of Microbial Elution and Growth Media. Comparison of Handwashing, Sonication time, Tween[®] 80 and Growth Media. All conditions are sonication unless otherwise noted. The error bars represent ± 1 SE. The conditions tested are as follows: 1. TSA-Handwash 2. R2A-Handwash 3. TSA-2 min. 4. R2A-2 min. 5. TSA-Tween-2 min. 6. R2A-Tween-2 min. 7. TSA-4 min. 8. R2A-4min. 9. TSA-Tween-4 min. 10. R2A-Tween-4 min. 11. TSA-7 min. 12. R2A-7 min. 13. TSA-Tween-7 min. 14. R2A-Tween-7 min. 15. TSA-9 min. 16. R2A-9 min. 17. TSA-Tween-9 min. 18. R2A-Tween-9 min.

The results from figure B.1 indicated that sonicating for two minutes yielded superior or equivalent microbial counts than all the other time points assayed. The addition of 0.1% Tween[®] 80 did not improve microbial counts. Additionally, the growth media did not have an effect on microbial counts.

To verify that handwashing and sonication were equivalent, an additional experiment comprising of the 2-minute hand wash and the 2 minute sonication was repeated. The results of the experiment are in figure B.2. The results indicated that hand washing and sonication were equivalent and growth media did not have an effect on total culturable microbial cell yields.

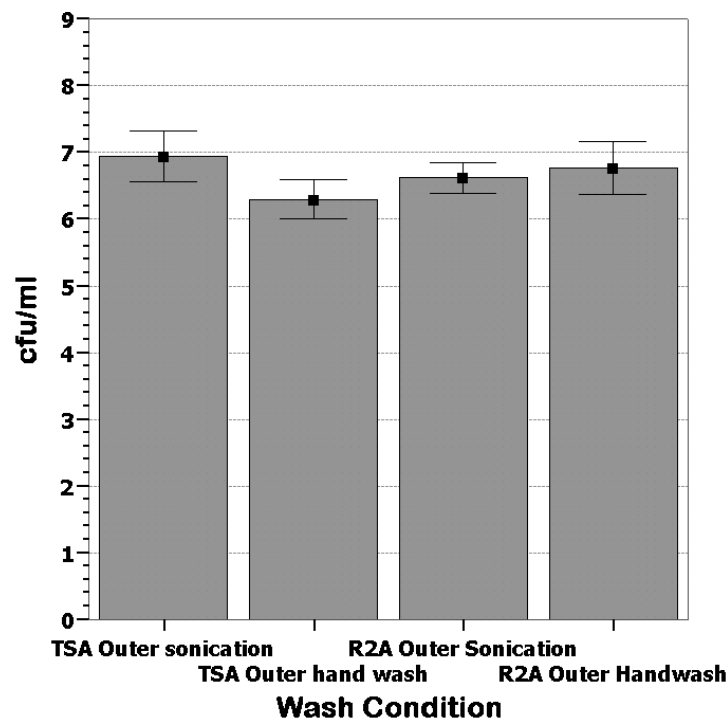


FIG. B.2. Comparison of Microbial Elution Second Experiment. The bar graph represents the obtained cell counts after the lettuce leaf is washed with either sonication or by hand. The error bars represent ± 1 SE. The first word represents the type of media used, the second word represents the location of the lettuce leaf and the third word is the wash method.

In addition to obtained total microbial cultural counts, sonication and hand washing were compared using the Live/Dead® BACLight™ bacterial viability assay. Total bacterial counts were obtained by staining the lettuce wash, filtering the rinsate and counting the number of bacteria under a fluorescence microscope. Live bacteria appeared green or yellow. Dead bacteria appeared red or orange. After total counts were recorded the total counts per sample was calculated using the equation:

$$T = N[(A/a)/V]$$

T = total number of bacteria
 N = Average number of bacteria per field viewed
 A = Surface area of filtration
 a = Area of microscope field
 V = Volume of sample

The constants used in the equation included the surface area of filtration, surface area of filter, 13 mm², and the area of the microscope field at 100x, 12.6 μm². The results of the total bacterial counts using fluorescence microscopy are listed in table A.1. The results from the total counts verified that sonication and hand washing were similar. Though either method could be used with confidence, sonication was used for all subsequent experiments to reduce human error.

TABLE B.1. Total Microbial Counts Using Fluorescence Microscopy. The log bacterial counts are listed in the table A.1. The counts have been adjusted to represent the whole 25 cm² of lettuce.

	Live Cells	Dead Cells	Total Cells	Ratio (live/dead)
Sonication				
Leaf 1	6.92	6.80	7.15	1.15
Leaf 2	6.64	6.59	6.92	1.11
Leaf 3	7.36	7.40	7.68	.94
Mean±SE	6.97±0.21	6.93±0.24	7.25±0.23	1.07±0.06
Handwash				
Leaf 1	7.15	7.08	7.41	1.16
Leaf 2	-	-	-	-
Leaf 3	6.40	5.89	6.52	1.09
Mean±SE	6.77±0.38	6.49±0.60	6.97±0.45	1.13±0.04

TABLE B.2. Total Microbial Culturable Counts on Iceberg Lettuce.

Sample Number	cfu/25 cm²
1	6.31
2	10.11
3	10.66
4	5.2
5	6.6
6	5.4
7	8.2
8	3.9
9	8.77
10	5.9
11	5.25
12	6.59

A survey of 25 cm² lettuce pieces was assayed using the previously described methodology. Results indicate that there was a large variation in total microbial counts on lettuce leaves. Total counts ranged from 3.9 cfu/25 cm² to 10.66 cfu/25 cm².

Conclusions from the total count survey was that the total amount of microorganisms present on the surface of a leaf could not be predicted based on previously obtained microbial count data. Each lettuce portion used had the potential of being unique in total cell counts and in the composition of the community. To investigate the latter conclusion more thoroughly a culture and culture independent methods were used to identify the microbial normal flora.

DGGE Analysis. The epiphytic community was assayed based on the 16s rDNA v6-v8 region to obtain a more complete survey of the lettuce microbial community. Lettuce from different geographical regions, seasons, growing conditions and varieties was assayed. Analysis of DGGE gels was analyzed using the Bionumerics software version 4.5 (Applied Maths, Austin, TX). Cluster analysis of the bacterial community indicated that there were three major clusters based on seasonal changes and geographic location, but spatial parameters were the most important (Figure B.3). The summer and winter clusters were more closely related than the fall clusters even though the summer and fall samples were obtained within a closer time period. Varieties did seem to have a greater similarity but it secondary to location.

The DGGE data indicated large variations were present within samples obtained from the same location, time and same plant (Field-Iceberg and Romaine samples). Additionally, the largest variation was also found in plants obtained from the field before processing. The results from the 16s rDNA analysis revealed that the epiphytic community was diverse and contained a large diversity even within the same plant.

FAME. Spread plates were saved and isolated microbial colonies were chosen for further analysis. Colonies were selected based on colony morphology and on the number of representative colonies. Isolated microbial colonies were grown in TSB broth at room temperature. After culture tubes were turbid or had noticeable growth, cultures were streaked on a TSA plate to verify that a pure culture was present. If a broth exhibiting growth consisted of a pure culture, the microorganisms were grown in a TSA slant. The slants were analyzed by fatty acid methyl ester analysis (FAME) at the Texas Plant Disease Diagnostic Laboratory (College Station, TX). Results and morphology of the microorganisms are in table A.2.

TABLE B.3. Identification of Microorganisms by FAME. Observed morphologies of microbial colonies and their subsequent identification using fatty acid methyl ester analysis. The score represents the similarity index.

Colony Morphology/Color	Identified Microorganism	Score
Round convex/yellow-white	<i>Micrococcus lutus</i> GC subgroup C	0.67
Flat slightly irregular/white	<i>Bacillus cereus</i>	0.85
Round convex/yellow-white	<i>Micrococcus kristinae</i>	0.87
Round mountainous/white	<i>Bacillus pumilus</i> GC subgroup B	0.76
Round concentric circles/white	<i>Pseudomonas putida</i>	0.63
Irregular flat mucous/yellow	<i>Pseudomonas sp.</i>	0.24
Irregular flat mucous/white	<i>Staphylococcus sp.</i>	0.56
Round flat/orange	<i>Paenibacillus sp.</i>	0.42
Round flat/beige	<i>Paenibacillus sp.</i>	0.10
Round flat/yellow-white	<i>Bacillus megaterium</i> GC subgroup A	0.91
Round concave/clear	<i>Pseudomonas fluorescence</i> biotype C	0.72
Round concave/clear	<i>Pseudomonas fluorescence</i>	0.64
Round convex mucous/red-brown	<i>Pseudomonas sp.</i>	0.23
Round mucous/white	<i>Pseudomonas sp.</i>	0.35
Round flat/yellow	<i>Pantoea agglomerans</i>	0.61
Round small/pink	Unknown	-
Round irregular/white	<i>Bacillus pumilus</i> GC subgroup B	0.75
Round irregular/white	<i>Bacillus pumilus</i> GC subgroup B	0.76

The fatty acid analysis had many limitations. The most important was a complete database with known fatty acid profiles. The microorganisms most commonly found in the database were known plant pathogens. The majority of the normal flora on the surface of a plant are non-pathogenic (89). This bias is evident with the higher scores from bacteria, which are known to be plant pathogens, like *Pseudomonas* spp., or very common bacteria like the *Bacillus* spp. Nevertheless, important information was obtained from the FAME profiles. The primary conclusion from the FAME analysis was that colony morphology was irrelevant in determining microbial diversity and that *Pseudomonas* spp. and closely related bacteria are common epiphytic inhabitants.

ESEM. The identification of the density and diversity of the microbial community was important to obtain an overview of the microbial community in the phyllosphere, but it was also necessary to determine if the microbial community was evenly distributed on the surface of the leaf or if there are isolated colonies. A diagram of a cross section of a leaf is shown in figure B.4.

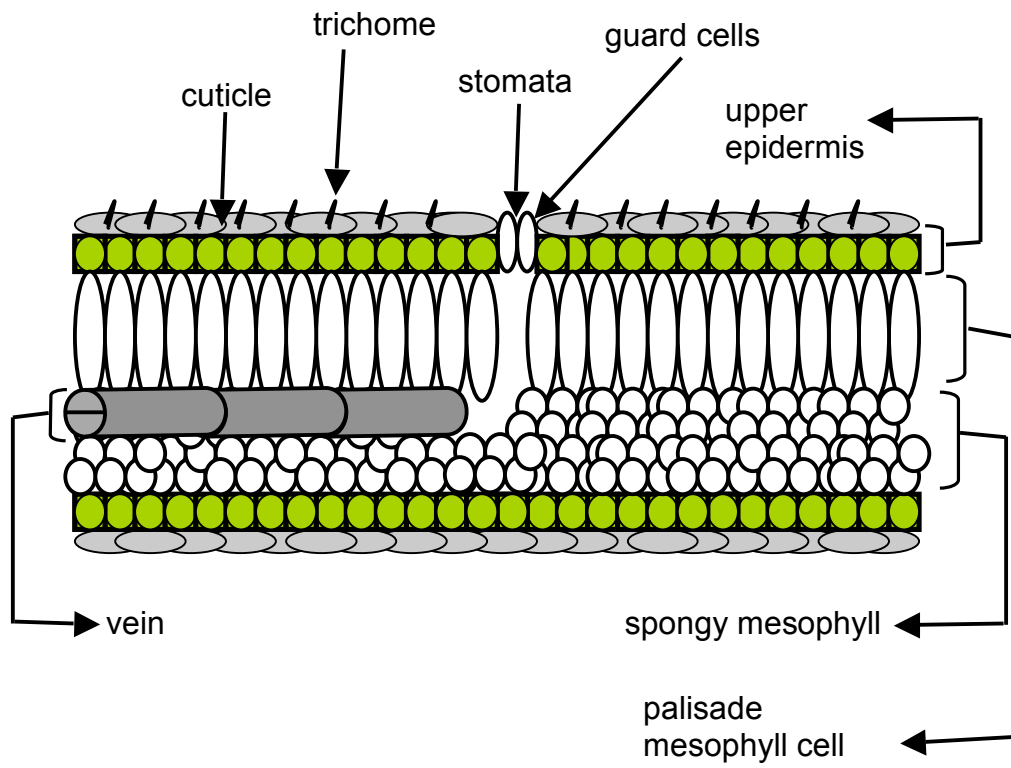


FIG. B.4. Generic Cross Section of Leaf. Diagram representing a cross section of a generic leaf.

To determine the spatial characteristics of the microbial flora, the surface of the lettuce leaves were scanned with in an electron scanning environmental microscope (ESEM).The lettuce leaves were cut and then viewed at the electron microscopy center at Texas A&M University (College Station, TX) (Fig A.9). The normal flora was sporadic on the surface of the lettuce leaf. The majority of the surfaces were uninhabited. Microorganisms tended to cluster together and were usually present near or around the stomata.

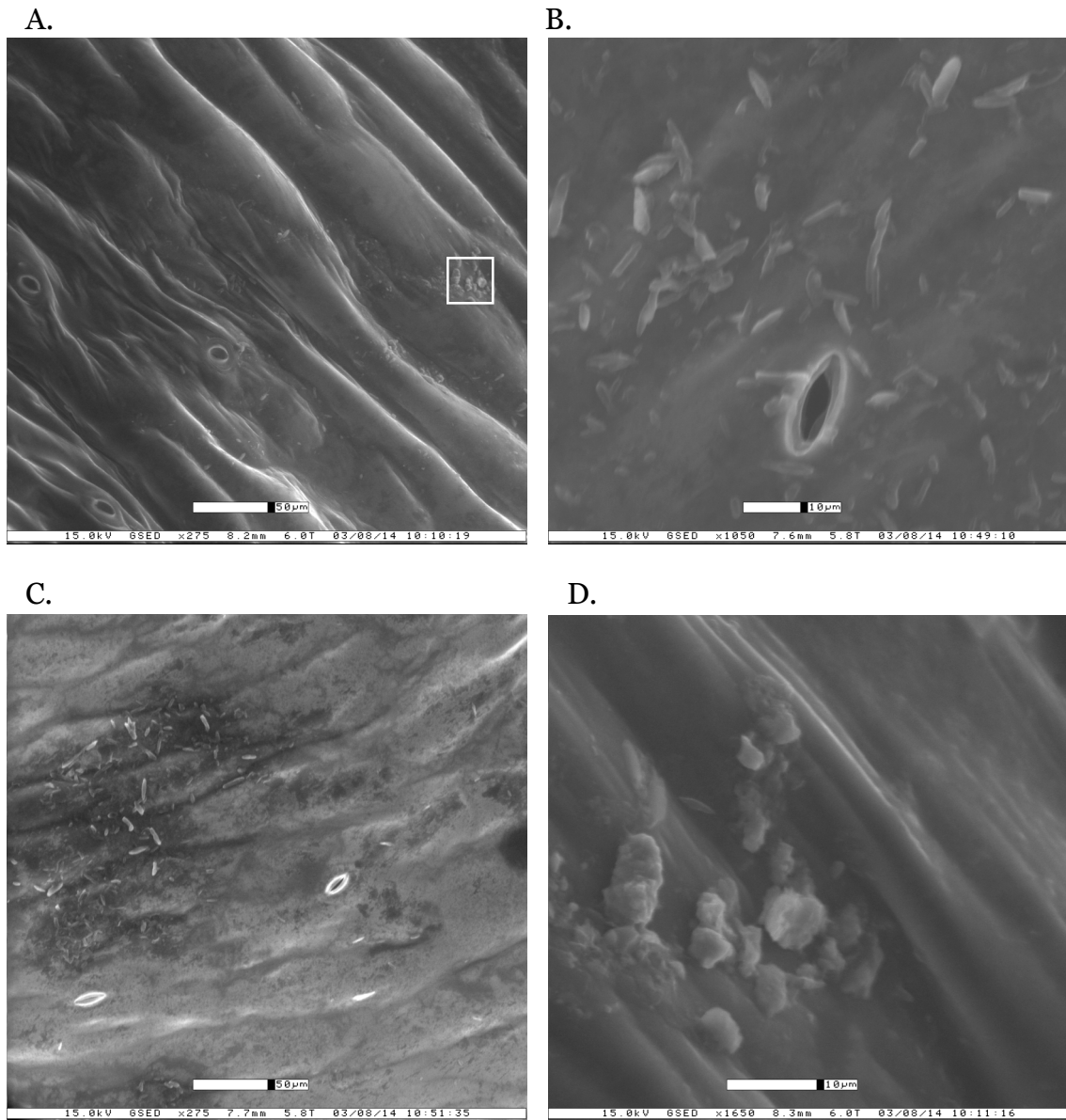


FIG. B.5. ESEM Surface of Lettuce. Representative ESEM photographs of the surface of lettuce. Figure A is a the surface of Bibb lettuce, figure B is the surface of Iceberg lettuce, Figure C is the surface Iceberg lettuce and Figure D is a close up of the Bibb lettuce represented by the white box in Figure A.

Summary of the Microbial Flora Supporting Experiments

The background microbial flora on the surface of the lettuce was highly diverse and had a large amount of variability. The community on the surface of the lettuce exhibited seasonal changes in community structure but was also highly dependent on the immediate location of the lettuce. These results indicated that a large portion of the normal flora were transient and may not offer significant contributions to the functioning of the community.

Virus Recovery. The experimental design for determining viral attachment considered all other interactions that could directly effect a decrease in viral titers, like viral attachment to the bags, effect of buffer and temporal factors. One factor that would be difficult to control in the attachment experiments were the effect of lettuce exudates on viral titers. Even though it is not a likely that a lettuce exudate will decrease the virus titer, the probability is nevertheless present. To determine if virus exudates can cause a decrease in virus titers within the experimental conditions, an additional experiment was developed. A 25 cm² piece of bibb lettuce was placed in a bag as previously described. Twenty ml of buffer (0.1 M NaPO₄, pH 8.0) was aspirated into the bag with lettuce. Buffer at pH 8.0 was chosen because pH 8.0 caused the greatest attachment or greatest loss of virus. The bag with lettuce and buffer was heat sealed and incubated as previously described (see Chapter III). After a half hour a total of 10⁴ pfu of virus was inoculated into the bag containing the lettuce wash. An additional bag with only the buffer was also inoculated with the same amount

of virus this was the control bag. After an additional half hour incubation, the bags were cut open and the virus titer was determined for each bag. The “% recovery” was calculated by dividing the titer from the lettuce wash with the titer from the control. This assay was run with echovirus 11 and MS2 bacteriophage. The results are in Figure A.10.

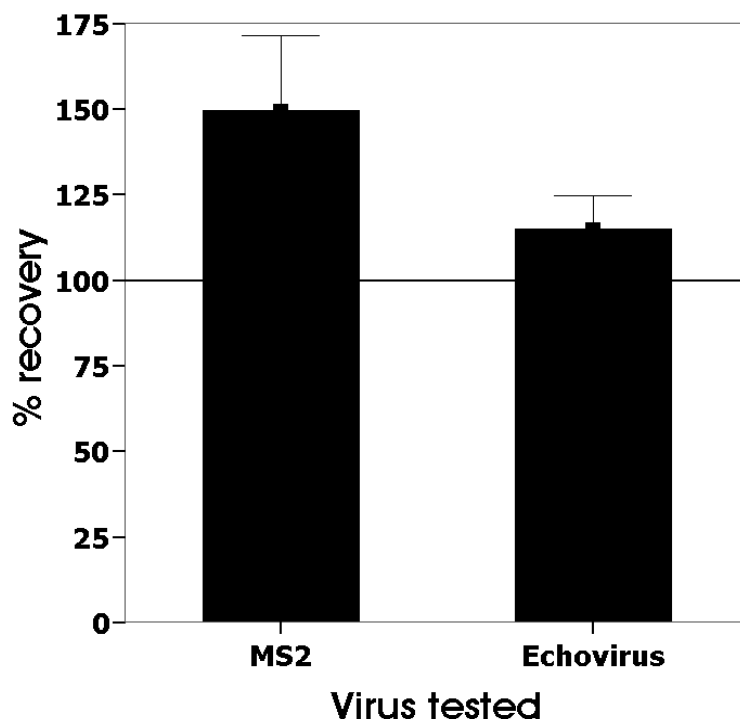


FIG. B.6. Antiviral activity of Lettuce Exudate. The “% recovery” for MS2 bacteriophage and echovirus 11 exposed to lettuce rinsate are represented by the bar graphs. The error bars represent ± 1 SD. The horizontal line represents 100% recovery.

The lettuce wash did not cause a decrease in viral titers, and did have an effect on the titer. When the virus was exposed to the lettuce wash viral titers increased. Since viruses cannot replicate outside of their host the increase of titer must be due to de-aggregation. Statistical analysis indicates a statistical difference is present between the lettuce wash exudates and control, but the difference is a positive difference. These results indicate that lettuce exudates do not adversely affect virus titers, and that decreases in titers during the experiment are due to viral adsorption to lettuce.

TABLE B.4. Statistics of Antiviral Lettuce Exudate. Statistical data for virus recoveries. The *P* value was obtained using the Students t-test, tested against 100% recovery.

Virus	n	Mean ± SE	SD	<i>P</i>-value
MS2	9	150 ± 10.9	32.6	0.02
Echovirus	6	115 ± 4.9	11.9	0.03

VITA

Everardo Vega

Education

Doctor of Philosophy, Texas A&M University August 2006

Bachelor of Science, University of Texas at El Paso, Microbiology 2000

Selected Distinctions

American Society for Microbiology (ASM)
National Center for Infectious Diseases (NCID)
Post-doctoral Fellowship 2006

NASA Graduate Student Research Fellowship 2002

Peer Reviewed Journal Articles

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