

GROWTH BEHAVIOR OF *ALICYCLOBACILLUS ACIDOTERRESTRIS* AS A
FUNCTION OF STRAIN AND CULTURE CONDITIONS

By

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ABSTRACT OF THE THESIS

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Alicyclobacillus acidoterrestris is an acidophilic, spore-forming spoilage organism of concern for the fruit juice industry. The occurrence of bacterial spore-formers in low pH foods was thought to be insignificant. However, in recent years, spoilage of acidic juice by *Alicyclobacillus* was recognized and the seriousness of this situation began to be appreciated. *A. acidoterrestris* has been associated with commercially pasteurized fruit juices as well as other low pH, shelf-stable products such as bottled tea and isotonic drinks. It has been isolated from garden and forest soils and may be introduced into the manufacturing process through unwashed or poorly washed fruit. If spores are not destroyed by processing, they can germinate, grow, and spoil product. The purpose of this study was to develop an understanding of *A. acidoterrestris* growth as a function of strain, pH and temperature so that growth of *A. acidoterrestris* spores might be inhibited by environmental control and product formulation. Four strains of *A. acidoterrestris* were used to investigate the growth kinetics in response to pH (3.0, 4.0, 5.0) and temperatures (26, 30, 37.5, 45°C) by measuring the optical density (OD) every hour for 48 hours using a microtitre plate reader to develop the growth curves. The growth rates were calculated using the software program DMFit and were used to illustrate the relationship between the growth kinetics of *A. acidoterrestris* strains. When examining the generated data, we

observed that there were some differences between growth kinetics of tested strains at various pH and temperature conditions. The differences were random and did not follow any specific trend. The use of a reference strain allows for greater availability and reproducibility. Accordingly, the ATCC 49025 strain is representative of the food isolates (strain N1100, N1102 and N1139) and can be used as a reference strain.

Dedication

*To my beloved family
for your love, support and compassion.*

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Table of Contents

Abstract of the Thesis.....	ii
Dedication.....	iv
Acknowledgements.....	v
Table of Contents.....	vi
List of Tables.....	viii
List of Illustrations.....	ix
List of Abbreviations.....	x
Chapter I – Literature Review.....	1
I.1. <i>Alicyclobacillus acidoterrestris</i>	1
I.2.a. History.....	2
I.2.b. Source.....	3
I.2.c. Spoilage.....	4
I.2.d. Guaiacol production.....	5
I.3. Classification of <i>Alicyclobacillus</i>	6
I.3.1. ω -cyclohexanoic fatty acid membrane.....	7
I.4. Spores.....	8
I.4.a. Pathogenicity.....	10
I.5. Fruit Juice	10
I.5.a. Microbial contamination.....	11
I.5.b. Sterilization and Pasteurization overview	12
I.5.c. Pasteurization of Fruit Juice.....	13
I.6. Inactivation methods of spores.....	14
I.6. a. Ultraviolet (UV) radiation.....	15
I.6.b. Gamma irradiation.....	16
I.6.c. Pulse electric field (PEF)	16

I.6.d. High pressure processing (HPP).....	17
I.6.e. Chemicals.....	18
I.6.f. Use of nisin.....	20
I.7. Predictive modeling.....	21
I.7.a. Growth modeling.....	22
I.8. Use of conditions in which spores will not grow.....	23
I.9. Significance of a reference strain.....	23
I.10. Structure of this thesis.....	24
Chapter II- Hypothesis.....	26
Chapter III- Materials and Methods.....	27
III.1. Culture and culture conditions.....	27
III.2. Growth measurements.....	27
III.3. Growth kinetics.....	28
Chapter IV- Results and Discussion.....	29
IV.1. Response to pH and temperature.....	29
IV.2. Lag Time of <i>A. acidoterrestris</i> strains.....	32
IV.3. Maximum Growth Rate (μ_{\max}) of <i>A. acidoterrestris</i> strains.....	34
IV. 4. Conclusive Discussion.....	36
Bibliography.....	45

List of Tables

Table		Page
1	Final cell density of <i>A. acidoterrestris</i> strains	38
2	Growth kinetic data at 30°C	39
3	Growth kinetic data at 37.5°C	40
4	Growth kinetic data at 45°C	41

List of Illustrations

Figure		Page
1	Growth of <i>A. acidoterrestris</i> strains at 30°C	42
2	Growth of <i>A. acidoterrestris</i> strains at 37.5°C	43
3	Growth of <i>A. acidoterrestris</i> strains at 45°C	44

List of Abbreviations

ATB	Acidophilic thermophilic bacteria
ATCC	American Type Culture Collection
DPA	Dipicolinic acid
FA	Fatty acid
HACCP	Hazard Analysis and Critical Control Point
HPP	High Pressure Processing
NFPA	National Food Processors Association
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PEF	Pulse Electric Field

Chapter I – LITERATURE REVIEW

I.1. *Alicyclobacillus acidoterrestris*

Alicyclobacillus spp. are gram-positive, non-pathogenic, obligately aerobic, rod-shaped, thermophilic, and acidophilic spore-forming bacteria (Baumgart, 1999; Chang et al., 2004). *Alicyclobacillus spp.* have been isolated from soil, spoiled fruit juices, and several thermal environments. *Alicyclobacillus* are sometimes called acidophilic thermophilic bacteria (ATB) and grow well in acidic environments, surviving at pH levels as low as 2.5 (Cerny et al., 1984). This bacterium can grow in a pH range from 2.2-5.8 as well as at temperatures ranging from 23-55°C (Baumgart, 1999). Its ability to survive at high temperatures and low pH levels has been attributed to the unique cellular membrane composition containing ω -cyclohexanoic fatty acids (Krischke and Poralla 1990; Murakami et al., 1998; Alpas et al., 2003). All *Alicyclobacillus spp.* metabolize sugars with acid production, but no gas production (Sawaki, 2007). Water activity greater than 0.9 is required for growth. Some species have been reported to grow in fruit juice with up to 18.2 °Brix (Splittstoesser et al., 1994). Of all species in the genus, *A. acidiphilus*, *A. acidoterrestris*, *A. herbarius* and *A. pomorum* have been reported to be associated with spoilage of fruit juices and beverages (Matsubara et al., 2002; Cerny et al., 1984; Goto et al., 2002; Goto et al., 2003).

Alicyclobacillus acidoterrestris is of concern for the fruit juice industry due to the spoilage of commercially pasteurized fruit juices and has also been associated with bottled tea, isotonic drinks, and other low pH, shelf-stable products (Chang et al., 2004; Sapers et al., 2005). The spores can survive the pasteurization treatment given to most shelf-stable, glass-packaged fruit juices. The heat treatment induces germination of the

spores (Peña et al., 2009; Sapers et al., 2005). Since *A. acidoterrestris* spores have been shown to resist high temperatures and acidic environments, they have been suggested as the target microorganism for the design of a thermal process for fruit juices (Chang et al., 2004).

I.2. History, Source, Spoilage, and guaiacol production

I.2.a. History

Until the mid-1980's, the occurrence of bacterial spore-formers in low pH foods was thought to be insignificant because it was assumed that gram-positive, spore-forming bacteria could not germinate and grow to any great degree at pH levels below 4.5 (Sapers et al., 2005). In 1982, contamination of pasteurized apple juice occurred in Germany on a large scale. This was the first reported incidence of spoilage by *Alicyclobacillus*. The cause was attributed to an organism related to *Bacillus acidocaldarius*. However, later studies showed that the cause was *Alicyclobacillus acidoterrestris* (Yokota, 2007). In 1989, an incident of deterioration of an acidic juice product was reported in Japan. The causative agent was found to be similar to the bacterium identified in Germany. By the mid-1990's spoilage of acidic juice products by members of the recently named genus *Alicyclobacillus* was recognized and the seriousness of this situation began to be appreciated (Sapers et al., 2005).

The ability of spores of *A. acidoterrestris* to grow under highly acidic conditions makes it a good candidate for spoilage of shelf stable fruit juices and beverages. Following the first spoilage incident caused by *A. acidoterrestris* reported in 1982, when aseptically packaged apple juice in Germany was contaminated (Cerny et al., 1984),

several spoilage incidents have been reported over the past two decades. Several spoilage cases were reported in Japan, Europe, and the USA in the 1990's (Splittstoesser et al., 1994; Jensen, 2000). In addition, more food products have been reported spoiled by *A. acidoterrestris*, including isotonic water and lemonade (Yamaziki et al., 1996), carbonated fruit juice drinks (Pettipher and Osmundson, 2000), fruit pulps, shelf-stable iced tea containing berry juice (Duong and Jensen, 2000), and canned diced tomatoes (Walls and Chuyate, 1998).

In 1990 and 1995, an unpleasant odor was detected in 40% of apple juice samples in Australia. It was reported that this bad odor was not caused by any additives or preservatives added to the juice, but was due to microbial contamination. Splittstoesser and co-workers (1994) reported the strain isolated from pasteurized apple juice in 1990 was a thermoacidophilic bacterium. This was the first mention of the isolation of this organism from contaminated fruit juice in the United States (Yokota, 2007). Though spoilage by *Alicyclobacillus spp.* was previously regarded as sporadic, the survey by the National Food Processors Association (NFPA) in 1998 showed the large scale of fruit juice spoilage associated with *Alicyclobacillus spp.* (Chang et al., 2004). Results of the survey indicated that 35% of the fruit juice manufactures who responded experienced spoilage unconfirmed but consistent with growth of acidophilic sporeformers (Chang et al., 2004).

I.2.b. Source

Soil is considered to be the major source of *A. acidoterrestris* and also the most important source of contamination of acidic products. Studies have suggested that contamination of fruit juices is most likely caused by fruit contaminated by soil during

harvest or by unwashed or poorly washed raw fruit used in processing facilities (Chang and Kang, 2004). Another possibility is that soil can be carried into the manufacturing facilities by employees. Water has also been proposed to be another important source of contamination. McIntyre et al. (1995) isolated a strain of *Alicyclobacillus* from spoiled juice product and found the same strain in a sample of ingredient water from the processing facility.

I.2.c. Spoilage

Spoilage can cause substantial economic losses in the food industry, and the presence of spoilage agents can be indicative of conditions that are suitable for the proliferation of more serious disease-causing agents (Brown, 2000). Most sporeforming bacteria cannot germinate at low pH, and therefore pose little threat of spoilage in acidic foods; however *A. acidoterrestris* spores are problematic due to their ability to germinate and grow at low pH (Black et al., 2007; Splittstoesser et al., 1994). *Alicyclobacillus spp.* pose a new challenge to the fruit juice industry. Elimination and control of this organism from the processing environment has been challenging and little information is available on how certain manufacturing practices and processing treatments affect *Alicyclobacillus* during fruit concentrate production (Bahceci et al., 2005; Steyn et al., 2011; Walker and Philips, 2008^a).

The impact of *Alicyclobacillus spp.* may not only be widespread, but may also cause a great loss to the manufacture (Chang et al., 2004). Not until consumer complaints were received was spoilage looked at as a concern. Consumers have become more conscience about food quality and have an increased expectation of consistency. With the

large scale of the modern food industry and the nationwide distribution of products, a small number of viable organisms have the potential to contaminate large volumes of juice, resulting in severe financial losses for the producers affected. The reported spoilage of juice products occurred long before the expiration date, and no obvious defect, such as swelling in the container was observed (Chang et al., 2004). There were no gases produced and there were no substantial changes in fruit juice pH detected. The major complaints were based on off flavors described as “medicinal”, “antiseptic” or “hammy” (Chang et al., 2004). The major compounds associated with the off-flavors and the spoilage bacterium are guaiacol and the halophenols, including 2,6-dibromophenol (2,6-DBP) and 2,6-dichlorophenol (2,6-DCP). Guaiacol is the predominant metabolite and is associated with the smoky taints in fruit juices (Chang et al., 2004).

1.2.d. Guaiacol production

Guaiacol is a well-known compound that can either enhance or detract from flavor in a variety of products. The smoky/phenolic odor of guaiacol is commonly used as a component of synthetic flavorings in processed foods. Guaiacol contributes to the characteristic odor of some roasted foods including barley malt, and as an off-odor in wine, fruit juices, chocolate ice cream, and vanilla yogurt (Chang et al., 2004). In roasted products, guaiacol is formed by thermal decomposition of phenolic precursors. In fruit juices and dairy foods, guaiacol is a product of microbial metabolism (Chang et al., 2004). Vanillic acid was the immediate precursor of guaiacol identified in the microbial synthetic pathway.

The supposed pathway of guaiacol formation from vanillic acid is by nonoxidative decarboxylation (Chang et al., 2004). The ability to decarboxylate vanillic acid to guaiacol is commonly seen among soil bacilli. Vanillic acid can be present in fruit juices due to contamination, but is also naturally derived from the plant polymer lignin (Chang et al., 2004). Microorganisms like *Alicyclobacillus* spp. further convert vanillic acid to vanillyl alcohol, guaiacol, catechol, methoxyhydroquinone (Chang et al., 2004). Tyrosine is another possible precursor for guaiacol formation. Apple juice contains approximately 4.1µg tyrosine/ml juice, and orange juice contains 3-13.5µg tyrosine/ml (Jensen, 1999). Storage temperature and oxygen concentration are important in forming guaiacol from tyrosine. Little is known about this reaction since it has not been widely investigated and the guaiacol synthetic pathway mainly recognized is that of lignin degradation (Chang et al., 2004).

I.3. Classification of *Alicyclobacillus*

Wisotzkey and co-workers (1992) focused on the proper taxonomic placement of *Bacillus acidocaldarius*, *Bacillus acidoterrestris*, and *Bacillus cycloheptancius* using the 16S rRNA comparative sequence analysis. It was found that *B. acidocaldarius* and *B. acidoterrestris* were almost identical (98.8%), clearly belonging to the same genus, where *B. cycloheptancius* was more distant (93.2% and 92.7%) using primary sequence comparisons (Wisotzkey et al., 1992). The secondary structures of 16S rRNA belonging to the three microorganisms were also examined and were found to be identical or very

similar to each other, but differed from other *Bacillus* spp. (Wisotzkey et al., 1992).

Based on the results of this study it was determined that *B. acidocaldarius*, *B. acidoterrestris*, and *B. cycloheptanicus* should be reclassified as the genus *Alicyclobacillus*. This reclassification was mainly in part to the unusual ω -alicyclic fatty acids in their cell membrane (Wisotzkey et al., 1992). Wisotzkey suggested the reclassification of *B. acidocaldarius*, *B. acidoterrestris*, and *B. cycloheptanicus*, as species of *Alicyclobacillus* based upon their thermoacidophilic phenotype and alicyclic fatty acids in the cellular membranes (Sapers et al., 2005). The presence of ω -alicyclic fatty acids is thought to be the factor that enables these organisms to survive under acidic and thermal conditions, but it is not an essential condition for survival (Yokota, 2007).

1.3.1. ω -cyclohexanoic fatty acid membrane

The most distinctive characteristic of *Alicyclobacillus* spp. is the presence of ω -alicyclic fatty acids as the major membrane component. Researchers suggest that ω -alicyclic fatty acids are associated with the exceptional resistance of *Alicyclobacillus* spp. to acidic conditions and high temperatures (Chang et al., 2004). Kannenberg and co-workers (1984) demonstrated that ω -cyclohexane fatty acid-containing lipids pack densely, resulting in low diffusion at high temperatures. Wisotzkey et al. (1992) proposed that this property provides an advantage when cultures are grown at high temperatures or low pH. Closely packed rings of the ω -alicyclic fatty acids may form a protective coating for the cell membrane, and contribute to the resistance of *Alicyclobacillus* spp. to acidic conditions and high temperatures (Chang et al., 2004). Kannenberg and co-workers (1984) stated that lipids containing fatty acids with a cyclohexane ring may stabilize the

membrane structure and help maintain barrier function of prokaryotic membranes at high temperatures. These fatty acids contribute to the heat resistance of *Alicyclobacillus* by forming a protective coating with strong hydrophobic bonds. These hydrophobic bonds stabilize and reduce membrane permeability in extreme acidic and high temperature environments (Kannenberg et al., 1984; Jensen, 1999; Wisotzkey et al., 1992). Thus, ω -cyclohexane lipids may be an aspect of thermoacidophilic adaptation of bacterial membranes (Chang et al., 2004).

I.4. Spores

Spores are formed by some bacteria a sporulation process that is initiated in response to harsh environmental conditions (Black et al., 2007). Spores have the ability to survive for long periods without nutrients, are metabolically dormant, contain little or no high energy compounds such as ATP and NADH, exhibit no detectable metabolism of endogenous or exogenous compounds and, little if any, enzyme activity in the spore core (Setlow, 2006). Due to their lack of enzyme action and metabolism, the dormant spore cannot repair damage to macromolecules such as DNA or protein. As a result, spores have acquired a multitude of mechanisms to protect macromolecules from damage during their periods of dormancy. Spores are extremely resistant to potentially lethal treatments such as exposure to moist and dry heat, UV and γ - radiation, and toxic chemicals that rapidly kill growing cells (Black et al., 2007; Nicholson et al., 2000; Setlow, 2006). Due to their ability to survive and since many sporeformers are soil microorganisms, spores are present at high levels in soils and are commonly associated with most foods (Black et al., 2007). Their resistance to stress, in conjunction with the ubiquity of spores in the

environment, makes these microbes' major agents of food spoilage and food-borne disease (Setlow, 2006).

A. acidoterrestris spores can germinate and grow at pH less than 4.0 and have shown strong heat resistance, which presents a potential threat to the fruit juice and beverage industry. D-values of *A. acidoterrestris* in juices at 95°C have been reported to range from 0.06 to 5.3 minutes, and z-values range from 7.2 to 12.9°C (Silva and Gibbs, 2001). Little is still known about the characterization of spores of the thermoacidophilic bacterium *A. acidoterrestris* (Yamazaki et al., 1997). It has been known that the pH heat treatment medium influences heat resistance of bacterial spores. The combination of heat and acidification was one of the first treatments used to reduce the heat resistance of some heat resistant microorganisms (Chang et al., 2004). However, because of the specific characteristics of *A. acidoterrestris*, no complete sterilization of vegetative cells and spores can be expected by acidification (Yamazaki et al., 1997). While little detail is known about the mechanisms of heat resistance of bacterial spores, it has been known that the heat resistance of spores is associated with dehydration, dipicolinic acid (DPA) content, presence of heat-stable proteins, and mineralization (Yamazaki et al., 1997).

Yamazaki et al. (1997) conducted a study to determine the influence of divalent cations on the heat resistance of spores of the thermoacidophilic spoilage bacterium. The heat resistance of *A. acidoterrestris* spores was not affected by the presence of the different divalent cations, Ca^{2+} , Mg^{2+} , Ba^{2+} , Mn^{2+} , and Sr^{2+} in the sporulation medium nor was it affected by the demineralization or remineralization (Yamazaki et al., 1997). However, the Ca^{2+} content in *A. acidoterrestris* spores of the different forms were greater

than the DPA content. In contrast, the DPA content in the *Bacillus subtilis* spores was greater than the Ca^{2+} content (Yamazaki et al., 1997). These findings suggest that the presence of constant amounts of Ca-DPA and a stronger binding characteristic of divalent ions, especially Ca^{2+} and Mn^{2+} , is reflected in the specific heat resistance of *A. acidoterrestris* spores (Yamazaki et al., 1997).

1.4.a. Pathogenicity

Wall and Chuyate (2000) tested the pathogenicity of *A. acidoterrestris* by either injecting spores directly into mice or by feeding spore-inoculated juice to guinea pigs. No death or illness symptoms were found in the mice or guinea pigs, indicating that *A. acidoterrestris* lacks pathogenicity at the level tested. There were also no cases of human illness attributed to the consumption of different juices spoiled by *A. acidoterrestris* that have been reported. Therefore, although *A. acidoterrestris* can cause serious spoilage problems, it is not a safety concern.

1.5. Fruit Juice

Juice is defined as “the aqueous liquid expressed or extracted from one or more fruits or vegetables, purees of the edible portions of one or more fruits or vegetables, or any concentrates of such liquid or puree” (FDA, 2001). Fruit juices are consumed widely around the world, mainly because they are considered a healthy natural source of nutrients. However, the high water activity and high carbohydrate content along with other nutrients of fruit juices favor microbial growth. Microbial contamination of fruit juice and juice products have caused several cases of foodborne illnesses and spoilage

incidents, which not only results in threats to human health, but also leads to huge economic losses.

1.5.a. Microbial contamination

Outbreaks of illnesses associated with consumption of fruit juices have been an increasing public health problem since the early 1900's. It was originally thought that the pH of fruit juice is below that which supports growth and even survival of pathogenic bacteria. However, in 1991, contamination of fresh pressed apple cider with *Escherichia coli* O157:H7 resulted in foodborne illnesses and subsequent cases of hemolytic uremic syndrome (Besser et al., 1993). From 1995 to 2005, according to the CDC's Foodborne Outbreak Reporting System, there were 21 juice-associated outbreaks that caused 1,366 cases of illnesses. Of the reported outbreaks, 13 were of known etiology, among which *E. coli* O157:H7, *Salmonella*, *Cryptosporidium*, and shiga toxin-producing *E. coli* O111 were confirmed as causative agents of illness (Vodjdani, et al., 2008).

With the emergence of juice-associated outbreaks, the US Food and Drug Administration (FDA) published the juice hazards analysis and critical control point (HACCP) regulation in 2001. This regulation requires that juice processors include in their HACCP plan measures to provide at least a 5-log reduction in the pertinent pathogens most likely to occur (FDA, 2001). The juice HACCP regulation only applies to pathogens, and there is no regulation for controlling juice spoilage. It is necessary for the juice and beverage industries to take measures to ensure the quality of their products.

Since pH is typically less than 4.0, fruit juice spoilage is caused by yeasts, mold, and acid tolerant bacteria, such as lactic acid bacteria. Spoilage may result in the formation of haze and sediment in the juice and the presence of gas and/or undesirable

flavors or odors (Worobo and Splittstoesser, 2005). Contamination of juices with an initially small number of organisms has the potential to cause wide-spread spoilage that can result in significant economic loss.

1.5.b. Sterilization and Pasteurization Overview

Sterilization and thermal pasteurization are predominately used in the food industry for their effectiveness and product safety record. Sterilization is used to prevent spoilage of food and other substances by destroying all forms of microbial life. This can be achieved by applying physical agents, such as heat and irradiation, or by chemical substances. Heat sterilization is the most common method of sterilizing foods. Moist heat or dry heat can be used, depending upon the nature of the substance to be sterilized. Moist heat is also used in pasteurization, which is not considered a true sterilization technique because not all microorganisms are killed. Only certain pathogenic organisms and other undesirable bacteria are destroyed. Sterilization requires higher temperatures for longer periods to ensure destruction of all bacteria and spores, but it often spoils the product in the process.

The basis of pasteurization is the application of heat to prolong the storage time of foods for a limited time, by reducing the number of targeted organisms to what is considered an acceptable level. Many bacteria cannot survive exposure to the range of temperatures used in pasteurization. The energy of the heating process is disruptive to the membranes that enclose the bacteria. The bacterial enzymes that are vital for the maintenance of growth and survival of the bacteria are denatured, or lose their functional shape, when exposed to heat. The disruption of bacteria is usually so complete that recovery of the cells following the end of the heat treatment is impossible.

Pasteurization processes are designed to control the level of microorganisms present in foods (Silva and Gibbs, 2004). In low acid foods, sterilization is needed to kill spores of pathogenic and spoilage organisms. The spores of the pathogen *Clostridium botulinum* are the target of the heat treatment for low acid foods (pH > 4.6) since the spores may germinate and grow under these conditions (Silva and Gibbs, 2004). Most fruits have a low pH (< 4.6) and no *C. botulinum* germinate, grow, or produce toxin under these conditions (Lopez, 1987; Silva and Gibbs, 2004). No target and criteria have been defined regarding pasteurization of high acid foods, however, many researchers are acknowledging *Alicyclobacillus spp.* as a spoilage agent and important target in the quality control of acidic beverages due to their resistance to pasteurization temperatures and low pH as well as their ability to produce off flavors (Chang et al., 2004).

1.5.c. Pasteurization of Fruit Juice

Pasteurization is designed to inactivate the vegetative cells of pathogens, including the vegetative cells of sporeformers. FDA has not defined pasteurization in terms of juice and juice products and has not specified the parameters for juice pasteurization due to the unique characteristics of the various types of juice and juice products (FDA, 2001). Current pasteurization conditions vary by processor. Juice processors choose appropriate pasteurization conditions for their particular types of juices based on literature that provides data on pasteurization times and temperatures. The nature of the product container and the physical properties of the fruit juice often dictate the processes that should be used (Worobo and Splittstoesser, 2005). Pasteurization temperatures between 80 and 100°C are normally used (Silva and Gibbs, 2004). The process is much less severe than those used for low-acid foods and if refrigerated storage

is intended, a milder pasteurization would be used to obtain the same shelf-life.

Typically, the fruit juice industry applies a hot-fill-hold pasteurization process, where the product is heated to 86-96°C, held for approximately 2 minutes to destroy mesophilic spoilage microbes, and filled into the containers and sealed (Oke and Paliyath, 2006; Chang and Kang, 2004; Jay et al., 2005a). The containers are closed while the product is hot and then cooled to develop a vacuum which reduces the oxygen available as well as prevent microbial growth (Oke and Paliyath, 2006).

Spoilage by *A. acidoterrestris* occurs when the spores are induced and germinate at high temperatures and in acid conditions (Peña et al., 2009). Aside from its ability to survive pasteurization, *A. acidoterrestris* does not grow in juice concentrates and survives mainly as spores (Bevilacqua et al., 2008). The fact that spores are not inactivated by heat treatment is of major concern for the food industry, because when a concentrate is diluted to produce juice, *A. acidoterrestris* will rapidly start to multiply (Lee et al., 2006; Bevilacqua et al., 2008).

I.6. Inactivation of spores

New technology provides different methods to inactivate spores. For a long time it was believed that thermal processing was the only way to reduce the initial spore concentration of *A. acidoterrestris* spores and prevent spoilage. New methods have been proposed to inhibit and/or control spore germination. In many cases, more research is necessary on this organism to ensure the efficiency in killing and/or inactivation of spores in these new methods.

1.6.a. Ultraviolet (UV) radiation

Ultraviolet (UV) energy is a non-ionizing radiation with germicidal properties at wavelengths in the range of 200-280 nm (Bintsis et al., 2000; Lado and Youself, 2002). UV radiation damages microbial DNA and to a lesser extent denatures proteins (Lado and Yousef, 2002). Potentially lethal DNA lesions are scattered randomly through the cell population during UV radiations. Cells that are unable to repair their radiation-damaged DNA die. Sub-lethally injured cells are often subjected to mutations. UV energy at 254 nm induces the formation of pyrimidine dimers which distorts the DNA helix and blocks cell replication (Lado and Yousef, 2002). UV radiation also cross-links aromatic amino acids at their carbon-carbon double bonds (Lado and Yousef, 2002). The resulting denaturation of proteins contributes to membrane depolarization and abnormal ionic flow.

UV radiation has a high potential to inactivate a wide variety of microorganisms and its application can be used to decontaminate foods, equipment, building and the environment (Rice and Ewell, 2001). However, spores are 10- to 50-fold more resistant than growing cells to UV radiation at 254 nm, which is the most efficient wavelength for spore killing (Setlow, 2006; Nicholson et al., 2000, 2005). The major reason for spore resistance to 254 nm radiation is the photochemistry of the DNA in spores. The major DNA products generated by 254 nm irradiation of growing cells are cyclobutane dimers and (6-4)-photoproducts formed between adjacent pyrimidines in the same DNA strand (Setlow, 2006). UV radiation at wavelengths longer than 254 nm will also kill spores, but is less effective.

1.6.b. Gamma irradiation

Gamma irradiation works by using high-energy photons that are emitted from an isotope source (Cobalt 60) producing ionization throughout a product. In living cells, these disruptions result in damage to the DNA and other cellular structures. Ionizing radiation generates hydroxyl radicals from water, which remove the hydrogen atoms from the sugar and the bases of the DNA strands (Lado and Yousef, 2002). These photon-induced changes at the molecular level cause the death of the organism or render the organism incapable of reproduction (Bintsis et al., 2000). The gamma process does not create residuals or impart radioactivity in processed products. Gamma irradiation is effective against vegetative and sporulated bacteria (Farkas, 1998). Irradiation with long-wave UV (320-400 nm) causes the formation of hydroperoxides radicals in the membrane's unsaturated fatty acids, which induces changes in membrane permeability (Lado and Yousef, 2002; Bintsis et al., 2000).

Gamma irradiation has high potential even though its development and commercialization has been held back in the past by unfavorable public perception (Ross et al., 2003; Resurreccion et al., 1995). Another drawback from an industrial standpoint is that this process tends to proceed relatively slowly (Newton, 2007).

1.6.c. Pulsed electric field (PEF)

Pulsed electric field (PEF) treatment is based on the delivery of pulses at high electric field intensity (5-55kV/cm) for a few milliseconds (Jeantet et al., 1999; Lado and Yousef, 2002). Destruction of microbial cells by PEF is due to irreversible electroporation of the cell membrane, which leads to leakage of intracellular contents and

eventually lysis of the cell (Qin et al., 1995; Barbosa-Canovas et al., 1999). PEF treatment has lethal effects on various vegetative bacteria, mold, and yeast. A series of short, high-voltage pulses breaks the cell membranes of vegetative microorganisms in liquid media by expanding existing pores (electroporation) or creating new ones. Pore formation is reversible or irreversible depending on factors such as the electric field intensity, the pulse duration, and number of pulses. The membranes of PEF-treated cells become permeable to small molecules; permeation causes swelling and eventual rupture of the cell membrane.

Emerging non-thermal processes, such PEF, are gaining commercial application most rapidly with juices and other fruit-derived products because their naturally low pH provides an additional hurdle to growth (Mermelstein, 1997; Qiu et al., 1998; Jia et al., 1999; Leistner and Gould, 2002; Ross et al., 2003). Application of PEF technology has been successfully demonstrated for the pasteurization of foods such as juices, milk, yogurt, soups, and liquid eggs. Efficacy of spore inactivation by PEF in combination with heat or other hurdles is a subject of current research. Much more research is needed before these technologies can be adapted for the production of shelf-stable low acid foods (Yousef, 2001).

1.6.d. High Pressure Processing (HPP)

High Pressure Processing (HPP) is a non-thermal process that generates high quality foods. Microbial growth is retarded at pressures in the range of 20-180 MPa. These pressures also inhibit protein synthesis (Lado and Yousef, 2002). Loss of cell viability begins around 180 MPa, and the rate of inactivation increases exponentially as the pressure increases (Lado and Yousef, 2002). At lethal high pressure treatments,

microbial death is considered to be due to changes in permeability of the cell membrane and denatured proteins (Farr, 1990; Lozano, 2006).

Non-thermal processes are well-suited for the destruction of vegetative cells but have limited effectiveness in killing spores. While dormant spores are extremely resistant to harsh conditions, such as heat and radiation, germinated spores lose much of this resistance. This appears to be the major reason that HPP kills spores, since HPP causes spore germination (Black et al., 2007). The germinated spores are subsequently killed by exposure to pressure and elevated temperature. Unfortunately, these treatment temperatures are not lethal to dormant spores (Black et al., 2007). In order to inactivate the spores, strong heat treatment (95°C for a few minutes) or HPP (> 600MPa) would be needed, resulting in loss of nutritional and sensorial quality (Bevilacqua et al., 2008).

1.6.e. Chemicals

Spores are generally significantly more resistant than growing cells to a wide variety of toxic chemicals, including acids, bases, phenols, aldehydes, alkylating agents, and oxidizing agents (Bloomfield and Arthur, 1994; McDonnell and Russell, 1999; Russell, 1982; Russell, 1990; Setlow, 1999; Tennen et al., 2000). In many cases the reasons for spore resistance to these types of agents are not known, and for many chemicals, such as acids, bases, aldehydes, and oxidizing agents, the target for spore killing is not known. It appears that some chemicals, in particular oxidizing agents, damage the spore's inner membrane so that the membrane ruptures upon spore germination and outgrowth, although there are some data implicating protein damage in killing by oxidizing agents (Palop et al., 1996; Setlow, 2006; Palop et al., 1998).

However, for other agents, such as alkylating agents, it is clear that the target for spore killing is spore DNA (Setlow et al., 1998).

Since *A. acidoterrestris* most likely enters processing facilities on fruits that come in contact with contaminated soil, raw materials must first be washed thoroughly to prevent *A. acidoterrestris* contamination. However, bacteria cannot be reduced sufficiently by washing with just cold or warm water, so detergents or bactericides need to be added to the wash water. Orr and Beuchat (2000) studied the effectiveness of selected disinfectants for inactivating *A. acidoterrestris* spores; their results showed that when spores of a five-strain mixture were suspended in 200 ppm chlorine, 500 ppm acidified sodium chlorite, or 0.2 % H₂O₂ for 10 minutes at 23°C, spore populations were reduced by 2.2-, 0.4- and 0.1- logs, respectively. More than a 5-log reduction of spores was observed after treatment with 1,000 ppm chlorine or 4% H₂O₂. The disinfectants were less effective at killing spores on the surface of apples. Both 500 ppm chlorine and 1,200 ppm acidified sodium chlorite reduced viable spores by less than 1 log. Chlorine dioxide was reported to be more effective at killing *A. acidoterrestris* spores. More than a 4-log reduction of spores was observed after treatment with 40 ppm chlorine dioxide for five minutes in aqueous suspension. Treatment with 80 and 120 ppm chlorine dioxide for 5 min reduced spores to undetectable levels. When chlorine dioxide was applied to apple surfaces, greater than a 4.8-log reduction of spores was observed after treatment with 40 ppm free chlorine dioxide for four minutes (Lee et al., 2004). Cortezzo et al. (2004) suggested that treatment with oxidizing agents results in damage to the spore inner membrane, which sensitizes spores to subsequent stress.

Chemicals have been considered as a hurdle to combine with the heat processing to inhibit *A. acidoterrestris* spores, as well as the inactivation of spores through commercial disinfectants or ethanol since the 1990's (Orr and Beuchat, 2000; Hsiao and Siebert, 1999; Shearer et al., 2000; Bevilacqua et al., 2008). In recent years, there have been trends for “going green” and “all natural” from the consumers. Use of synthetic additives has declined and more friendly compounds, such as bacteriocins are now being considered to control and/or inhibit *A. acidoterrestris* spore germination (Bevilacqua et al., 2008; Burt, 2004).

1.6.f. Use of nisin

Bacteriocins are legally considered natural if used in concentrations equal to or below what are found in foods naturally fermented with the bacteriocin-producing starter culture (Ross et al., 2003; Cleveland et al., 2001). The antimicrobial polypeptide nisin is currently the only bacteriocin approved for use in food by the World Health Organization (Ross et al., 2003; Cleveland et al., 2001; Pol et al., 2000). Nisin has a great effect against gram-positive bacteria and is particularly effective against spores. Nisin initially forms a complex with Lipid II, which is a precursor molecule in the formation of bacterial cell walls. The nisin-lipid II complex then inserts itself into the cytoplasmic membrane forming pores and allows the efflux of essential cellular components resulting in inhibition or death of the bacteria (Delves-Broughton, 2005).

Nisin can have a synergistic effect used with heating to inactivate microorganisms (Delves-Broughton, 1990; Adams and Nizo, 2007; Peña et al., 2009). It is not fully understood how nisin acts against spores, though the action against spores is predominantly sporostatic. It affects the post-germination stages of spore development

by inhibiting the pre-emergent swelling and therefore outgrowth and formation of vegetative cells (Komitopoulou et al., 1999). The more the spores are heat damaged the more they are susceptible to nisin, and nisin appears to bind to sulfhydryl groups on the spore surface (Delves-Broughton, 2005). The presence of nisin during heating increased the mortality of spores of *Bacillus cereus* and *A. acidoterrestris* (Beard et al., 1999; Komitopoulou et al., 1999; Penna and Moraes, 2002; Wandling, et al., 1999; Peña et al., 2009). It can be added directly to the juices, or incorporated in swellable polymers released in controlled manner during storage (Komitopoulou et al., 1999; Yamazaki et al., 2000; Buonocore et al., 2004; Bevilacqua et al., 2008). However, Yamazaki et al., (2000) reported that while nisin was able to inhibit spore germination in orange juice, it was not able to do so in a clear apple juice, most likely due to the competitive effect of phenols. Aside from regulatory control, the use of nisin in commercial food supplies is also restricted by high cost and by decreased bactericidal activity in complex food substrates (Garcia-Graells et al., 1999; Terebiznik et al., 2000; Ross et al., 2003).

1.7. Predictive Modeling

Predictive microbiology provides an estimate of the potential growth of particular microorganisms under a variety of conditions. The models used in predictive microbiology are developed from experimental work. These models are then extrapolated to foods. Predictive models can provide critical support in different relevant areas in the food industry. They are helpful tools for evaluating microbial responses and can help to identify potential problems for a product or process. Understanding the effect of various variables on the growth of microorganisms is essential in evaluating their survival

potential and identifying factors important in controlling their existence and minimizing potential risks (Grijnspeerdt and Vanrolleghem, 1999).

There are three levels among predictive microbiological models: primary level models describe changes of microbial numbers with time, secondary level models summarize the effect of environmental conditions and the tertiary level models combine the two levels (Whiting, 1995; Grijnspeerdt and Vanrolleghem, 1999). The most used level one predictive models are the modified Gompertz model (Zwietering et al., 1990) and the logistics model (Peleg, 1997). These models were based on theoretical considerations and were not originally developed for modeling bacterial growth (Baranyi et al., 1993; Grijnspeerdt and Vanrolleghem, 1999). Baranyi and coworkers (1993, 1994) developed a mechanistic model for bacterial growth which is being more adopted than the modified Gompertz model. This model is a truly dynamic model in that it can deal with time varying environmental conditions.

1.7.a. Growth modeling

Predictive modeling has been used to estimate growth in broth as a function of specific environmental factors. This approach allows food scientists to predict the effect of treatments on microbial growth and quality control of food products. In the present study, the software DMFit and the model of Baranyi and Roberts (1994) were used to calculate the growth kinetics from the growth curves. An understanding of the lag phase and growth rate is crucial to understanding conditions to inhibit growth. By understanding the behavior of *A. acidoterrestris* in the environment and how this spoilage organism is affected by various environmental growth parameters, such as pH and

temperature, reformulation of beverages could be developed for preventing growth of *A. acidoterrestris* and as a result, prevent spoilage of products.

I.8. Use of conditions in which spore will not grow

Temperature and pH are the major environmental growth factors which are studied most because of their importance in fundamental research and their practical importance, especially in the food industry (Rosso et al., 1995). In theory, there are time/temperature/pH combinations under which *A. acidoterrestris* cannot grow, just as there are conditions that will inhibit the growth of *Clostridium botulinum* spores. The pH of a food has an influence on the amount of heat necessary to kill the spores of *C. botulinum*, however, growth is inhibited at a pH lower than 4.6. In order to assess conditions in which spores will not grow, differences between industrial strains and those originating from culture collection need to be established.

I.9. Significance of a reference strain

An implicit concern of food microbiologist is the suitability of culture collection strains for use as substitutes for environmental isolates. The study of bacteria culture collection is essential for research and development. The American Type Culture Collection (ATCC) collection of bacteria represents the most diverse public assemblage of prokaryotes in the world. ATCC was established in 1925 when a committee of scientist recognized a need for a central collection of microorganisms that would serve scientist all over the world. The collection includes strains relevant to medicine, public

health, industrial microbiology, agriculture and environmental microbiology. It is also recognized as a repository for reference cultures. Reference strains are important for quality control of culture media and methods and for the development and validation of new methods.

I.10. Structure of this thesis

The focus of this research was to develop an understanding of *A. acidoterrestris* growth as a function of strain, pH, and temperature so that growth from *A. acidoterrestris* spores which survive high pressure processing might be inhibited by environmental control and product formulation. Data on the lag and exponential phases of growth for environmental and archival strains of *A. acidoterrestris* were evaluated and used to understand microbial growth under a continuous variable set of conditions in beverages.

The identification and characterization of strains is an integral function of culture collections. The importance of a particular strain may be as a reference for medical or taxonomic research, as an assay organism for testing or screening, or as an essential component of a patent application for a product or process in which it is involved. In this thesis, four strains of *A. acidoterrestris* were investigated to determine if the culture collection strain could be used as a reference strain for the industrial isolates.

Growth curves were measured at OD 650 using a microtitre plate reader. The ability to examine a large matrix of experimental samples in a short period of time has made the use of microtitre plate readers an important tool in generating the large amount of data required to construct useful models. Growth curves were generated as a function of three pH levels (3.0, 4.0, and 5.0) and four temperature levels (26, 30, 37.5, and 45°C).

This information is necessary to understand the growth behavior of the strains and culture collections. This will be addressed by generating growth kinetics using times and temperatures which *A. acidoterrestris* is likely to encounter in beverages.

The present research builds on the available data and uses different growth parameters and growth kinetics to understand the behavior of strains under different conditions. In this study, the vegetative cells of four strains of *A. acidoterrestris* were measured using a microtitre plate reader to better understand the behavior of the bacterial population at different pH and temperatures. Afterwards, the data generated from the growth curves were used to calculate the growth kinetics of each strain to determine if the culture collection strain is representative of the food isolates.

Chapter II- HYPOTHESIS

This study tests the hypothesis that specific combinations of pH and temperature can be used to inhibit *Alicyclobacillus acidoterrestris* growth, and furthermore that the ATCC 49025 strain is representative of food isolates. This hypothesis will be tested by addressing three objectives.

Objectives:

1. Determine the growth characteristics of *A. acidoterrestris* as a function of pH and temperature for each strain
2. Calculate the growth constants by using the Baranyi and Roberts (1994) model for determining kinetic constants from the data collected in objective 1.
3. Compare these kinetic constants among strains to evaluate the suitability of *A. acidoterrestris* ATCC 49025 as the reference strain for future studies.

Chapter III- MATERIALS AND METHODS

III.1. Culture and culture conditions

Four strains of *A. acidoterrestris* were used in this study: N1100, N1102, N1139 (industrial isolates obtained from Ginny Scott at the Grocery Manufacturers Association, Washington D.C.) and ATCC 49025 (American Type Culture Collection, Rockville, MD). Stock cultures were maintained through monthly transfers on potato dextrose agar (PDA, [Difco, MI]) slants adjusted to pH 3.5 with filter sterilized 10% tartaric acid and stored at 4°C. The cultures were grown in potato dextrose broth (PDB [Difco, MI]) adjusted to pH 4.0 with filter sterilized 10% tartaric acid at 40°C for 48 hours. Cells (100 µl) were inoculated into 10 ml of PDB at pH 4.0 for 48 hours before being used.

III.2. Growth measurements

Cells (300 µl) were inoculated into 30 ml of PDB at pH 3.0, 4.0 and 5.0, vortexed, and then 300 µl of PDB at pH 3.0, 4.0 and 5.0 were pipetted into triplicate wells of the 96 well plate. To avoid condensation, desiccant was added to empty wells and the sealing film was sealed only around the edge of the plate to allow for gas exchange. A 20 gauge needle was used to poke a hole for each sample to equilibrate with the dry atmosphere. The samples in the microtitre plate reader were incubated at 30, 37.5 and 45°C. Growth was monitored before incubation (0 h) and every hour for 48 hours. Growth was determined by measuring the optical density (OD) at 650nm in a THERMOmax microplate reader (Molecular Devices Corporation, USA). All experiments were done in triplicate.

III.3. Growth Kinetics

Growth curves were constructed with the software DMFit 1.0 (Institute of Food Research, Norwich Research Park, Norwich, UK; <http://www.ifr.bbsrc.ac.uk>) and an Excel (Microsoft, Redmond, WA) spreadsheet add-in program, and the model of Baranyi and Roberts (1994) were used to calculate the growth kinetics. L = the lag time (hours) and μ = the maximum growth rate (1/seconds) achieved. L and μ were compared for statistical differences using the t-test: paired two samples for means. The data handling was carried out using Excel. Significance was determined at the $P < 0.05$ level. Results were replicated to ensure reproducibility.

Chapter IV- RESULTS AND DISCUSSION

IV.1. Response to pH and temperature

Alicyclobacillus acidoterrestris can grow at high temperatures and low pH with optimum growth around 40°C and pH 4.0. These characteristics are attributed to the ω -cyclohexyl fatty acids in the cell membrane. Spoilage of beverages is problematic in the food industry since products are formulated at low pH and *A. acidoterrestris* thrives in the acidic environment. In order to assess the behavior of growth pH value of *A. acidoterrestris*, we performed growth curves for each strain at pH 3.0, 4.0 and 5.0 that are not only typical for growth, but are also conditions which beverages are formulated. Spoilage of beverages is also problematic to the food industry since the products are pasteurized under high temperatures, which can induce germination of *A. acidoterrestris* spores. In order to assess the minimal growth temperature, we generated growth curves for each strain at 26, 30, 37.5 and 45°C.

The purpose of this study was to evaluate the growth behavior of *A. acidoterrestris* strain N1100, N1102, N1139 and ATCC 49025 in acidified Potato Dextrose Broth (aPDB) over time to determine how the strains behave under different pH and temperature conditions and how the strains behave compared to each other at a given pH.

In order to acquire detailed data about the growth of *A. acidoterrestris* under certain pH conditions, 96-well plates were used to test the four strains under pH 3.0-5.0. None of the strains grew at 26°C and any pH. Under other conditions, the growth and final cell density decreased as the pH values increased. Figure 1 shows growth of strains

N1100, N1102, N1139 and ATCC 49025 over a 48-h period in aPDB at 30°C. As shown in the figure, the population of the cultures increased after about a 10-h lag phase and then reached the stationary phase by the end of the 48-h period. Table 1 shows the final OD for all strains under the various pH and temperature conditions. As pH increased, the final OD for all strains decreased. Strain N1102 was the only strain that had a higher final OD at pH 5.0 compared to the other strains at 30 and 37.5°C (Table 1).

To examine the effect of temperature on the strains of *A. acidoterrestris*, 96- well plates were used to test the four strains at pH 3.0-5.0 over three temperature ranges. Initially temperatures of 26, 33, and 40°C were examined, but no growth was observed at 26°C (data not shown). For the purpose of this experiment different temperature ranges needed to be explored. The next set of temperature ranges that were explored were 30, 37.5 and 45°C. The four strains of *A. acidoterrestris* tested, at pH 3.0, 4.0 and 5.0 showed some differences in the final cell density as seen in Table 1. It is evident that under lower temperature conditions the bacteria need a longer time to adapt (Figures 1, 2 and 3). In all cases, the final OD for the four strains was low, as is typical of *A. acidoterrestris*.

To examine the effect of pH on the four strains, 96-well plates were used to measure the optical density (OD) over 48 hours. There were no differences among the final cell densities of the four strains at pH 3.0, 4.0 and 5.0 at 30°C. However, significant differences ($P < 0.05$) were observed when comparing the final cell density and strains at each pH with the exception of strain N1100 (Table 1). At pH 3.0, strains N1102, N1139 and ATCC 49025 had significantly different final cell densities. Differences occurred between strain N1102 and N1139 at pH 4.0 and between N1139 and ATCC 49025 at pH

5.0. Strains N1139 and ATCC 49025 showed no significant differences in the final cell density at the various pH conditions at 37.5°C (Table 1). The final cell density of strains N1100 and N1102 were significantly different at pH 4.0 and 5.0. Strain N1102 was significantly different from the other strains having a higher final cell density at pH 5.0. There were no significant differences of the final cell density between strains at pH 3.0. At 45°C, strain N1139 showed no significant differences at the various pH conditions. Strains N1100 and ATCC 49025 were significantly different at pH 3.0 compared to pH 4.0 and 5.0. Strain N1102 was significantly different at pH 3.0 and 5.0. There were no significant differences of the final cell density between strains at pH 5.0 (Table 1). The final cell density decreased with increasing pH and increased with increasing temperature and was highest for all strains at pH 3.0 and 45°C.

IV.2. Lag Time of *A. acidoterrestris* strains

The main kinetic parameters (lag time and maximum growth rate) of the bacterial curves are shown in Tables 2, 3 and 4 using DMFit and the model of Baranyi and Roberts (1994). For the four strains of *A. acidoterrestris* tested, the lag time of growth within each strain at pH 3.0, 4.0, and 5.0 did not show any difference. It is evident that under these conditions *A. acidoterrestris* needs a long time to adapt. Lag time is the parameter that most preservation methods aimed at retarding microbial growth rely on (Baty and Delignette-Muller 2004). The slowest lag times varied amongst the different strains when different pH was used in the culture medium.

At all temperatures, significant differences were observed ($P < 0.05$) among kinetic growth parameters of the four *A. acidoterrestris* strains tested. At 45°C there were no significant differences between lag time and strain at pH 3.0 and 4.0. Strain N1100 was the one with the longest lag time at all temperatures, except at 37.5°C where strain N1102 had the longest lag time. In general strain ATCC 49025 showed the shortest lag time at pH 5.0 for all three temperatures compared to the other pH conditions. Strain N1139 showed the shortest lag time for all three temperatures compared with the other strains.

To explore whether the origin of the strain significantly influences their behavior at different conditions, the food isolates were compared to the culture collection strain. When comparing the lag times of strains N1100, N1102 and N1139 to strain ATCC 49025 at a given pH only a few random differences were observed.

At 30, 37.5, and 45°C, there were no significant differences among lag times of the food isolates were compared to strain ATCC 49025 at pH 3.0. However, at 30°C strain N1102 at pH 4.0 and strain N1139 at pH 5.0, the lag times were significantly different ($P < 0.05$) compared to ATCC 49025 (Table 2). At 37.5°C strain N1139 at pH 4.0 and strain N1102 at pH 5.0, the lag times were significantly different compared to ATCC 49025 (Table 3). At 45°C there were no significant differences between lag time and strain at pH 3.0 and 4.0, however, the lag times of strains N1102 and N1139 at pH 5.0 were significantly different compared to ATCC 49025 as seen in Table 4.

Overall, the few significant differences that were observed were random and did not follow any specific trend. Our results also suggest that growth of *A. acidoterrestris* strains is favored at 45°C with decreasing lag times compared to lower temperatures.

IV.3. Maximum Growth Rate (μ_{\max}) of *A. acidoterrestris* strains

The main kinetic parameters (lag time and maximum growth rate) of the bacterial curves are shown in Tables 2, 3 and 4 as calculated by DMFit and the model of Baranyi and Roberts (1994). For the four strains of *A. acidoterrestris* tested, the maximum growth rate (μ_{\max}) within each strain at pH 3.0, 4.0, and 5.0 did not show any difference. In general, the highest growth rates (μ) were obtained when pH 3.0 was used in the culture medium, and lowest with pH 5.0.

The highest growth rates (μ) varied with strain and pH condition. For all cases, the maximum growth rates for all strains were low. There were no significant differences found between the μ_{\max} and the four strains at 30°C for all pH conditions (Table 2). In general, strain ATCC 49025 had the lowest μ_{\max} at pH 5.0 than at pH 3.0 or 4.0. This was true at all three temperatures. Strain N1139 showed the lowest μ for all three temperatures compared with the other strains.

At 30°C there were no significant differences observed between μ_{\max} of the food isolates compared to strain ATCC 49025. At 37.5°C no significant differences were observed at pH 3.0 and 4.0. The maximum growth rate was significantly higher for strain N1102 at pH 5.0 compared to ATCC 49025. Strain N1100 was the only strain at this temperature that has a significantly lower growth rate at pH 5.0 compared to pH 4.0. At 45°C the maximum growth rate for strain ATCC 49025 was significantly higher than strain N1100 and N1139 at pH 3.0. At pH 4.0, strain N1102 had significantly higher maximum growth rate compared to ATCC 49025. Strains N1139 and ATCC 49025 both

display a maximum growth rate that was significantly higher at pH 3.0 compared to pH 4.0 and 5.0.

Overall the few significant differences that were observed were random and did not follow any specific trend. Our results also suggest that growth of *A. acidoterrestris* strains is favored at 45°C with a higher μ_{\max} compared to 30 and 37.5°C.

IV.4. Conclusive Discussion

Fruit based products are a major problem for spoilage because of the unique physical properties of fruits. *A. acidoterrestris* has been isolated from garden and forest soils as well as in fruit juices and may be introduced into the manufacturing process through unwashed or poorly washed fruit (Komitopoulou et al., 1999; Deinhard et al., 1987). It has also been recently isolated from water used as an ingredient in a finished fruit beverage (Komitopoulou et al., 1999; McIntyre et al., 1995). Recognition of *A. acidoterrestris* as a spoilage problem in fruit juices has been increasing and its potential to grow in a variety of fruit juices and other beverages at low pH have been established by many researchers. Although spoilage does not cause illness, it can however, cause consumer rejection of products which results in significant economic loss to the juice and beverage industry. In order to prevent potential spoilage, *A. acidoterrestris* growth characteristics need to be investigated. We have demonstrated that the environmental isolate, ATCC 49025, can be used as a reference strain for the food isolates (N1100, N1102 and N1139).

In this thesis, four *A. acidoterrestris* strains were characterized by examining the growth at various pH and temperature conditions. Comparing the data sets between temperature ranges, 45°C had the shortest lag times and the highest μ_{\max} . Our results suggest that low pH and temperature at 45°C favor the growth of *A. acidoterrestris* (with decreasing lag phase and increasing the maximum growth rate), which makes these conditions inadvisable for formulation. Thus, the conditions in which beverages are formulated and stored are fundamental aspects which need to be controlled to prevent spoilage by *A. acidoterrestris*.

A model strain was determined from the growth kinetics of *A. acidoterrestris* indicating that strain ATCC 49025 could be used as a representative of food isolates. Based on the results of this thesis, the environmental isolate, ATCC 49025, is similar to the food isolates, N1100, N1102 and N1139. ATCC 49025 can be the reference strain used in future work and can be representative of the food isolates.

TABLES

Table 1 Final cell density of *A. acidoterrestris* strains under different pH and temperature conditions.

	Temp	pH	N1100	N1102	N1139	ATCC 49025
Final Cell Density (OD_{650nm})	30°C	3.0	0.21 ± 0.08 ^{abc} _d	0.26 ± 0.04 ^b _e	0.22 ± 0.03 ^a _e	0.29 ± 0.04 ^c _e
		4.0	0.23 ± 0.06 ^{ab} _d	0.24 ± 0.04 ^b _d	0.19 ± 0.03 ^a _{de}	0.24 ± 0.03 ^{ab} _d
		5.0	0.13 ± 0.04 ^{ab} _d	0.26 ± 0.05 ^{ab} _{de}	0.17 ± 0.02 ^a _d	0.23 ± 0.02 ^b _d
	37.5°C	3.0	0.26 ± 0.05 ^a _{de}	0.26 ± 0.03 ^a _{de}	0.24 ± 0.05 ^a _d	0.27 ± 0.009 ^a _d
		4.0	0.28 ± 0.003 ^b _e	0.26 ± 0.02 ^{ab} _d	0.21 ± 0.02 ^a _d	0.23 ± 0.01 ^a _d
		5.0	0.20 ± 0.03 ^a _d	0.31 ± 0.02 ^b _e	0.20 ± 0.01 ^a _d	0.21 ± 0.03 ^a _d
	45°C	3.0	0.41 ± 0.02 ^{bc} _e	0.34 ± 0.03 ^b _e	0.29 ± 0.01 ^a _d	0.38 ± 0.02 ^c _e
		4.0	0.31 ± 0.03 ^b _d	0.26 ± 0.04 ^{ab} _{de}	0.23 ± 0.07 ^{ab} _d	0.24 ± 0.007 ^a _d
		5.0	0.22 ± 0.01 ^a _d	0.21 ± 0.005 ^a _d	0.20 ± 0.02 ^a _d	0.19 ± 0.006 ^a _d

Values are a reflection of experiments done in triplicate.

Means in the same row with no superscripts (a,b,c) in common are significantly different ($P < 0.05$).

Means in the same column with no subscripts (d,e) in common are significantly different ($P < 0.05$).

Table 2 Growth kinetic data for four strains of *A. acidoterrestris* under different pH conditions at 30°C.

	<u>Strain N1100</u>		<u>Strain N1102</u>		<u>Strain N1139</u>		<u>Strain ATCC 49025</u>	
pH	L	μ	L	μ	L	μ	L	μ
3.0	24.3 ^{ab} _d	-0.5 ^a _d	18.2 ^b _d	0.5 ^a _d	16.0 ^a _d	0.4 ^a _d	19.9 ^{ab} _d	1.3 ^a _d
4.0	20.7 ^{ab} _d	0.5 ^a _d	15.9 ^a _d	0.4 ^a _d	10.3 ^{ab} _d	0.2 ^a _d	21.2 ^b _d	0.6 ^a _d
5.0	ND	-1.3 ^a _d	18.0 ^b _d	0.6 ^a _d	13.7 ^a _d	0.4 ^a _d	18.0 ^b _d	0.6 ^a _d

Values are a reflection of experiments done in triplicate.

L = lag time (hours); μ = maximum growth rate (1/hours x 10⁻²).

Means in the same row (for the same kinetic parameter) with no superscripts (a,b,c) in common are significantly different ($P < 0.05$).

Means in the same column with no subscripts (d,e) in common are significantly different ($P < 0.05$).

ND = lag time not determined from DMFit software.

Table 3 Growth kinetic data for four strains of *A. acidoterrestris* under different pH conditions at 37.5°C.

	<u>Strain N1100</u>		<u>Strain N1102</u>		<u>Strain N1139</u>		<u>Strain ATCC 49025</u>	
pH	L	μ	L	μ	L	μ	L	μ
3.0	17.8 ^a _d	1.4 ^a _{de}	18.6 ^b _d	1.8 ^a _d	13.0 ^{ab} _d	0.8 ^a _d	18.0 ^{ab} _d	1.9 ^a _d
4.0	16.8 ^{ab} _d	1.4 ^b _e	15.6 ^{ab} _d	1.6 ^{ab} _d	11.8 ^a _d	0.6 ^a _d	15.5 ^b _d	1.9 ^{ab} _d
5.0	14.3 ^{ab} _d	0.4 ^a _d	18.8 ^b _d	1.9 ^b _d	ND	0.2 ^a _d	15.1 ^a _d	0.5 ^a _d

Values are a reflection of experiments done in triplicate.

L = lag time (hours); μ = maximum growth rate (1/hours x 10⁻²).

Means in the same row (for the same kinetic parameter) with no superscripts (a,b,c) in common are significantly different (P < 0.05).

Means in the same column with no subscripts (d,e) in common are significantly different (P < 0.05).

ND = lag time not determined from DMFit software.

Table 4 Growth kinetic data for four strains of *A. acidoterrestris* under different pH conditions at 45°C.

pH	<u>Strain N1100</u>		<u>Strain N1102</u>		<u>Strain N1139</u>		<u>Strain ATCC 49025</u>	
	L	μ	L	μ	L	μ	L	μ
3.0	11.0 ^a _d	1.1 ^a _d	12.2 ^a _d	1.9 ^{ab} _d	9.5 ^a _d	0.8 ^a _e	13.9 ^a _d	3.6 ^b _e
4.0	13.5 ^a _d	4.5 ^{ab} _d	11.8 ^a _d	2.9 ^b _d	9.4 ^a _d	0.4 ^a _d	ND	0.6 ^a _d
5.0	13.4 ^{abc} _d	0.4 ^a _d	11.2 ^b _d	1.2 ^a _d	16.7 ^c _e	0.3 ^a _d	7.4 ^a _d	0.5 ^a _d

Values are a reflection of experiments done in triplicate.

L = lag time (hours); μ = maximum growth rate (1/hours x 10⁻²).

Means in the same row (for the same kinetic parameter) with no superscripts (a,b,c) in common are significantly different ($P < 0.05$).

Means in the same column with no subscripts (d,e) in common are significantly different ($P < 0.05$).

ND = lag time not determined from DMFit software.

FIGURES

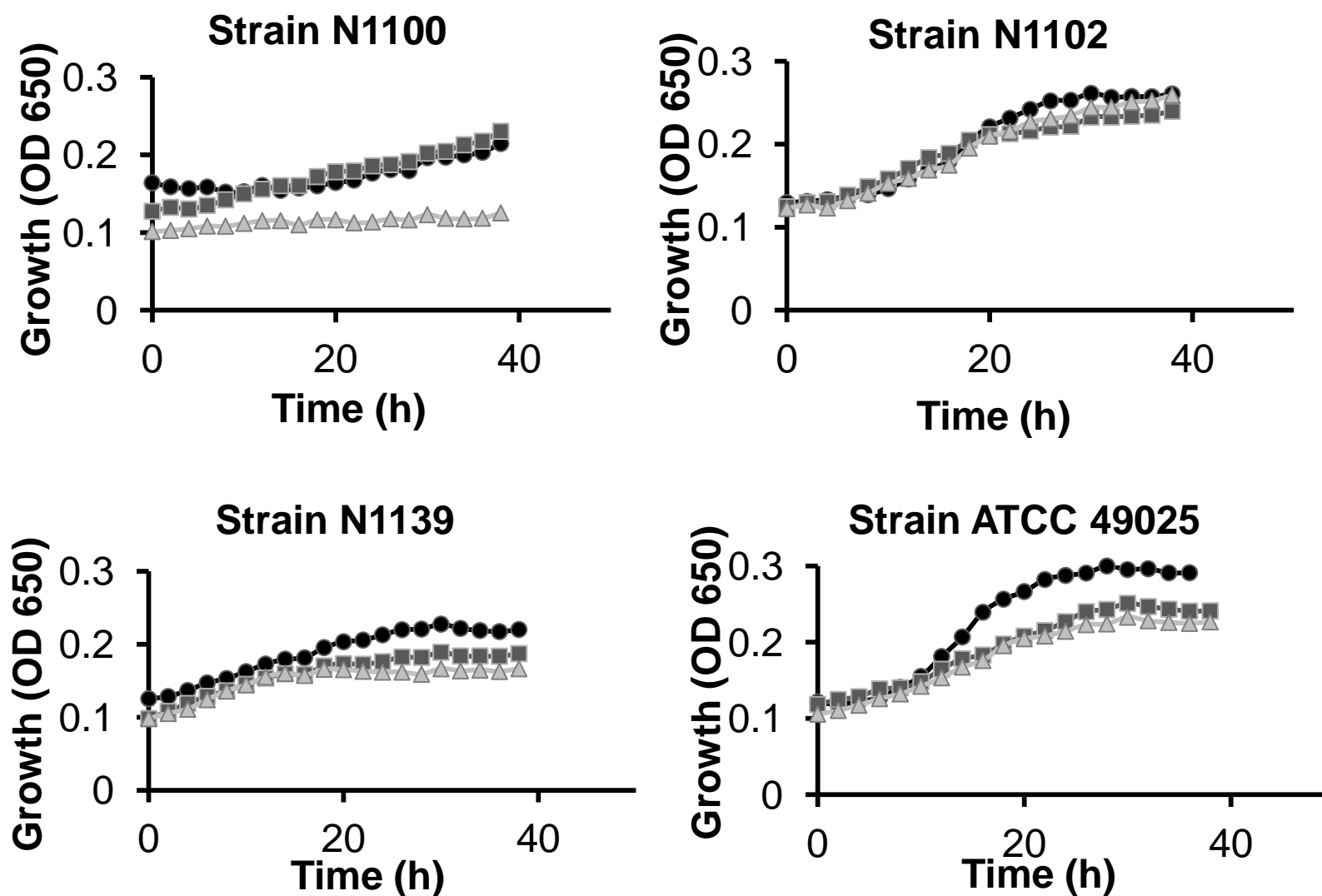


Figure 1: Growth of *A. acidoterrestris* strains at pH 3.0 (●), 4.0 (■), and 5.0 (▲) at 30°C. Values are reflective of experiments performed in triplicate.

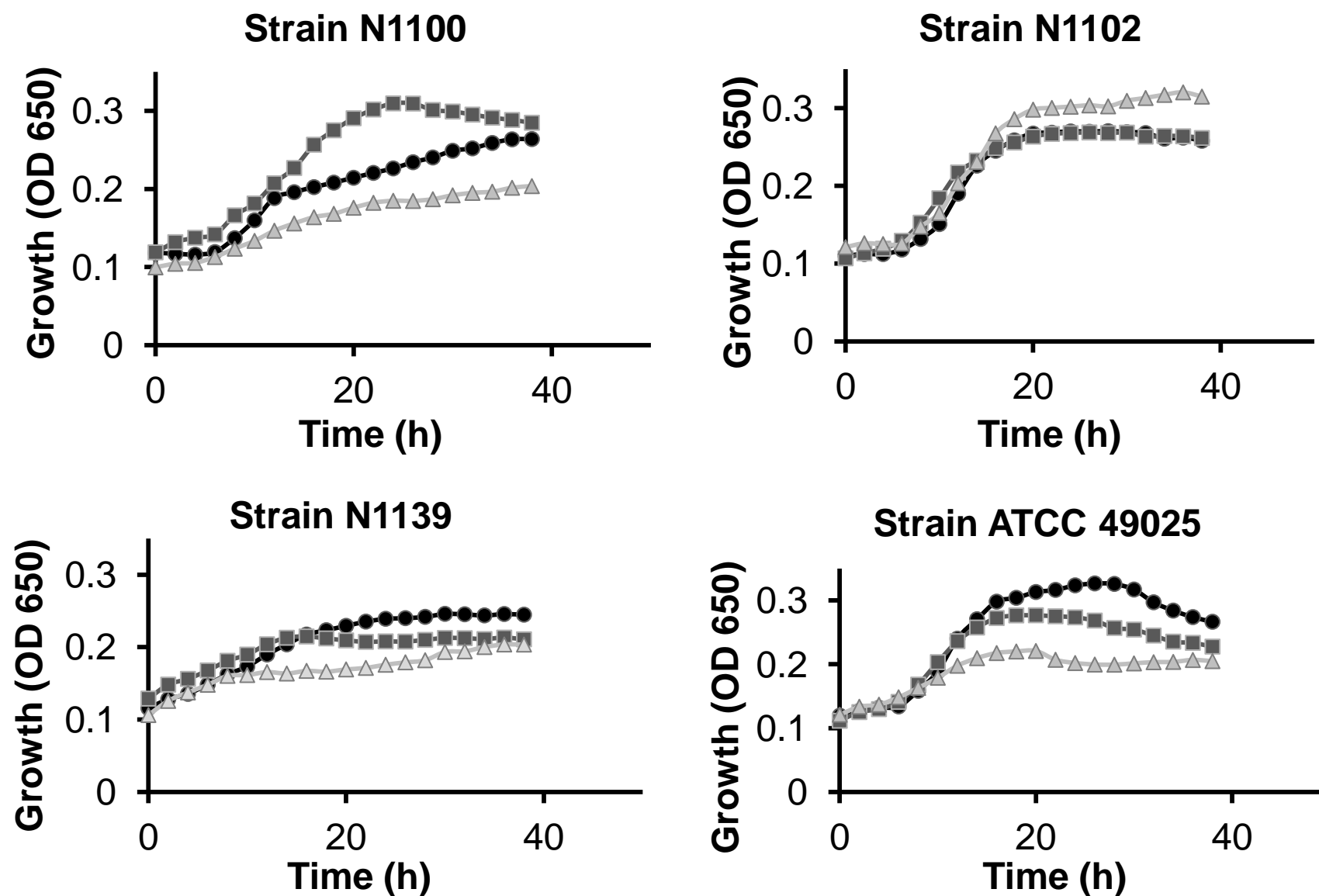


Figure 2: Growth of *A. acidoterrestris* strains at pH 3.0 (●), 4.0 (■), and 5.0 (▲) at 37.5°C. Values are reflective of experiments performed in triplicate.

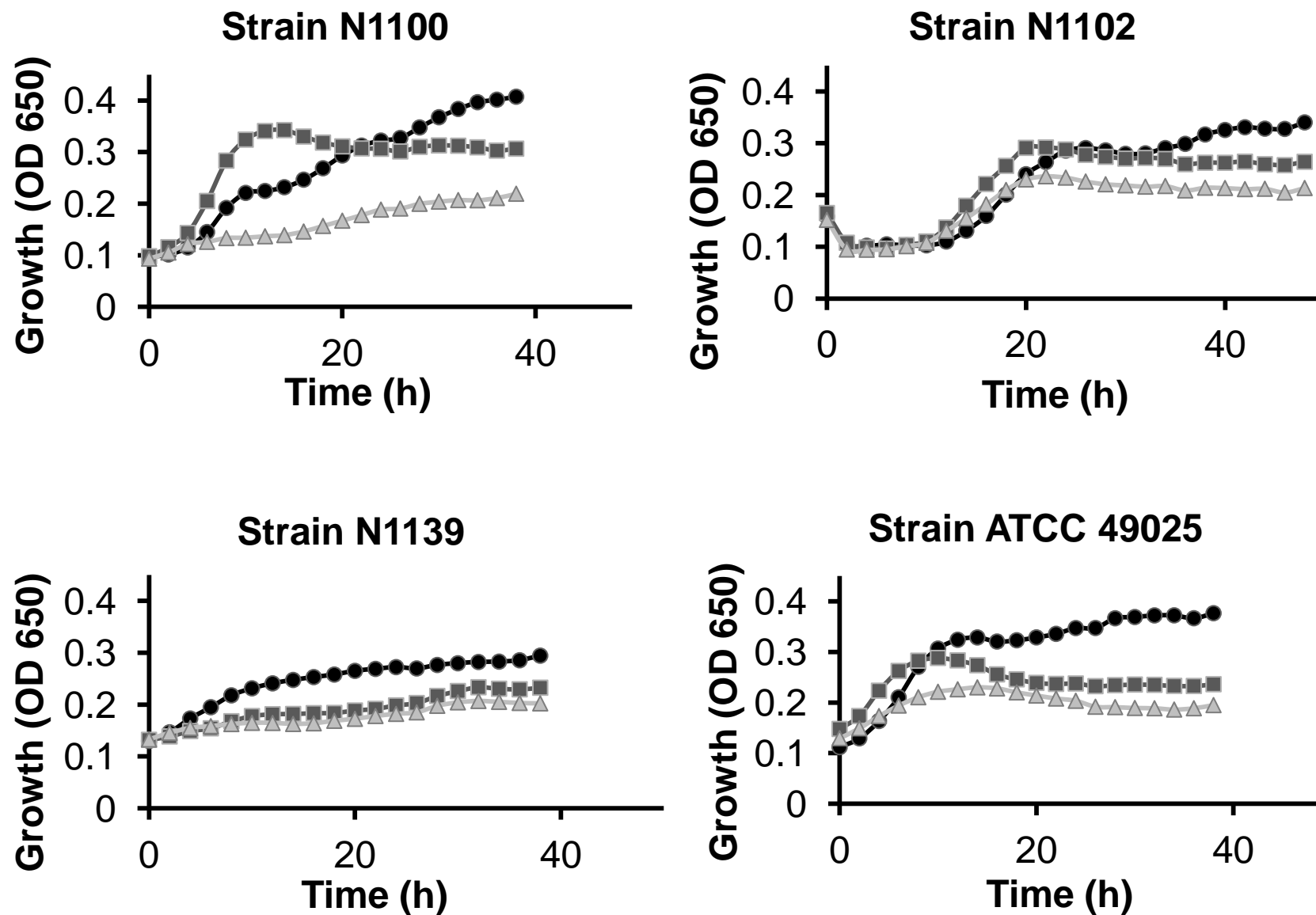


Figure 3: Growth of *A. acidoterrestris* strains at pH 3.0 (●), 4.0 (■), and 5.0 (▲) at 45°C. Values are reflective of experiments performed in triplicate.

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