CLAUDIA MARIA MATUTE CORNWELL
Fate of *Salmonella* Montevideo, *Salmonella* Gaminara, and *Salmonella* Poona in
Homemade Unpasteurized Fruit and Vegetable Juices
(Under the direction of MARK A. HARRISON)

The effects of acid adaptation on the survival of *Salmonella* spp. in acidic

unpasteurized fruit and vegetable juices stored at 3 temperatures, and the survival of *S.*

Poona in unpasteurized cantaloupe juice were studied. Juices with pH ranges of 3.0 to 4.3

were inoculated with 10^8 CFU/ml of acid adapted (grown in tryptic soy broth

supplemented with 0.4% dextrose overnight) and non-adapted (grown in TSB overnight)

cells, stored at 4, 10, and 20°C, and sampled every 24 h up to one week. Samples were

enumerated on bismuth sulfite (BSA) and plate count agar supplemented with nalidixic

acid. The same procedure was followed with cantaloupe juice except: only non-adapted

cells were used and samples were enumerated only on BSA. Adapted and non-adapted

cells had no significant difference on survival. The highest lethality of *Salmonella*

occurred in the most acidic juice. In cantaloupe juice, *S.* Poona was able to multiply at

20°C.

INDEX WORDS: Acid tolerance response, Acid adaptation, *Salmonella* Montevideo,

Salmonella Gaminara, *Salmonella* Poona, Unpasteurized fruit and

vegetable juices, Cantaloupe juice

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by

CLAUDIA MARIA MATUTE CORNWELL

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Approved:

Major Professor: Mark A. Harrison

Committee: Joseph F. Frank
                  Ronald R. Eitenmiller

Electronic Version Approved:

Gordhan L. Patel
Dean of the Graduate School
The University of Georgia
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DEDICATION

I dedicate this thesis to my parents: Marco Matute and Maria del Carmen de Matute, and to my husband Robert Cornwell for all the love, support, and motivation they have given me throughout my college years.

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First of all, I would like to thank my parents for all the love and support you have given me throughout the years. I know you had to work very hard and sacrifice a lot for me to be here and I want you to know how much I appreciate it. I'm proud to have you as my parents!

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CHAPTER I

INTRODUCTION

The standard American diet is failing to meet the recommended daily levels for the consumption of fruits and vegetables. Nutritionists say that an easy way to reach these levels is to simply drink a 16 oz glass of fresh fruit and/or vegetable juice each day. Unfortunately, these freshly made juices are not pasteurized and, therefore, have a higher risk for presence of pathogens.

Over the past years, the number of foodborne outbreaks involving fresh fruit and vegetables as well as unpasteurized fruit juices has been rising. These "untreated" juices account for an estimated 6,200 illnesses each year in the U. S. alone. Pathogens such as *Salmonella* spp. have been found in orange juice causing foodborne salmonellosis amongst consumers and in some cases death. This contradicted the previous beliefs that citric juices contained high enough levels of acidity to make them a safe product for consumers. Several studies have shown that certain species of bacteria such as *Salmonella* spp. and *E. coli* O157:H7 have the ability to adapt to highly acidic conditions. This survival mechanism is called acid tolerance response and it activates when cells are first grown at mild external pH values of 5.5 to 6.0 and then transferred to severely acidic conditions.

Salmonella related outbreaks have also occurred in low acid fruits and vegetables. Many outbreaks involving *S*. Poona in cantaloupe have occurred in the past decade. The biggest one was in 1991 and more than 400 people were affected. The last cantaloupe

outbreak took place earlier this year. Melons are particularly susceptible to pathogen contamination since they grow on the ground.

Given the incidence of *Salmonella* spp. in unpasteurized juices and in cantaloupe, the purpose of this project was to compare the survival pattern of acid adapted versus non-acid adapted *S.* Montevideo in homemade tomato juice and *S.* Gaminara in a variety of highly acidic homemade fruit and vegetable juices stored at 4, 10, and 20ºC; as well as study the fate of *S.* Poona in homemade cantaloupe juice stored at the same temperatures.

CHAPTER II

LITERATURE REVIEW

Homemade juices:

According to the U.S. Second National Health and Nutrition Examination survey, the standard American diet is lacking adequate levels of fruit and vegetables. Less than 10% of Americans met the recommended 2 fruit and 3 vegetable daily servings. Only half of the U.S. population ate one garden vegetable a day and an alarming 41% did not eat any fruits nor fruit juices during the day (*11*). However, in 1998, DeRoever reported a 27% increase in the consumption of fresh produce in the U.S. (*29*). Americans are finding new ways to change their dietary habits and consume more fruits and vegetables daily. Drinking more fresh fruit and vegetable juices is one effective way. The popularity of juices is reflected by the growing number of juice bars appearing across the nation as well as the expanding variety of juices seen on the menus of trendy restaurants (*64*). This trend is more evident among health conscious and/or vegetarian individuals.

The idea of home juicing became popular, in part, due to individuals like Jay Kordich, best known as "The Juiceman", who claims his cancer was cured after drinking large amounts of homemade carrot/apple juice each day (*55*). Since then, he has created his own line of Juiceman juice extractors and advertised the many benefits of juicing.

Advantages of juicing:

Over the past few years, "juicing" has become more and more popular amongst the American household. Juice lovers claim that drinking fresh fruit and vegetable juices

has various health benefits such as increased energy, strengthened immunity, reduced risk of heart disease, cancer, and stroke, stronger bones, and improved skin complexion. Juicing also provides a quick and easy way to meet the daily recommended servings of fruits and vegetables; a 16 oz glass of carrot juice contains the equivalent amount of nutrients present in approximately 3 pounds of carrots (*55*). Since fresh juices are not pasteurized, nor do they go through any other type of heat treatment, people that consume them get a higher amount of vitamins than those that consume commercial pasteurized juices.

Disadvantages of Juicing:

Consuming homemade and/or unpasteurized juices also has its disadvantages. As earlier explained, one of the main reasons people choose to juice their own fruits and vegetables, or drink unpasteurized juices is for the high amounts of nutrients they posses. However, some of them may not know that in order to get the maximum health benefits, fresh juices must be consumed immediately since they may loose some nutritious value during storage (*55*). People that prepare juices at their home, may not give the proper attention to safety (such as hand washing, appropriate method for washing of produce, and cross-contamination) increasing the risk of contaminating the juice. Since the juices do not go through a heat treatment, nor do they have preservative substances, they have a higher amount of microorganisms present. If foodborne pathogens are present then they have a higher risk of causing foodborne illness. Another disadvantage unpasteurized juices have is a shorter shelf life. Generally, commercially pasteurized orange juice lasts at least a month, while fresh orange juice lasts only 7 to 9 days if properly stored at 4°C (*71*).

*Salmonella***:**

      Salmonella was first discovered and named after Daniel E. Salmon, an American veterinary scientist in 1885 (*21, 2*). It is a gram-negative, rod-shaped, non-sporeforming bacterium belonging to the Enterobacteriaceae family. It is facultatively anaerobic in nature and, with the exceptions of *S*. Gallinarum and *S*. Pullarum, nonmotile. Today the number of known strains of *Salmonella* total over 2,700 and new strains are continually recognized (*9*).

      Salmonella grows at an optimum temperature of 37°C and may survive at temperatures as high as 54°C, nevertheless it has proven to posses some psychrotrophic qualities since it has the ability to grow at temperatures as low as 2°C (*27*). It is also able to propagate at a pH ranging from 4.5 to 9.5 (6.5 – 7.5 optimum) (*31*); however, studies have shown that if *S*. Typhimurim is exposed to a mild pH (5.5 - 6.0) for a short period of time and subsequently transferred to pH values lower than 4.5, its chances of survival will increase (42). This behavior is called acid tolerance response (ATR) and will be further discussed later on in this chapter. *Salmonella* spp. may not grow in foods with an a_w value less than 0.93 (*31*).

      The disease caused by *Salmonella* spp. is generally known as salmonellosis. The infectious dose for *Salmonella* in some cases is as large as 10^8 to 10^9 cells. However, doses as low as 1-10 cells have been reported to cause symptoms (*23*). The infectious dose amount depends on a variety of factors such as type of strain, food, and stomach contents, health, and age of host. Acute symptoms such as diarrhea, nausea, abdominal cramps, and fever usually occur within 6 to 48 hours of consumption and generally last for 1 to 2 days; however, this time may be lengthened depending on the factors

previously mentioned (*38*). Children, the elderly, and other persons with immune system deficiencies such as AIDS and cancer patients are most at risk. Persons with severe diarrhea may require rehydration, often with intravenous fluids. Antibiotics are not usually necessary unless the infection spreads from the intestines.

A more severe form of *Salmonella* infection which accounts for less than 2.5% of all cases in the U.S. is typhoid fever. It usually lasts for about 1 to 8 weeks and is primarily caused by *S.* Typhi. Serious symptoms immerge such as septicemia, high fevers, and in most cases, pink spots in chest and trunk, and blood from nose and bowel appear. Enteric fever is a less severe form of typhoid fever. Spots and bleeding do not occur, and it only lasts for up to three weeks (*23*). Ten percent of typhoid fever cases result in death as opposed to less than 1% for other forms of salmonellosis cases (*38*).

Salmonella spp. are naturally found in animal and human gastrointestinal tracts in the environment in contaminated water, soil, manure, insects, food contact surfaces, seafood, reptiles and birds. Other sources of infection may be person to person contact, cross-contamination, or consumption of raw meats, eggs, and poultry (*23*).

Infiltration of *Salmonella* in Produce:

Although *Salmonella* is better known for its association with poultry and egg products, the number of foodborne outbreaks involving *Salmonella* in fresh produce, and in unpasteurized fruit and vegetable juices has been rising. Therefore, several studies have been conducted to further investigate the ways *Salmonella* as well as other human pathogens come in contact with fruit and vegetables.

The natural structure of certain produce such as the calyx and stem in the apple, are where the highest amounts of microorganisms are present (22). These may penetrate produce through the calyx, or through tissue damage such as punctures, wounds, cuts, and splits caused during ripening, harvesting or processing (*36*). The presence of bacterial soft rot in produce may increase the chances of pathogen contamination. In a 1997 study, Wells and Butterfield detected *Salmonella* spp. in 18 to 20% of soft-rotted samples of cantaloupe, carrots, tomatoes, sprouts, beans, broccoli, lettuce, onions, peppers, and potatoes as opposed to only 9 to 10 % of intact vegetables samples (*70*). Vegetables such as celery and tomatoes have proven to be at higher risk for bacterial soft rot (*63, 65*).

Insects such as houseflies may act as vectors for pathogens in damaged fruits and vegetables. Houseflies may carry as many as 100 different pathogens and can spread 65 of them. One of the human pathogens isolated from these insects is *S.* Typhimurium (*58*).

Another way *Salmonella* may infiltrate fruits and vegetables is by absorption of contaminated water. Several studies have shown that if certain fruits are placed in water several degrees cooler than the fruit itself, they absorb some of it. For example, in a 1995 study a higher amount of *Salmonella* cells were found in tomatoes that had been placed in contaminated water 15ºC cooler than the fruit (*71*). The same results were obtained when apples were submerged in cooler water inoculated with *E. coli* O157:H7- another human pathogen closely linked to fresh fruits and vegetable as well as juice outbreaks (*13*). This contact with water may occur during processing or washing of the produce, on the field with heavy rain, or even at home.

Cross contamination of produce may also take place in restaurants, supermarkets, or households. One very common mistake is using the same utensil to cut meat and fruit.

***Salmonella* outbreaks involving fruits and vegetables and unpasteurized juices:**

The very first record of a *Salmonella* outbreak related to an unpasteurized fruit drink dates back to 1923, when 24 people became ill with typhoid fever after drinking apple cider. It was later found that the apples used to produce the cider had been previously rinsed in water contaminated with *S*. Typhi taken from a nearby stream (*59*). Cross-contamination is one of the many ways produce may become contaminated with *Salmonella*. In 1944, an asymptomatic worker with typhoid fever, prepared some orange juice at a hotel's restaurant in Ohio. As a result, 18 people became sick and one died (*60*).

During the summer of 1995, several people (63 cases in 21 states) became sick with salmonellosis by drinking unpasteurized orange juice at a popular theme park in Orlando, Florida. The source was traced back to a citrus juice processing plant in Florida were *S*. Hartford, *S*. Gaminara, and *S*. Rubislaw were found in unopened bottles of juice, as well as in different parts of the processing facility (*17, 24*). This outbreak is of significant importance since it was the first time that a *Salmonella* outbreak was associated with a citrus processing facility. It was previously believed that citrus juices contained a high enough level of acidity to make it a safe product for consumption (*61*).

Since then, several other *Salmonella* outbreaks associated with citrus juices have come about. Perhaps the biggest one occurred in June 1999. People in 15 U.S. states and 2 Canadian provinces were affected by *S*. Muenchen. Three hundred people became ill and one died after drinking frozen or fresh unpasteurized orange juice produced by the Sun Orchard Company in Arizona (*19*). Isolates were found in unopened bottles of juice, and in storage vats from the packaging plant. However, the suspected source of contamination was orange juice containing melted ice that was imported from Mexico

(*56*). Another outbreak linking citrus juice to *Salmonella*, was the April 2000 outbreak of *S.* Enteritidis in 7 U.S. states. Seventy-four people were sick after consuming unpasteurized lemonade, grapefruit juice and orange juice produced by California Day-Fresh Foods.

Other acidic fruits have also been associated with *Salmonella* spp. outbreaks. Two outbreaks involving tomatoes occurred between 1990 and 1993. The first one was caused by *S*. Javiana, which resulted in 174 cases traced in 4 U.S. states. In the latter outbreak, 84 people were affected in 3 different states by *S*. Montevideo (*3*). Unfortunately, the source of contamination was never determined.

Foodborne salmonellosis has also been associated with low acid fruits such as watermelons and cantaloupes. These particular types of fruit are cause of concern because they present a higher risk of pathogen contamination since they grow on the ground. A field study conducted by the FDA showed that 0.76% (in 1990) and 1.06% (in 1991) of melon rinds from imported melon samples harbored a variety of *Salmonella* spp. on their surfaces (*45*). In 1990, the number of people affected by an outbreak involving cantaloupe could have been lowered if handler would have cleaned the rind thoroughly before cutting the fruit, instead, *S*. Chester infected 245 people (2 died) in 30 states (*18*). An outbreak of the same nature happened a year later. *Salmonella* Poona was the causative organism, and more than 400 people in 23 different U.S. states and Canada got sick after consuming cantaloupes grown in Texas (*3, 16*). Three *Salmonella* outbreaks (*S*. Oranienburg, *S*. Miami, and *S*. Bareilly) have been associated with pre-cut watermelons. In these cases, it is possible that the fruit was left out at room temperature for a long time,

thus, giving *Salmonella* cells time to multiply (*15, 44*). Again, the pathogen could have been present on the rind, or on the knife used to slice the watermelons.

Golden, et. al. conducted an experiment to study the growth of five different strains of *Salmonella* in the inside tissues of cantaloupe, watermelon, and honeydew melons stored at 5 and 23ºC (*45*). Their results showed that *Salmonella* cells from melons stored at 23ºC grew rapidly after 24 hours. Those in watermelon grew approximately one log more than the cells in cantaloupe and honeydew, and little or no growth resulted from 24 h incubation of *Salmonella* spp. in melons stored at 5ºC.

Many of these outbreaks, if not all, could have been avoided if good agricultural practices (GAPs) and/or good manufacturing practices (GMPs) had been followed. Simple safety measures such as washing fruits and vegetables prior to consumption or juicing, washing hands after going to the bathroom and before handling food, avoidance of food handling while sick, and keeping produce and homemade juices at the proper storage temperatures (4ºC) could greatly reduce the risk of food poisoning. Washing produce with a mild soap and/or mild bleach solution (1 tsp. bleach/ liter of water) will further decrease the numbers of potentially hazardous microorganisms (*4*).

A very effective way to eliminate the presence of pathogens in juices is to include a heat treatment step in the manufacturing process such as pasteurization. However, these juices would no longer be considered "fresh". Although pasteurization may destroy microorganisms present in the juice, this does not mean that they are 100% safe to drink; a slim possibility of cross-contamination still exists. An alternate mean of reducing risk of food poisoning, is irradiation of produce. Some orange juice processors are

implementing the use of ultraviolet irradiation technology to achieve the FDA's required

5-log pathogen reduction law (*37*).

FDA laws and regulations for safety of unpasteurized fruit and vegetable juices:

Of all juices produced annually in the U.S., 98% are pasteurized or treated in an

equivalent fashion, hence, posing little to no health risks to consumers. The remaining

2% are non-pasteurized, and although the percentage seems low, it accounts for 38

million gallons. If this amount is converted to individual servings, the number increases

to more than 600 million per year. These "untreated" juices are responsible for an

estimated 6 to 6.2 thousand illnesses per year (*50*).

Taking these statistics into consideration, and given the fact that the incidents of

outbreaks of foodborne salmonellosis and other pathogens related to unpasteurized juices

greatly increased, on November 1996 the U.S. Food and Drug Administration (FDA) met

to review the science currently available for the production of fresh juices and to consider

what measures should be taken into consideration to make them safer. A month later in a

public hearing, the National Food Processors Association (NFPA) and other associations

urged FDA to make pasteurization of fresh juices mandatory (*57*). After publishing a

notice of intent and taking all comments into consideration, FDA published two

proposals on April 24th 1998: a HACCP proposal (*34*), and a proposal to require a

warning label be placed on commercial unpasteurized fruit and vegetable juices (*33*).

Warning labels were proposed as a temporary mean to make consumers aware of

the risks taken when consuming unpasteurized juices since it would take time for the

HACCP plans to be fully implemented in juice processing plants. Juices that have been

processed to achieve a 100,000 fold (5 log) reduction in pathogenic microorganisms were exempted from the warning label proposal as were juices produced in establishments for immediate consumption (*33, 35*). Four months later, a final labeling rule was published which came into effect on September 8, 1998 for apple juice and cider producers, and on November 5, 1998 for all other unpasteurized juices. On January 1, 2000, FDA required all warnings to appear on the information panel of all unpasteurized juices except for juice processors with less than 500 employees (*33*) whom would have to enforce the rule a year later. The warning label chosen states: "This product has not been pasteurized and therefore may contain harmful bacteria that can cause serious illness in children, the elderly, and persons with weakened immune systems" (*33*). This label proved to be the most effective since it had understandable language by all consumers, it stated the risks that unpasteurized juices posses, as well as the population at highest risk for illness.

On January 19, 2001 the final rule on HACCP for juice processors was published. This rule requires all juices and juice ingredients in beverages be processed under a HACCP plan. The term "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" (*1*). This final rule will become effective one year after the publication date for large processors, after two years for small plants, and three years for very small juice manufacturers. Juices not processed according to regulation after the effective dates will be considered adulterated. This final rule, however, does not apply to harvesting, picking, or transporting raw ingredients of juice products nor those retail stores that make and sell juice directly to consumers and are addressed in the FDA Food Code (*1*).

These FDA regulations have caused a great deal of controversy amongst organizations such as the NFPA. The cause of disagreement revolves around the fact that NFPA wants pasteurization to be a requirement for all juices. Since the vast majority of juice outbreaks are attributed to the 2% of juices that are not pasteurized, NFPA believes that pasteurization would solve this problem. However, FDA refuses to make pasteurization a requirement because it claims that it would stop all incentives to develop alternative methods to pasteurization (*6*) and instead implemented the warning label which will only reduce the number of juice related illnesses by 5 – 16%. On the other hand, for the 98% of juices that are pasteurized and do not pose a health threat, FDA has, according to Kelly Johnston, the executive vice president of the NFPA, required "a whole new layer of inspection, regulation, and bureaucracy" with the proposal of the required HACCP plan for all juice processors (*50*).

ATR:

Foster and Hall (1990) define acid tolerance response, or ATR, as a survival mechanism that activates when certain bacterial cells are grown at mild external pH values of 5.5 to 6.0 which protects the cells from severe acid stress (*41*). There is evidence that this response to acid stress occurs with several species of bacteria including *Escherichia coli, Listeria, Streptococcus, Enterococcus* spp., and of course in *S.* Typhimurium (8*, 46, 51, 62, 40*). Foster and Hall found that when the growth medium was adjusted to a pH value of 3.3, the numbers of "adapted" cells (grown at pH 5.8) of *S.* Typhimurium LT2 exceeded the "unadapted" cells (those grown at pH 7.6) by three logs after only 80 minutes (*41*).

Although ATR's complete mechanism is not yet fully known, *S*. Tyhimurium experiences ATR in two steps: preshock, and acid shock (*39*). The preshock occurs when cells in the exponential phase are first adapted to a mild external pH for one or two cell doublings. During this stage, 12 "preshock ATR proteins" are produced and 6 repressed (*41, 39*). In acid shock, the cells are transferred from alkaline conditions immediately into a highly acidic environment. Throughout this stage, the bacterial cells stop reproducing and change the expression of at least 52 proteins (37 synthesized and 15 repressed). Forty- eight out of these 52 proteins are completely different than those expressed during preshock. Both of these steps in ATR need to be followed in order for *S*. Typhimurium cells to be able to withstand severe acid stress (*39*).

Several studies have shown that ATR not only protects *S*. Typhimurium against acid stress, it may also increase virulence of the pathogen and induces a cross-protection effect against other environmental stresses such as heat and higher salt concentrations (*40, 7*). Leyer and Johnson showed that after 20 minutes of exposure to 50°C temperatures, acid adapted cells experienced a 10- fold difference in cell death compared to non-acid adapted cells; this difference more than doubled after 60 minutes (*52*). Since *Salmonella* spp. have been known to adapt to high osmolarity environments with the help of starvation conditions (*26, 49*), Leyer and Johnson conducted an experiment to see if acid adaptation had an effect on osmotic stress protection. Their study showed that after *S*. Tyhimurium was exposed to 2.5 M of NaCl, non-acid adapted cells died off rapidly and no viable numbers were detected after 10 days. On the other hand, acid adapted cells were still present after 21 days.

No studies have been made to show the effect of acid adapted vs. non-adapted *Salmonella* spp. in unpasteurized fruit and vegetable juices. However, ATR may have an effect on the survival of certain pathogens in juices with highly acidic pH values.

Costs Associated with *Salmonella* Outbreaks:

A high number of salmonellosis cases are never reported, therefore, it is very difficult to estimate the actual financial costs. Years ago, estimates were made by the USDA's Economic Research Service (ERS). The numbers were not very accurate given the fact they only accounted for the reported annual numbers of illnesses and the medical and productivity costs associated with them. The Foodborne Diseases Active Surveillance Network (FoodNet) was created by the CDC, FDA, and USDA in 1995 to better monitor the incidence of foodborne illnesses in the U.S. It closely monitors the incidence of certain foodborne pathogens in 9 sites that account for 10% of the U.S. population. FoodNet was not only created to determine the amount of unreported cases of foodborne illness incidents, but also to pinpoint the source, and to develop an action plan against these pathogens (*20*).

An estimated 1.4 million cases of salmonellosis occur in the U.S. per year according to FoodNet, and it is estimated that 97% of these are not reported (*43*). Of these, 1.3 million cases (95%), according to the CDC, are food related. *Salmonella* infections account for 9.7% of the total amount of foodborne illnesses caused by known pathogens, 25% of all foodborne hospitalizations, and 30.6% of foodborne deaths. (*53*).

Patient related costs of salmonellosis cases range from $275 million to $1.1 billion per year in the U.S. Two approaches are available to estimate costs due to loss of

productivity: the human approach and the labor market approach. The first approach

estimates costs of a premature death to be $17,000 – $2.2 million depending on age at

time of death (*43*), while the labor market approach estimates the costs to be much

higher: $1.4- $8.3 million for males and $1.6 - $8.5 million for females also depending

on age (*69*). Economists have yet to agree on the best approach. Regardless of the

approach, the average costs for salmonellosis cases are $5,460 for each hospitalized case,

$315 per doctor visit, and $24 for each case recovering without the use of medical care

(*43*).

Objective:

Given the incidence of foodborne salmonellosis, the ability of *Salmonella* cells to

adapt to high acid environments, the added risks for potential contamination of fresh fruit

and vegetable juices, and the high costs associated with this foodborne pathogen, further

studies are needed to better understand its survival mechanisms. Therefore, the objective

of this project was to compare the survival of acid adapted versus non-acid adapted *S.*

Montevideo in homemade tomato juice and *S.* Gaminara in a variety of highly acidic

homemade fruit and vegetable juices stored at three different temperatures (4, 10, 20ºC);

as well as to study the fate of *S.* Poona in homemade cantaloupe juice stored at the same

temperatures.

CHAPTER III

MATERIALS AND METHODS

Bacterial Cultures:

Stock cultures of *S.* Montevideo G4639 (tomato outbreak isolate), *S.* Gaminara F2712 (orange juice outbreak isolate), and *S.* Poona (cantaloupe isolate), all obtained from the Center for Food Safety (Griffin, GA), were grown for 24 h on tryptic soy agar slants (Difco Laboratories, Detroit, MI, USA) and then stored at 4°C. The first two strains were used to conduct acid adaptation studies in various juices, while the latter was utilized in the cantaloupe juice study.

Preparation of Non-Acid Adapted Cultures:

Prior to inoculating the juices, *S.* Montevideo and *S.* Gaminara cells were adapted to be nalidixic acid (NA) resistant. A loop full of both *Salmonella* cultures was transferred from the stock culture slant to 9 ml of tryptic soy broth (TSB) without dextrose (Difco) supplemented with 0.05 ml/L of nalidixic acid (Sigma, St. Louis, MO, USA), and then incubated at 37°C for 18 – 24 h. One ml portions of these fresh cultures were transferred into another 9 ml of TSB without dextrose supplemented with NA and once again incubated at 37°C for 18 – 24 h (to 10^{7-8} cfu/ml). The final pH after growth was approximately 7.2 for both strains. Before inoculating juice with *S.* Poona, a loop full of the stock culture was inoculated into 9 ml of TBS without dextrose and grown for 24 h at 37°C. After inoculation, 1 ml was transferred into a fresh 9 ml tube of TSB without dextrose followed by incubation for 24 h at 37°C.

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Preparation of Acid Adapted Cultures:

After the first 24 h period, fresh cultures (1 ml) of *S*. Montevideo and *S*. Gaminara were also transferred from the TSB without dextrose (supplemented with NA) to 9 ml of TSB supplemented with NA and 0.4% dextrose (Fischer Scientific, Fair Lawn, N.J.) and incubated at 37°C for 18 – 24 h (to 10^{7-8} cfu/ml) to reach a pH of 5.5 – 6.0.

Both acid adapted and non-adapted cells were collected by centrifugation (Sorvall® Instruments RC-5B Refrigerated Superspeed Centrifuge, Dupont, Wilmington, DE, U.S.A.) at 8,300 x g for 15 min. After decanting, the remaining pellet was resuspended in 10 ml of 0.1% peptone water (Difco) and centrifuged for another 15 min. The pellet was again resuspended in 0.1% peptone and used to inoculate the designated juices.

Juice Preparation:

Five different types of juices were made using a homestyle fruit/vegetable juicer, Juice Extractor model #3165 (Oster®, Delray Beach, FL). All ingredients were purchased from the same local Athens, GA grocery store. The first four juices were prepared using directions described by Rodnitzky (*64*) while the cantaloupe juice was prepared according to the methods found in the booklet: *Fresh Juice Recipes and Menu Planer* (*5*). For each juice type prepared, 100 ml portions were dispensed into sterile, capped 100 ml media bottles.

Tomato Juice:

Vine-ripened tomatoes were hand washed with antibacterial soap and rinsed with warm water. The stem and butt were removed as well as any bruised flesh. The rest of the tomatoes were then cut into segments small enough to fit through the juicer and juiced.

Apple - Celery Juice Mix:

Six Granny Smith apples, 6 celery stalks, and 6 small limes were used per batch. Apples and limes were washed with antibacterial soap and rinsed with warm water. The apples were peeled, cored, and cut into eighths. Celery stalks were washed with warm water to remove visible impurities, leaves were discarded, and about 1cm was trimmed from the bottom and top of the stalks. They were then cut into 5 to 7 cm length pieces. All ingredients were added to the juicer except limes which were cut in half and juiced manually prior to mixing the juices together.

Carrot Juice Mix:

Nine small carrots, 3 Granny Smith apples, and 3 navel oranges were used per batch. Prior to juicing, the carrots were washed with warm water, the tops were removed, they were peeled, and cut into 5 – 7 cm length pieces. Apples were washed, peeled, and cut into eighths as described above. The carrot and apple pieces were juiced with the juicer. Oranges were washed with warm water and soap, cut into halves and juiced manually and combined with the carrot and apple juice.

Strawberry – Apple Juice:

Three cups of strawberries were washed with warm water, the leaves were removed, and any visible damage to the fruit was cut off while 6 Granny Smith apples

were prepared as previously described per batch with the apples and berries alternated when added to the juicer.

Cantaloupe Juice:

Ripe cantaloupe was scrubbed under warm water with a small brush and antimicrobial soap. The fruit was then cut into strips small enough to fit through the feeder tube of the juicer. Seeds were eliminated, however, the rind was not removed. The strips were then fed into the juicer and juiced.

Juice Inoculation and Sampling:

One-third of the tomato juice portions were inoculated with 1 ml portions of the resuspended acid adapted culture of *S*. Montevideo and mixed rapidly for approximately 10 sec. One ml samples of each portion were diluted into 9 ml peptone blanks and plated onto bismuth sulfite agar (BSA) plates (Difco) supplemented with 0.05 ml/L of nalidixic acid (Sigma, St. Louis, MO, U.S.A.) (BSA + NA) and onto TSA plates with 0.05 ml/L NA using the Autoplate® 4000 Automated Spiral Plater (Spiral Biotech Inc., Bethesda, MD, U.S.A.). Once inoculated, each plate was incubated at 37°C for 18-24 h. The three portions of the juice were then incubated at 4, 10, and 20°C, respectively. The 4°C portion was sampled and plated at times 0, 3 h, and at 24 h intervals through day 7 or until noticeable off odor or appearance developed. The portion incubated at 10°C was sampled and plated at times 0, 3 h, 5 h, and at 24 h intervals until off odor appeared. The 20°C portion was sampled and plated at times 0, 3 h, 5 h, 8 h, and day 1 – day 7 or until spoilage was noticed.

The procedure above was also used to inoculate one-third of the portions with non-acid adapted *S*. Montevideo. The remaining portions of tomato juice were uninoculated controls. The controls were only plated at 0, 24 h and 48 h. A pH measurement was taken every time the juices were sampled.

Acid-adapted and non-acid adapted *S*. Gaminara were used instead of the *S*. Montevideo for the apple-celery mixture, the carrot mixture, and for the strawberry-apple juice. The methodology previously described was followed.

One-half of the portions of cantaloupe juice were inoculated with 1 ml portions of resuspended non-acid adapted *S*. Poona. The remaining half was uninoculated controls. The same sampling procedure as the non-acid adapted juices was followed with a few exceptions: BSA plates without nalidixic acid supplementation were used to plate each sample, and no TSA plates were used.

All plates were incubated at 37°C after inoculation. TSA plates were incubated for 24 h, while BSA plates were counted after 48 h of incubation. At least three repetitions were made for each juice study.

Enrichment:

In the event that *Salmonella* spp. populations were present below detectable levels by direct plating, the particular juice samples, at the time of plating, were pre-enriched by transferring 1 ml portions of juice into lactose broth (Difco) tubes. The enrichment tubes were incubated at 37°C for 24 h. After incubation, 1 ml portions of the lactose pre-enrichment tubes were transferred into a selenite cystine (Difco) tube and into a tetrathionate (TT) broth, Hajna (Difco) tube. These selective enrichment tubes were

incubated at 37°C for 24 h, and subsequently streaked onto both XLD (xylose-lysine-deoxycholate) agar (Difco) and BSA plates (*32*). Twenty four hours later, the plates were checked for presence or absence of presumptive *Salmonella* colonies.

CHAPTER IV

RESULTS

Tomato Juice:

Initial pH values of all the tomato juice samples ranged from 4.3 - 4.2 and it remained the same throughout the entire sampling time (numbers not shown). *Salmonella* Montevideo numbers in juice stored at 4ºC slightly decreased by less than 1.0 log over a period of 120 h (Figure 1). Acid adapted *Salmonella* populations tended to be less than non-adapted populations. *S*. Montevideo counts in samples stored at 10ºC remained the same until about the third day of incubation when a very small increase in *Salmonella* numbers was observed (Figure 2). On this day an off odor, small bubbles, and visible separation were also present in the juice. Samples of tomato juice stored in the 20ºC incubator had a shorter shelf life of about 2 days. Shelf life limits were defined as the point where unacceptable sensory attributes (odor development, undesirable visible changes) where noted. *Salmonella* numbers started to increase after 8 h of inoculation (Figure 3). No difference was seen between acid adapted and non-acid adapted numbers in both juice samples stored at 10ºC and 20ºC.

Strawberry-Apple Juice:

This type of juice had a shelf life of 7-8 days if stored at 4ºC, 5-6 days if stored at 10ºC, and only 3 days when stored at 20ºC. Off odors, separation and gas bubbles were observed when shelf life expired in juices stored at 10 and 20ºC. The pH values were lower than those of tomato juice, ranging from 3.5 - 3.6. As illustrated on Figure 4, *S*. Gaminara numbers decreased up to day 6 and then proceeded to slowly increase. At day 6, acid adapted numbers had decreased about 3.2 logs and about 3.5 logs for non-acid

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adapted, however, 24 h later these numbers increased about 1 log for acid adapted and 1.5 for non-adapted populations. *Salmonella* numbers in juice stored at 10ºC steadily declined until day 6 when a sudden drop of more than 2 logs was observed (Figure 5). On strawberry-apple juice incubated at 20ºC, a decrease in population was also observed. Acid adapted *Salmonella* cells decreased 3.3 logs from first sampling time to day 3 and non-adapted cell numbers decreased by 3.4 logs (Figure 6).

Carrot Juice Mix:

A pH of 4.0 ± 0.1 was maintained in samples of carrot juice mix. Figure 7 shows a small drop in *S.* Gaminara numbers in juice stored at 4ºC. At 10ºC, *Salmonella* numbers remained constant in the juice mix throughout 7 days of sampling (Figure 8). On the other hand, juice incubated at 20ºC decreased slightly by 8 h after inoculation, and then increased by 24 and 48 h (Figure 9). There was no significant difference in the behavior of adapted and non-adapted cells.

Apple-Celery Juice Mix:

This juice had a pH value of 3.0 ± 0.1 throughout the entire experiment. Levels of *S.* Gaminara decreased below detectable levels after 24 or 48 h of incubation at all three temperatures. Results of juice stored at 4ºC show that acid adapted counts were slightly higher at 24 h than non-acid adapted counts (Figure 10). No differences were observed in the behavior of adapted and non-adapted cells except in juice stored at 10ºC where acid adapted counts enumerated on tryptic soy agar (TSA) were about 1.5 logs higher than the non-acid adapted TSA numbers 3 h and 24 h after inoculation. On the other hand, *Salmonella* numbers plated on bismuth sulfite agar (BSA) remained about the same until the 24 h sampling time when about 1.4 log of acid adapted cells still remained and no

non-adapted cells were detected with direct plating methods (negative enrichments) (Figure 11). A slight difference between acid adapted and non-acid adapted numbers was seen after 5 h in juice stored at 20ºC (Figure 12). BSA counts were undetectable by direct plating at 24 h, however, a positive enrichment indicated that a small number of *Salmonella* cells were still present. Acid adapted numbers enumerated on TSA were slightly higher compared to non-acid adapted populations. None of the apple-celery juice samples had any physical indications of contamination; no off colors nor odors were observed.

Cantaloupe Juice:

This type of juice had a very short shelf life 3, 2, and 1 days respectively for juice stored at 4, 10 and 20ºC. *S*. Poona populations in juices stored at both 4ºC and 10ºC experienced a decrease on the first day of inoculation followed by a slight increase to approximately the starting levels (Figure 13, 14). On juices incubated at 20ºC, bacterial counts started to increase much faster, at about 8 h after inoculation, and off odors and gas appeared at 24 h (Figure 13). The pH values of cantaloupe juice are higher than the other juices used (6.5 ± 0.1), therefore, no acid adaptation studies were done on it. A drop in pH to about 4.6 was observed after 2 days in juice stored at 10ºC, and after 1 day in juice stored at 20ºC, however, pH values did not change in juice stored at 4ºC.

Controls:

As stated on the materials and methods, uninoculated controls of each juice sample were plated at 0, 24, and 48 h. These were enumerated using BSA plates. No growth was seen as a result of direct plating any of the juice controls, and all enrichments were found to be negative (results not shown).

CHAPTER V

DISCUSSION

The results obtained in this study were not the expected. Given the extensive amount of research done on the effects of acid tolerance response (ATR) on the survival of *Salmonella* Typhimurium in highly acidic environments, one would have assumed acid adapted cells counts to decrease at a slower rate than the non-adapted cells. On the contrary, results show that adapting *Salmonella* spp. cells to a mild pH before placing them in the acidic juices did not seem to have an enhanced effect on their survival as compared to those that were not adapted. Several factors might have come into play to generate the results obtained. The organic acids present in juices might have an effect on *Salmonella* survival. However, *Salmonella* may also face pressures from the natural microbiota present in the unpasteurized juices and the metabolic by-products they produce.

Pasteurized juices are not only safer to consume, but they also have a much longer shelflife. This is due to the fact heat treatment administered to pasteurized juices destroys pathogens as well as most microorganisms naturally present. In a 1979 study, Goverd, et. al. studied the survival of *S.* Typhimurium and other serotypes in pasteurized apple juice and apple cider as affected by temperature and pH (*47*). They found that *Salmonella* could survive up to 30 days in the apple juice with a pH value of 3.6 if stored at 4ºC. They also observed that if an initial inoculum level of 2×10^5 cfu/ml was used, the survival rate was higher at 22ºC than at 4ºC. Comparing these results to those obtained for the

survival of *S*. Gaminara in strawberry-apple juice (pH 3.5 ± 0.1), samples stored at 20ºC were only sampled for up to three days because of spoilage, and those stored at 4ºC were sampled for 7-8 days because that is the normal shelf life for unpasteurized juices with low pH values (*72*). Bacterial numbers in juice decreased by more than 2 logs in less than a week. This may be due to the acid stress or to the ongoing competition for nutrients with other microorganisms present. Since Goverd et. al (*47*) proved that *Salmonella* spp. may survive up to a month in pasteurized apple juice regardless of its low pH, it is safe to say that the fast decrease of cells numbers in unpasteurized apple-strawberry juice may be attributed, in part, to the presence of other microorganisms. In a 1996 study, Buchanan and Edelson conducted a study to determine if *E. coli*'s ability to ferment glucose was an easy and effective method to induce acid tolerance; 7 enterohemorrhagic and 1 non-enterohemorrhagic strains were used (*12*). Adapted cells were grown on TSB with 1% glucose and non adapted cells were grown on TSB, both were incubated at 37ºC for 18 h. to obtain a final pH of 4.0-4.5 for adapted, and 6.9-7.0 for non adapted *E. coli.* After incubation, each set of inoculum was transferred into brain heart infusion broth with adjusted pH values of 3.0 and 2.5. Sampling times did not exceed 7 h. Acid adapted populations decreased at a much slower pace than non-adapted *E. coli* populations, however, medium used was sterilized in an autoclave, therefore, no competitive microorganisms were present to affect the results.

As stated earlier, the presence of organic acids may have an effect on the survival of *Salmonella* spp. Antimicrobial activities related to organic acids are well known (30). These acids are commonly found naturally in fruit juices and they may also be added as preservatives. It is well established that citric and malic acids are found in orange and

tomato juice, malic acid is also found in apples, and lemon juice contains high amounts of citric acid (*10, 67*). All of these were ingredients used in this project's juice mixtures. The degree of bacteriocidal activity of organic acids depends on the type of acid, its level of undissociation, as well as the pH of the medium (*25*). Mossel and De Bruin (*54*) studied the survival of *S*. Typhimurium and *E. coli* in apple, tomato, orange and lemon juices. Their results showed that at a pH of 3.8 – 4.1, the level of lethality of the organic acids present in their study were as follows: lactic> acetic> malic or citric, lactic being the most effective as an antimicrobial. However, other studies have concluded that citric acid has inhibitory properties against *S*. Typhimurium in media and on poultry carcasses (*66*). Various studies have been conducted to study the effects of organic acids on *Listeria* survival (*14, 48*). Buchanan and Golden (*14*) found that citric acid may inhibit *Listeria monocytogenes* cells if present in the undissociated form, or it may protect the bacteria if it is present in the dissociated form. If citric acid levels on the unpasteurized juices used in this experiment had an effect on the *Salmonella* cells, they might be able to affect the ATR mechanism.

Another factor that might have had an effect on the behavior of *Salmonella* cells in unpasteurized juices is the presence of heterofermentative microorganisms. These type of bacteria are known to be obligate fermenters that produce lactic acid, acetic acid, and other potential antimicrobials such as ethanol, and carbon dioxide (*68*). On the other hand, anaerobic microorganisms have the ability to use oxygen to produce hydrogen peroxide. If they were present in juices, the antimicrobial properties of hydrogen peroxide may have had an effect on *Salmonella* numbers.

S. Poona has been involved in several cantaloupe outbreaks. In comparison with the ingredients of the other juices, cantaloupe is a low acid fruit and it is grown in direct contact with the ground, consequently, the risks for potential contamination are high. Juicing fanatics claim that drinking cantaloupe juice with rind increases the nutritional quality of the juice and, thus, makes it "healthier". However, this increases its risk for pathogen contamination and it may also introduce more microorganisms to the juice. The juice made for this project included the rind, all of the other fruits and vegetables used (except celery) were peeled before extracting the juice, or skin was discarded (citrus). The addition of rind (more microorganisms present), and the difference in pH values may be the reason why cantaloupe had a much shorter shelf life than the other juices.

A study was conducted in 1994 to determine the fate of *E. coli* O157:H7 in cantaloupe and watermelon (*28*). The cantaloupe was sanitized prior to cutting, the rind was removed, and the entire experiment was conducted under aseptic conditions to minimize the number of natural microflora present in the fruit. Results were very similar to those observed by Golden, et. al (*45*). Populations were larger in watermelons than in cantaloupe, rapid growth occurred at 23/25ºC after 24 h, and little or no growth was observed at refrigeration temperatures. Bacterial cells used in these two studies were able to grow much faster than they were in cantaloupe juice, however, they sanitized the fruit and removed the rind before inoculation, so the number of competitors was much lower. In the *E. coli* study, the rind of the melons was also inoculated and sampled for bacterial enumeration over a period of 21 days at 5 and 25ºC. The *E. coli* O157:H7 population on melons stored at 25ºC increased by about 2 logs during the first 4 days and then remained constant throughout the rest of the experiment, while populations on melon samples

incubated at 5ºC decreased to $<10^1$ CFU/cm^2 after 8 to 14 days. Cantaloupes are usually maintained at room temperature in supermarkets, so if sufficient amounts of pathogenic cells are present, they have the potential to multiply and survive on the rind for up to 21 days posing a threat to all consumers.

CHAPTER V1

CONCLUSION

Salmonella continues to be the leading cause of foodborne related illnesses and deaths in the U.S. each year. The safety of unpasteurized juices has been a topic of concern in the commercial juice industry given the rising numbers of foodborne illness outbreaks related to their products. However, little information is available about homemade juices and their safety. Both high acid and low acid unpasteurized juices are at risk for bacterial contamination and have been linked to several illness outbreaks related to juice consumption.

Many investigations have been conducted to study the effects of ATR in *Salmonella* in laboratories; however, very few have been done on juices and none in unpasteurized juices. It was shown that there were no differences between acid adapted and non-acid adapted *S.* Montevideo and *S.* Gaminara survival patterns in highly acidic unpasteurized juices.

The apple-celery juice mix experienced a large drop in *S.* Gaminara numbers mainly because of its pH lethality (pH 3.0). However, a few cells were detected by enrichment even after 48 h. *Salmonella* Montevideo and *S.* Gaminara populations were maintained throughout the entire sampling period in tomato juice and in the carrot mix juice, and *S.* Poona was able to survive and grow in cantaloupe juice.

The results obtained in this study confirm that unpasteurized juices, regardless of their acidity, post a risk to either consumers who buying them, or make them at home.

APPENDICES

Figure 1: Comparison of the survival of acid adapted (AA) versus non-acid adapted (NA) *Salmonella* Montevideo in homemade unpasteurized tomato juice stored at 4ºC and enumerated on tryptic soy agar (TSA) and bismuth sulfite agar (BSA) supplemented with nalidixic acid (n = 3 for BSA counts, and n = 2 for TSA counts).
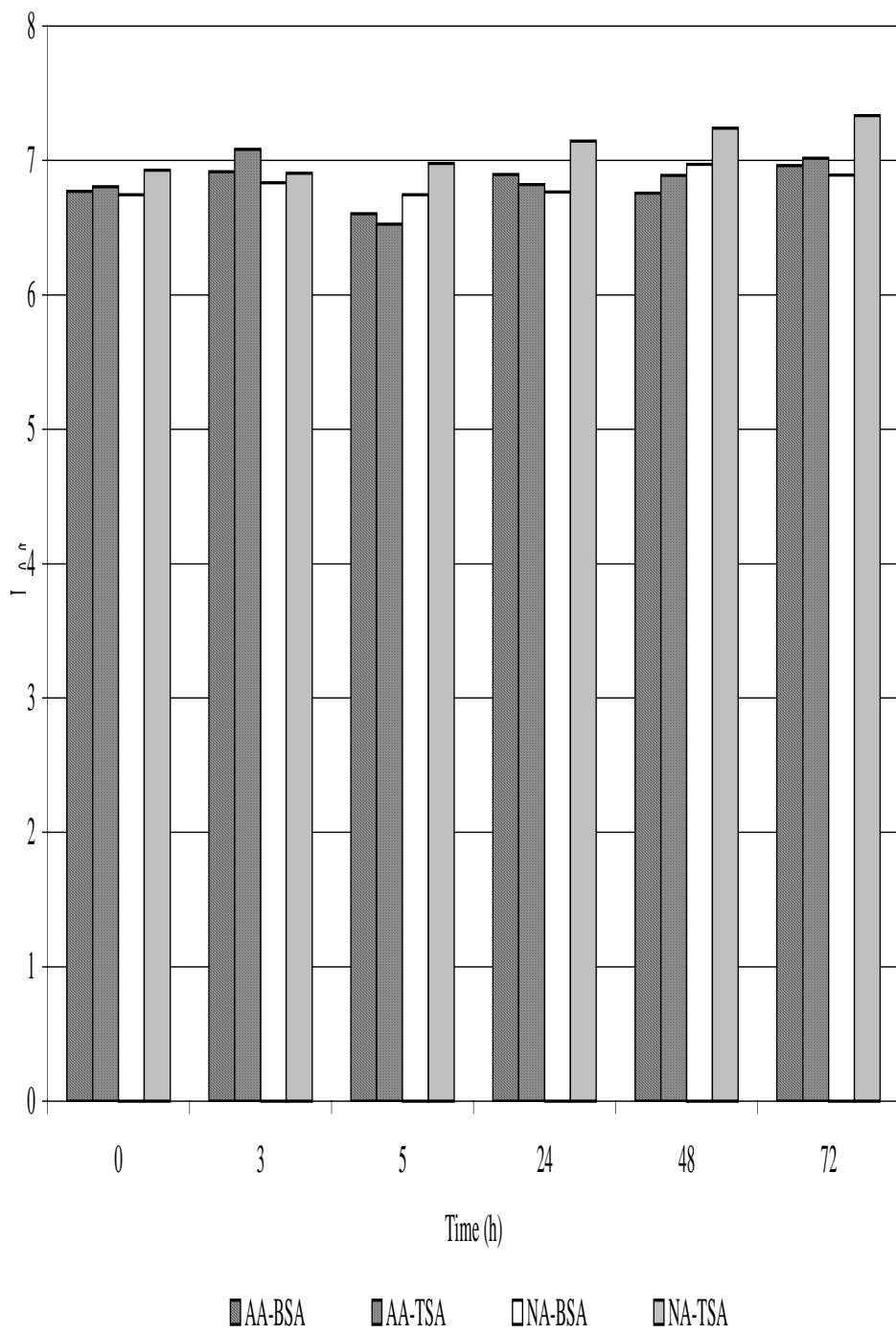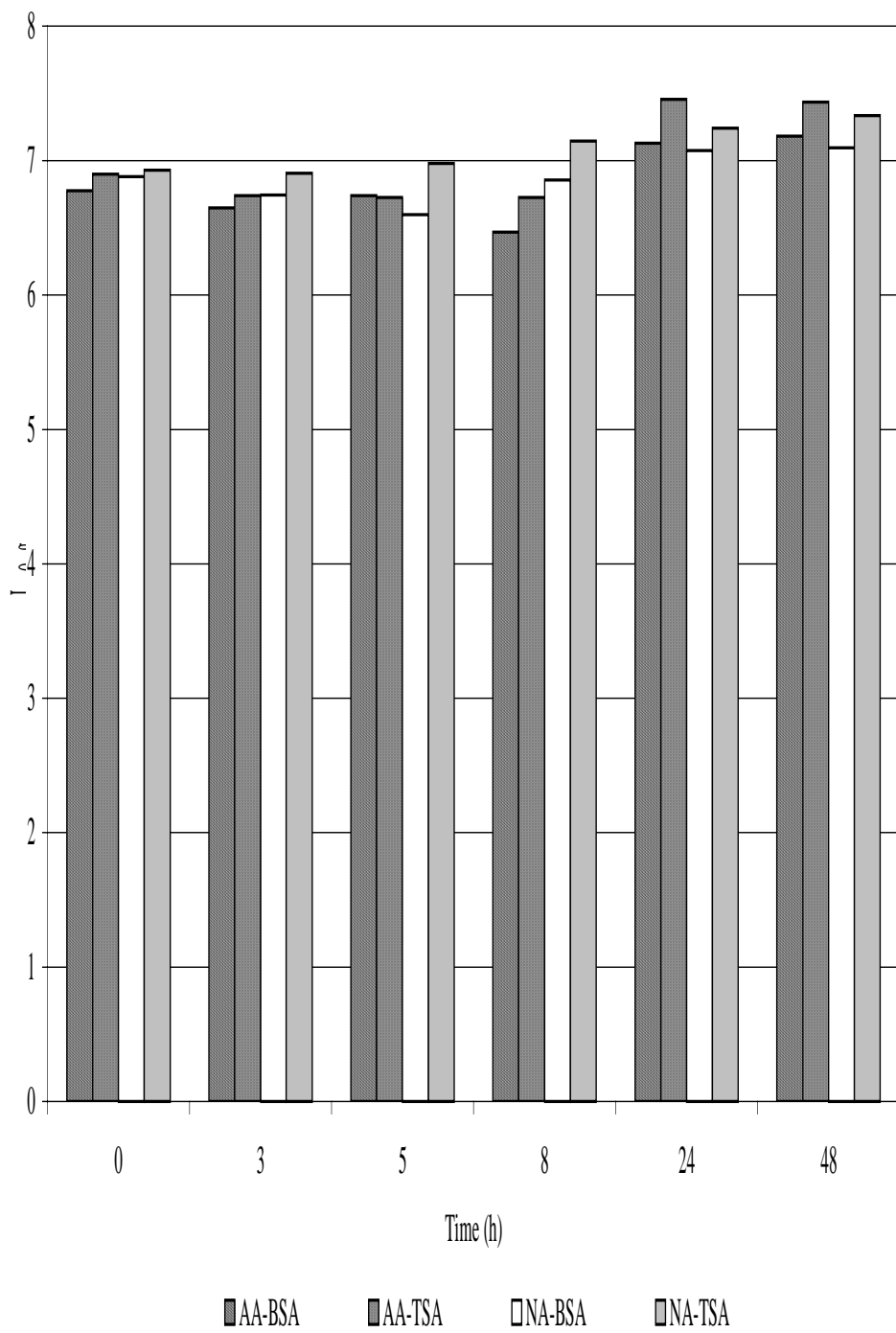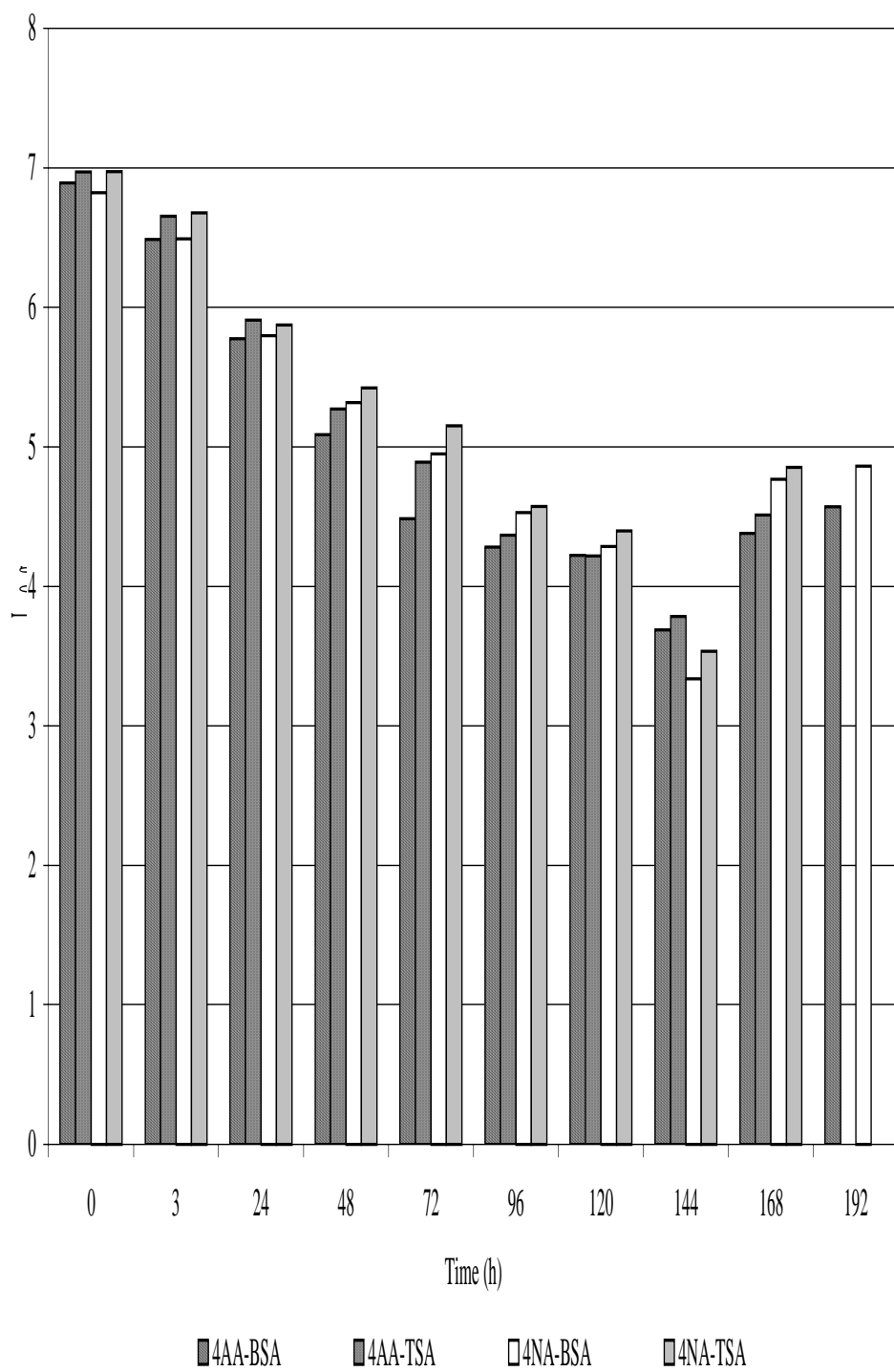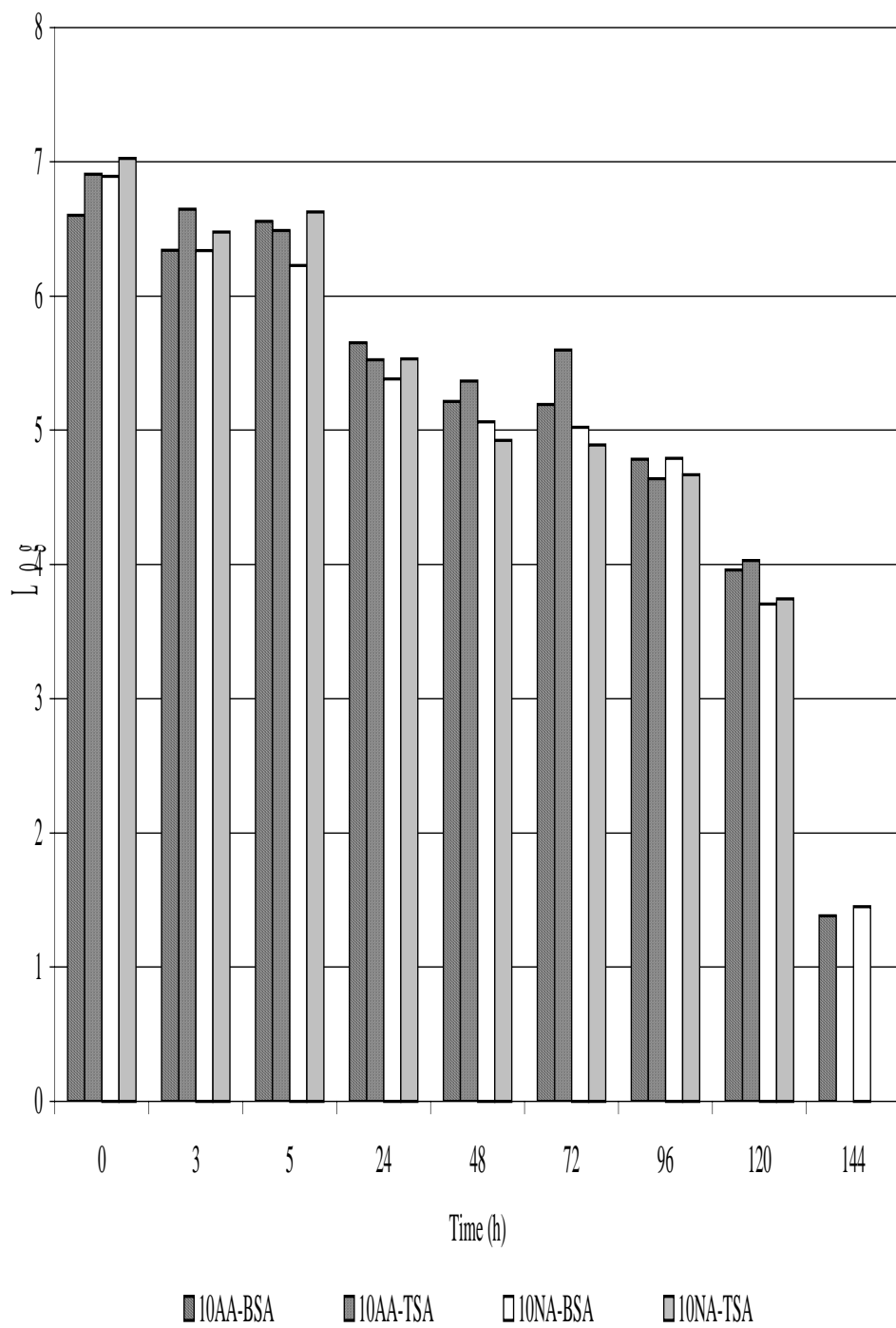
Time (h)

AA-BSA AA-TSA NA-BSA NA-TSA
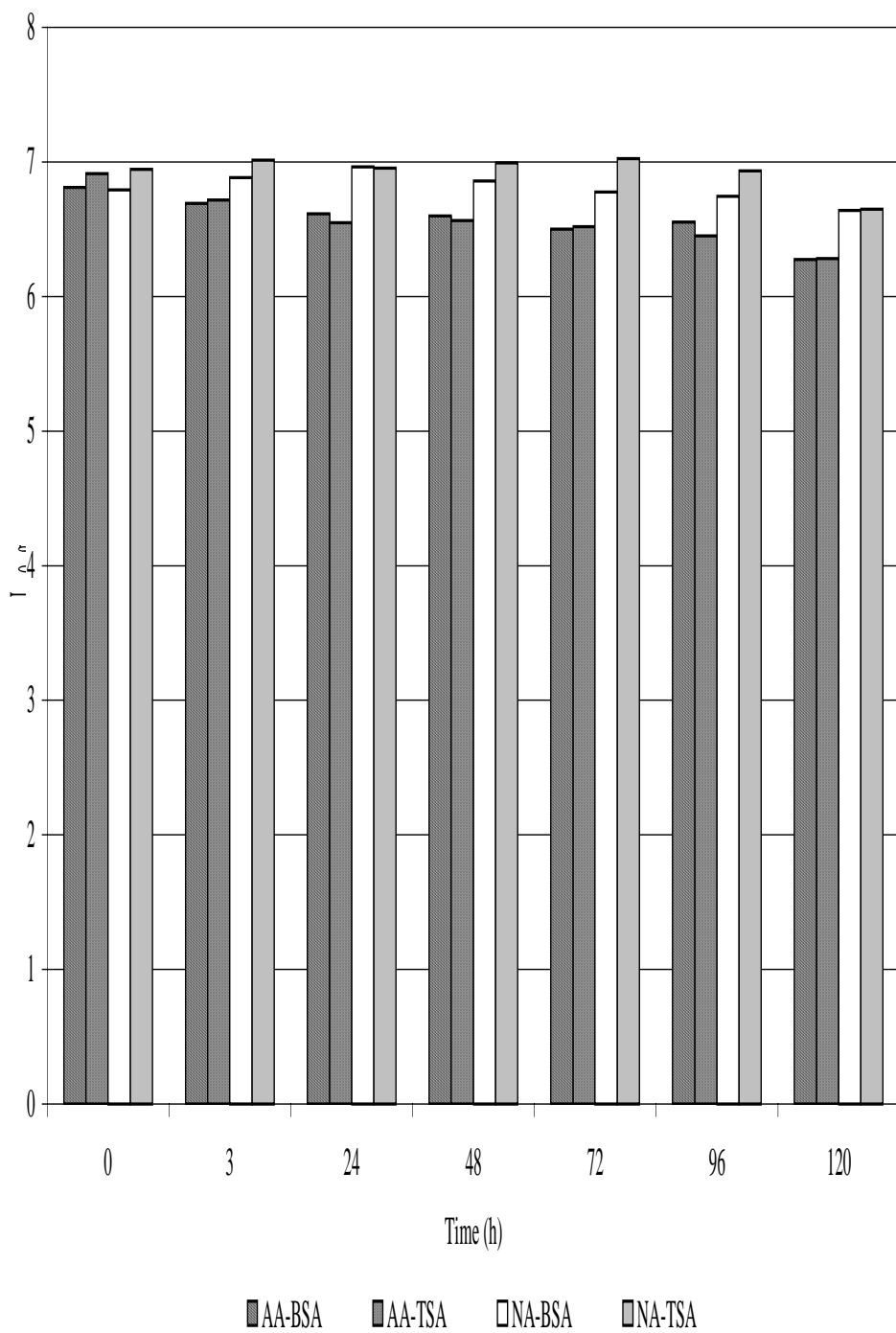
Figure 2: Comparison of the survival of acid adapted (AA) versus non-acid adapted (NA)

Salmonella Montevideo in homemade unpasteurized tomato juice stored at 10ºC and

enumerated on tryptic soy agar (TSA) and bismuth sulfite agar (BSA) supplemented with

nalidixic acid (n = 3 for BSA counts, and n = 2 for TSA counts).

Figure 3: Comparison of the survival of acid adapted (AA) versus non-acid adapted (NA) *Salmonella* Montevideo in homemade unpasteurized tomato juice stored at 20ºC and enumerated on tryptic soy agar (TSA) and bismuth sulfite agar (BSA) supplemented with nalidixic acid (n = 3 for BSA counts, and n = 2 for TSA counts).
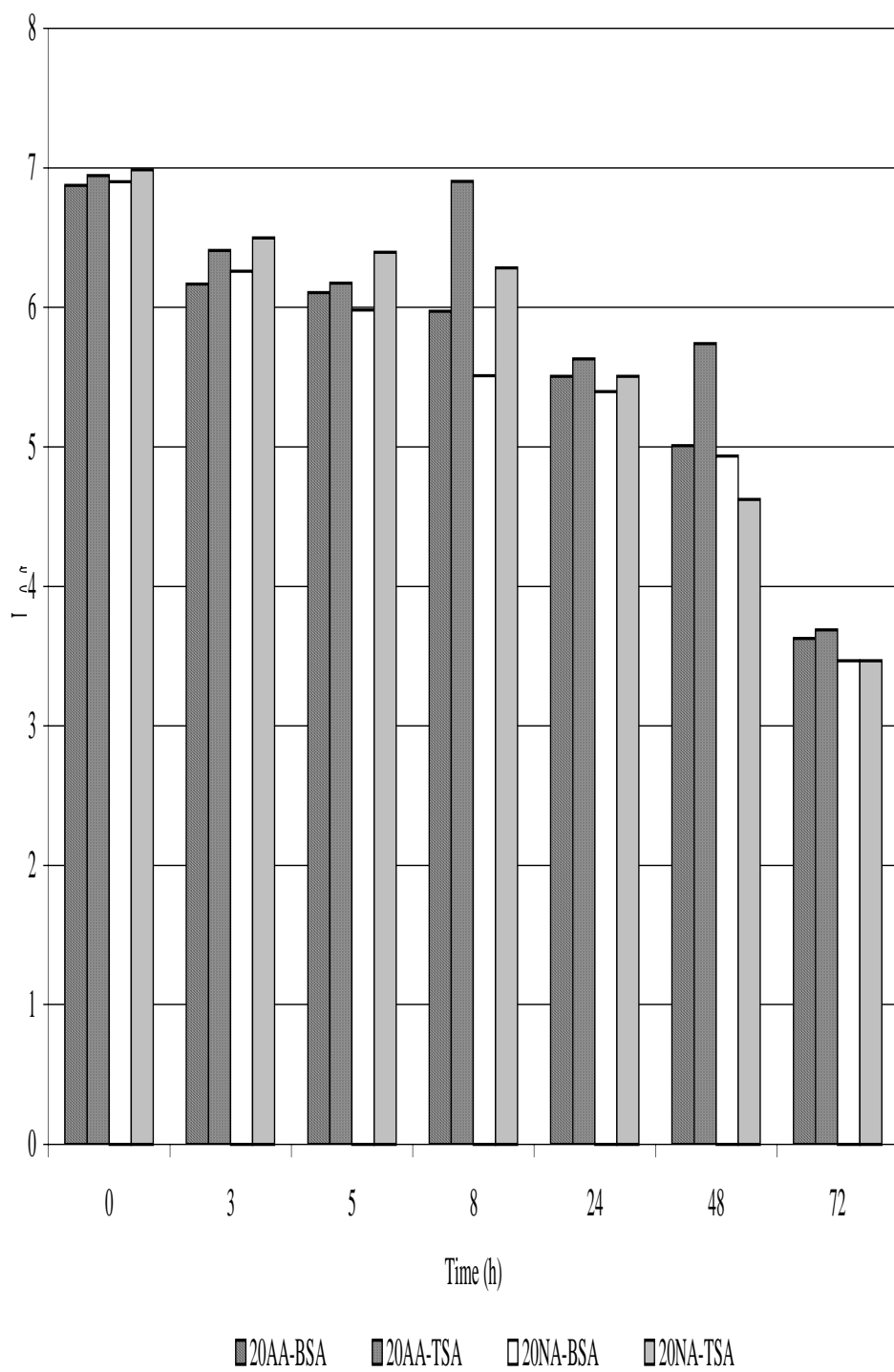
Time (h)

AA-BSA   AA-TSA   NA-BSA   NA-TSA

Figure 4: Comparison of the survival of acid adapted (AA) versus non-acid adapted (NA) *Salmonella* Gaminara in homemade unpasteurized strawberry-apple juice mix stored at 4ºC and enumerated on tryptic soy agar (TSA) and bismuth sulfite agar (BSA) supplemented with nalidixic acid (n = 3 for BSA counts except at 144, 168 h n = 2, and at 192 h n = 1; for TSA counts n = 3 except 144, 168 h n = 2).
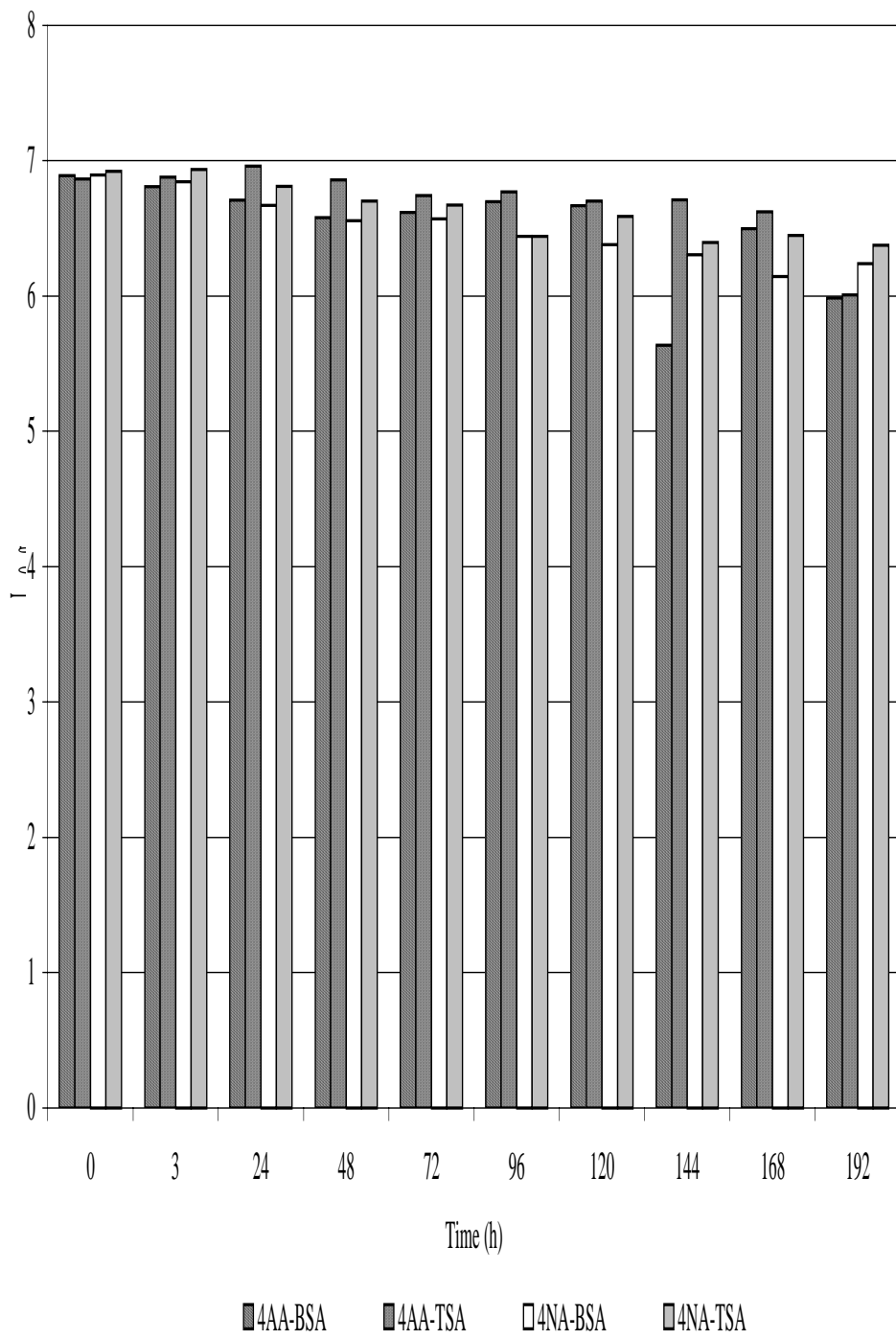
Figure 5: Comparison of the survival of acid adapted (AA) versus non-acid adapted (NA) *Salmonella* Gaminara in homemade unpasteurized strawberry-apple juice mix stored at 10ºC and enumerated on tryptic soy agar (TSA) and bismuth sulfite agar (BSA) supplemented with nalidixic acid (n = 3 for BSA counts except at 120 h n = 2, and at 144 h n = 1; TSA counts n = 3 except at 120 h n = 2).

Figure 6: Comparison of the survival of acid adapted (AA) versus non-acid adapted (NA) *Salmonella* Gaminara in homemade unpasteurized strawberry-apple juice mix stored at 20ºC and enumerated on tryptic soy agar (TSA) and bismut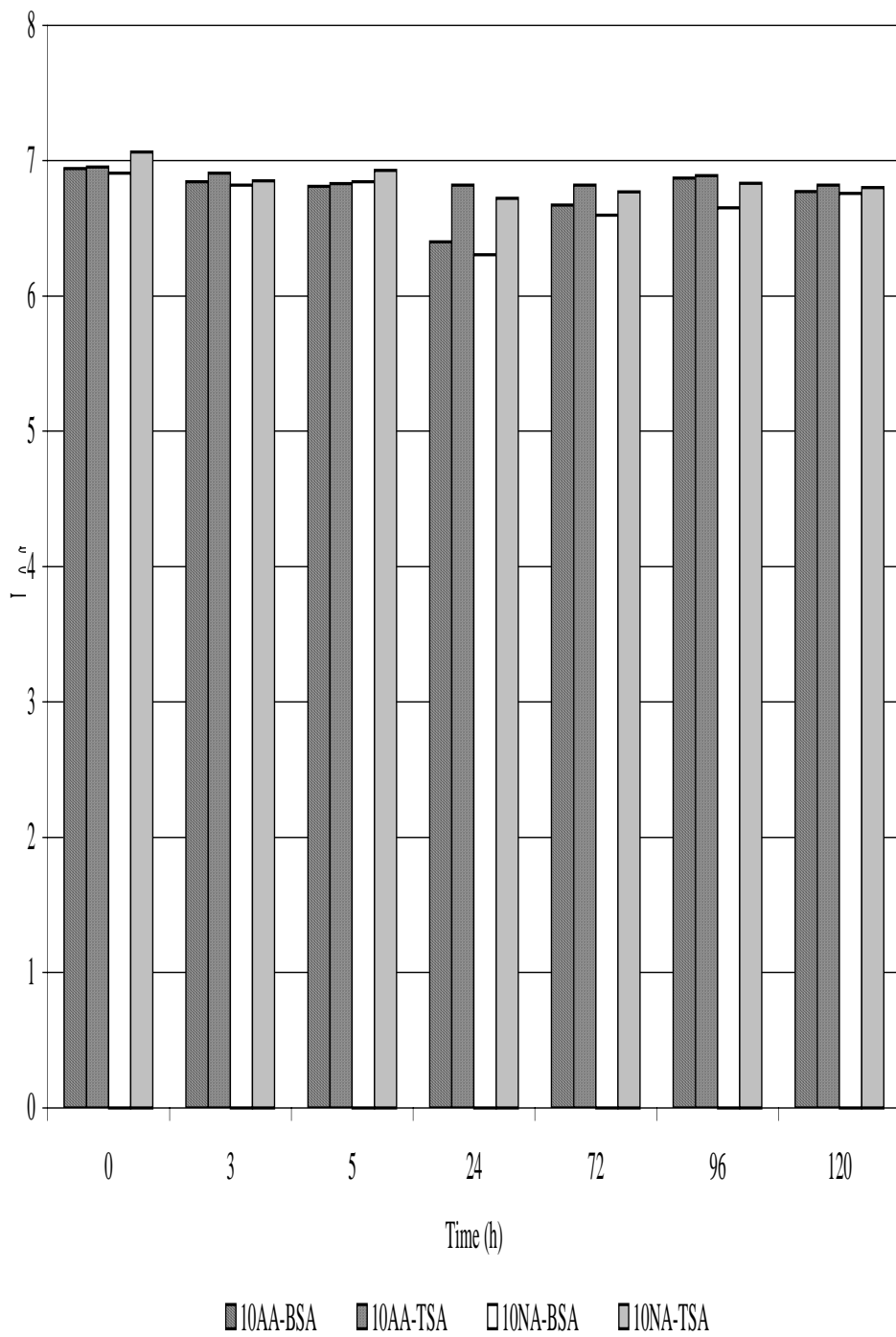h sulfite agar (BSA) supplemented with nalidixic acid (n = 3 for BSA counts except at 72 h n = 2; TSA counts n = 3 except at 24 and 72 h n = 2).

Figure 7: Comparison of the survival of acid adapted (AA) versus non-acid adapted (NA) *Salmonella* Gaminara in homemade unpasteurized carrot juice mix stored at 4ºC and enumerated on tryptic soy agar (TSA) and bismuth sulfite agar (BSA) supplemented with nalidixic acid (n = 3 except at 144 h n = 2 and at 192 h n = 1).
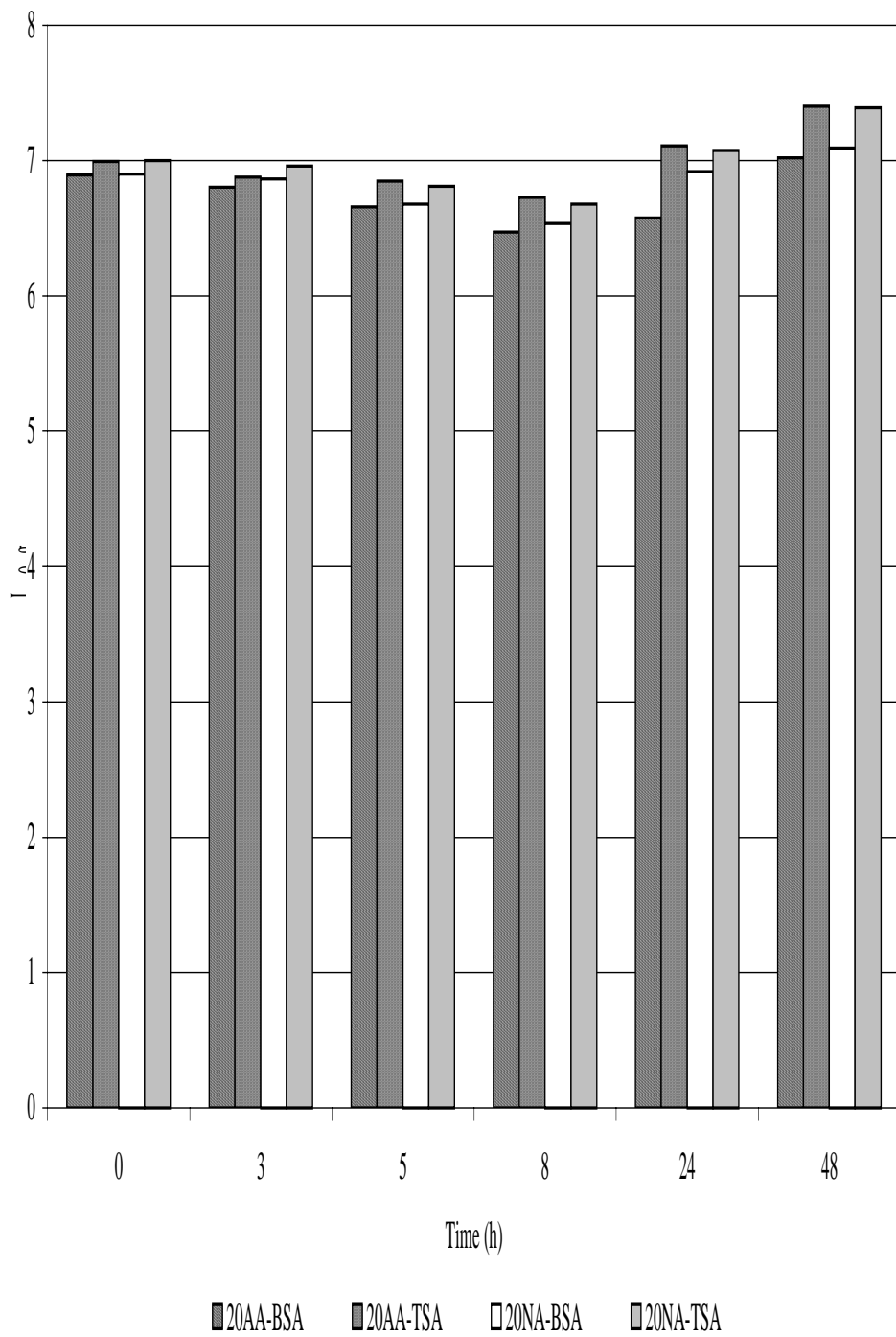
Figure 8: Comparison of the survival of acid adapted (AA) versus non-acid adapted (NA) *Salmonella* Gaminara in homemade unpasteurized carrot juice mix stored at 10ºC and enumerated on tryptic soy agar (TSA) and bismuth sulfite agar (BSA) supplemented with nalidixic acid (n = 3).

Figure 9: Comparison of the survival of acid adapted (AA) versus non-acid adapted (NA) *Salmonella* Gaminara in homemade unpasteurized carrot juice mix stored at 20ºC and enumerated on tryptic soy agar (TSA) and bismuth sulfite agar (BSA) supplemented with nalidixic acid (n = 3).
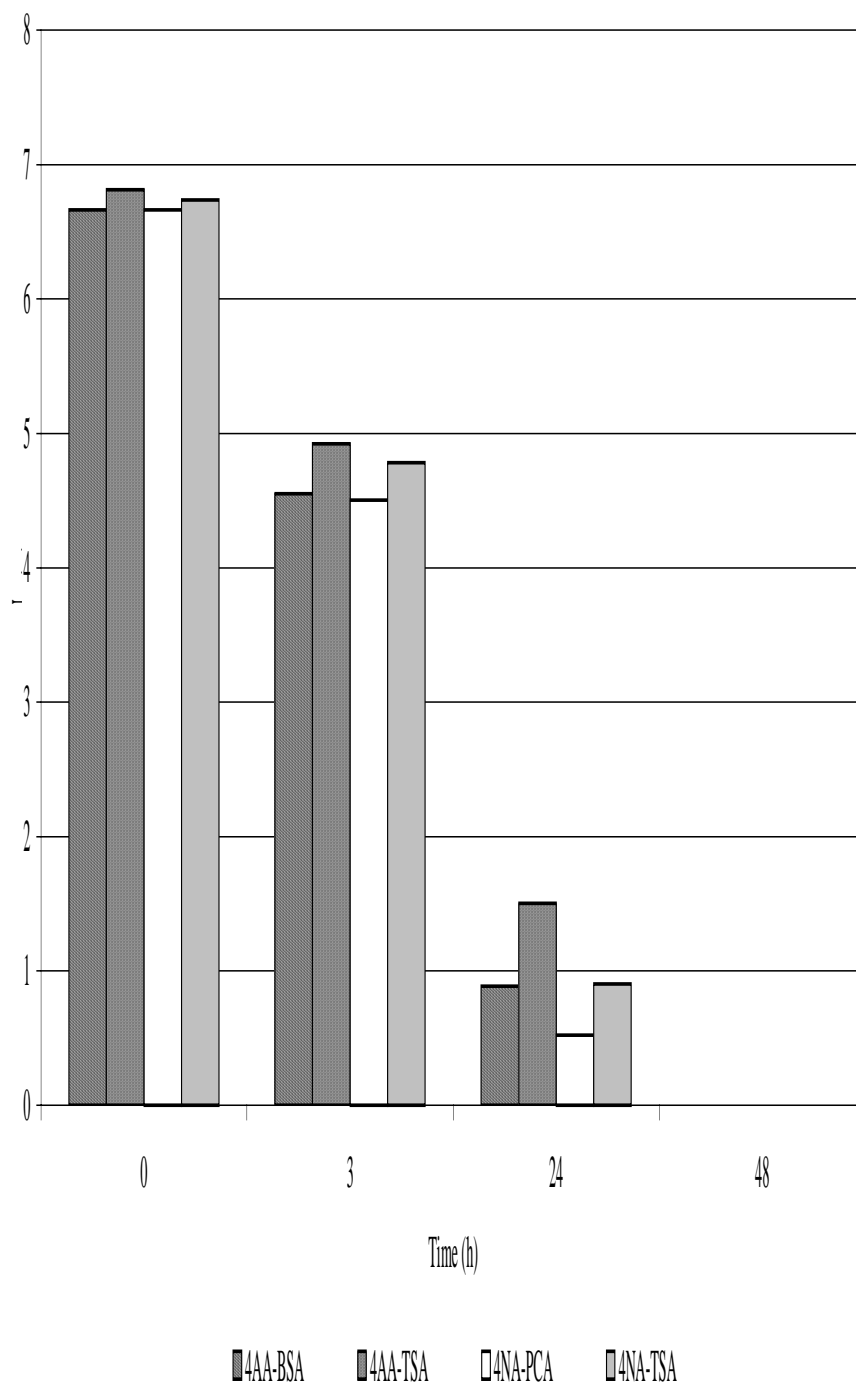
Figure 10: Comparison of the survival of acid adapted (AA) versus non-acid adapted (NA) *Salmonella* Gaminara in homemade unpasteurized apple-celery juice mix stored at 4ºC and enumerated on tryptic soy agar (TSA) and bismuth sulfite agar (BSA) supplemented with nalidixic acid (n = 3 for BSA counts except at 24 h n = 1; TSA counts n = 2).

Time (h)

4AA-BSA   4AA-TSA   4NA-PCA   4NA-TSA

Figure 11: Comparison of the survival of acid adapted (AA) versus non-acid adapted (NA) *Salmonella* Gaminara in homemade unpasteurized apple-celery juice mix stored at 10ºC and enumerated on tryptic soy agar (TSA) and bismuth sulfite agar (BSA) supplemented with nalidixic acid (n = 3 for BSA counts except at 24 h n = 1; TSA counts n = 2 except at 24 h n = 1).
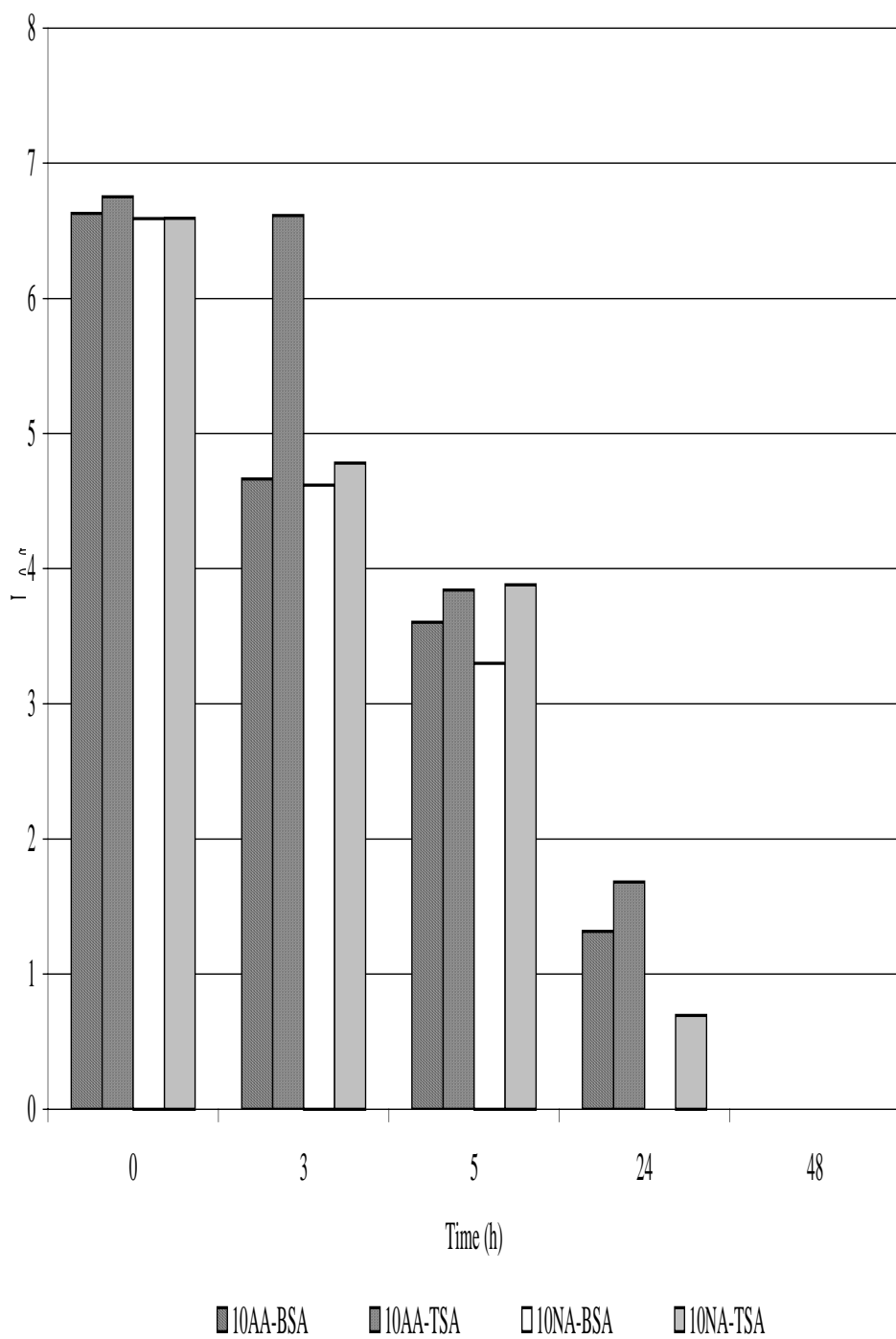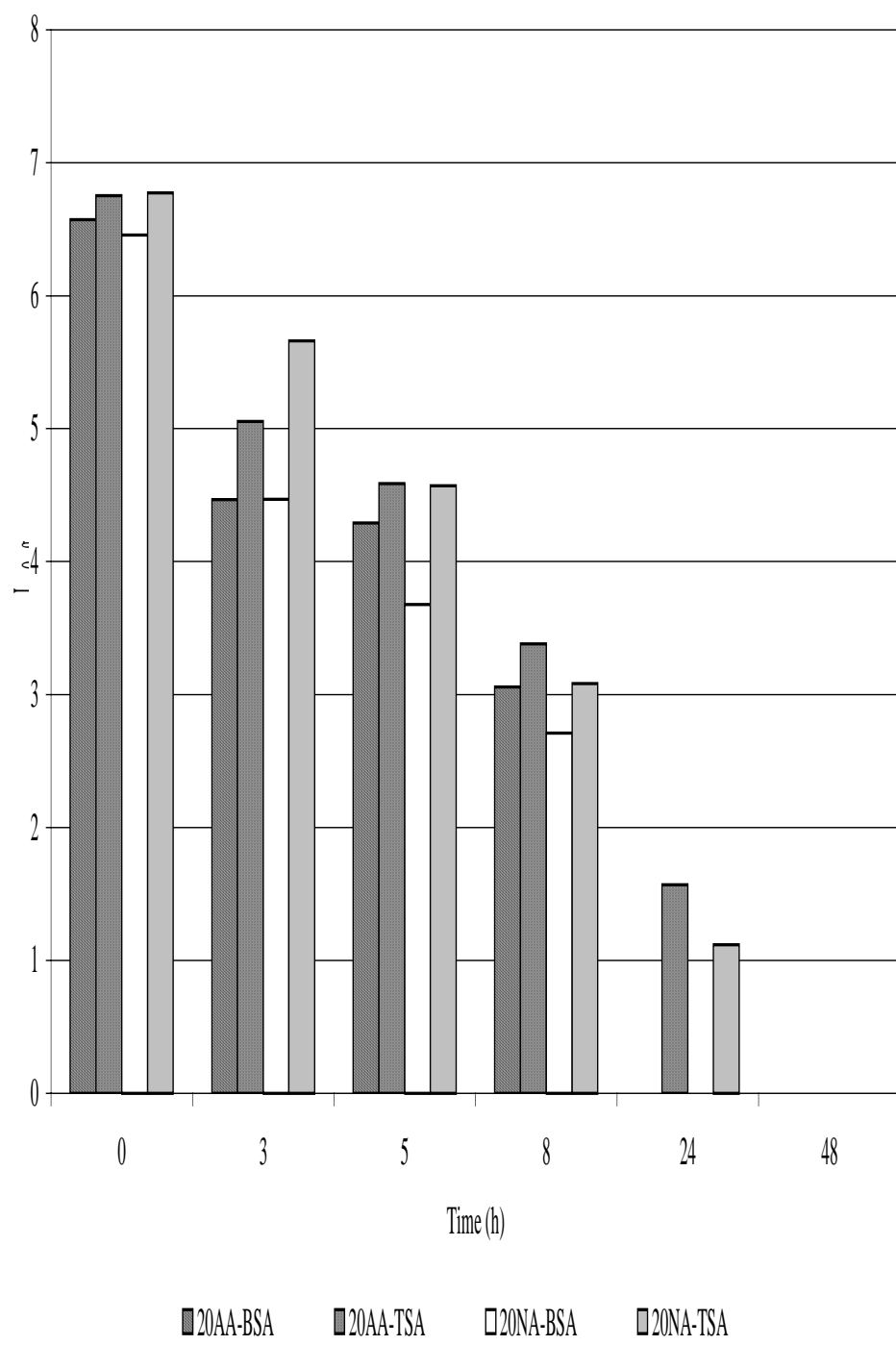
Time (h)

■10AA-BSA   ■10AA-TSA   □10NA-BSA   ■10NA-TSA

Figure 12: Comparison of the survival of acid adapted (AA) versus non-acid adapted (NA) *Salmonella* Gaminara in homemade unpasteurized apple-celery juice mix stored at 20ºC and enumerated on tryptic soy agar (TSA) and bismuth sulfite agar (BSA) supplemented with nalidixic acid (n = 3 for BSA counts except at 8 h n = 2; TSA counts n = 2).

Figure 13: Survival of *Salmonella* Poona in homemade unpasteurized cantaloupe juice stored at 4ºC and plated on bismuth sulfite agar plates (n = 3).
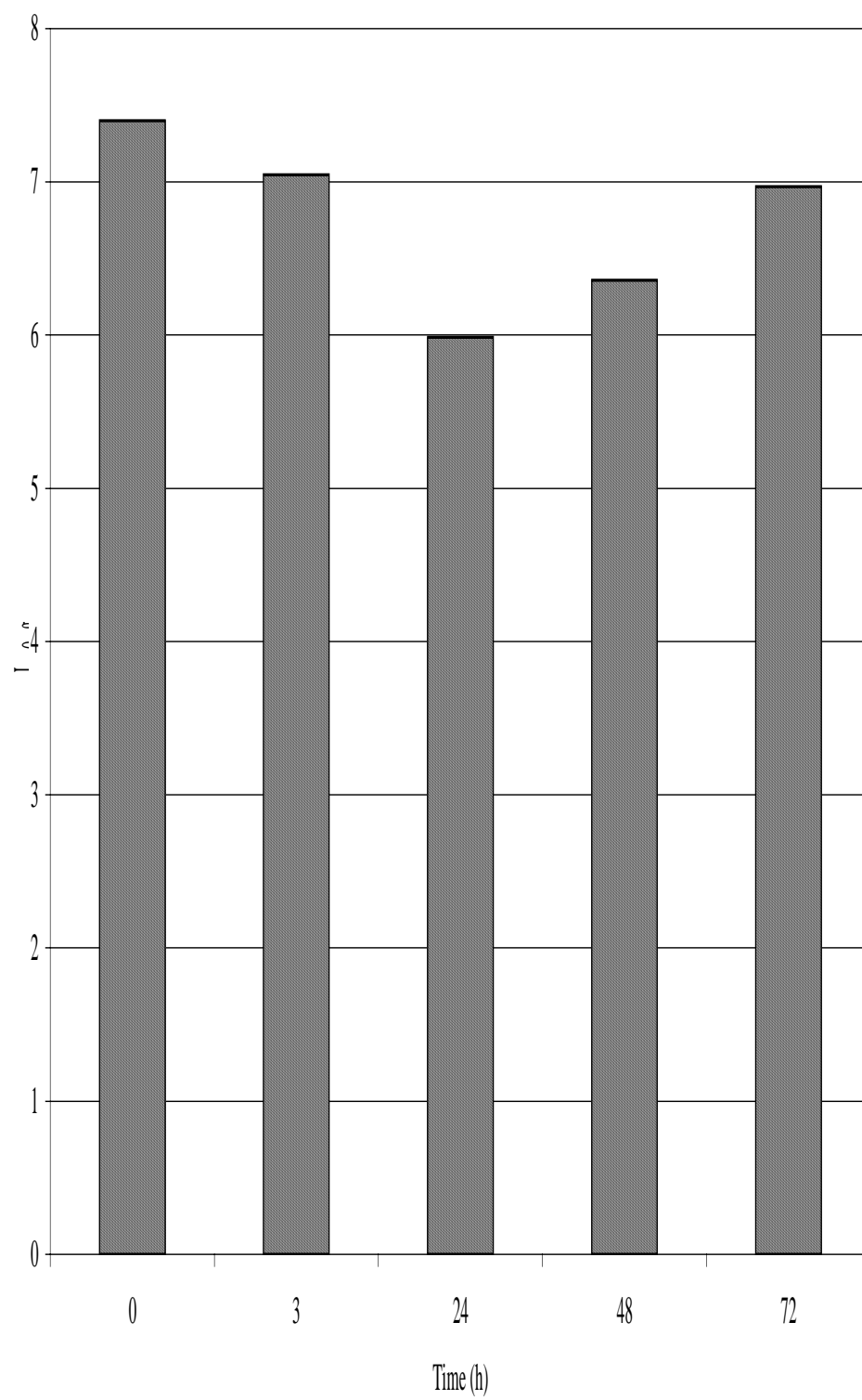
Figure 14: Survival of *Salmonella* Poona in homemade unpasteurized cantaloupe juice stored at 10ºC and plated on bismuth sulfite agar plates (n = 3).
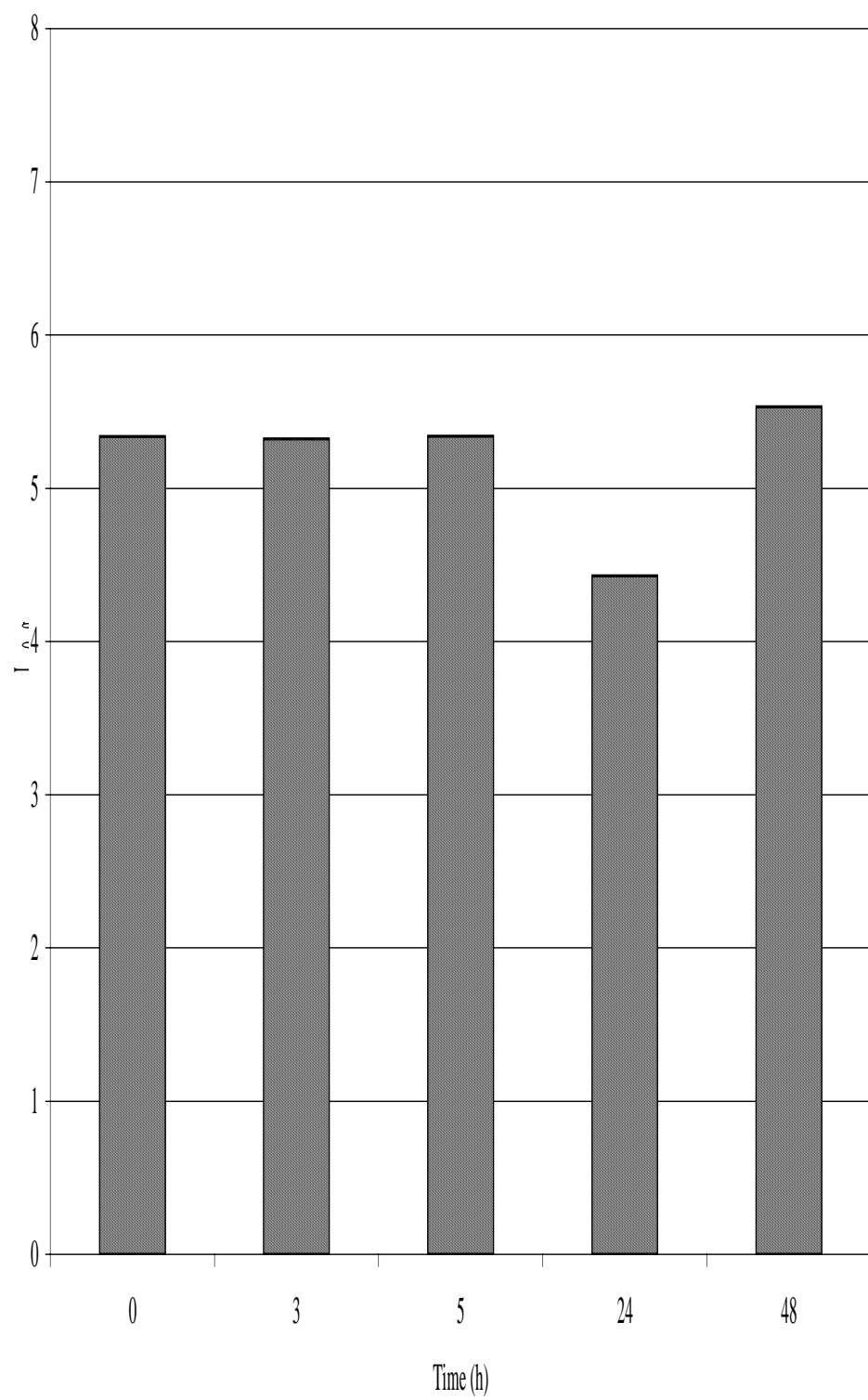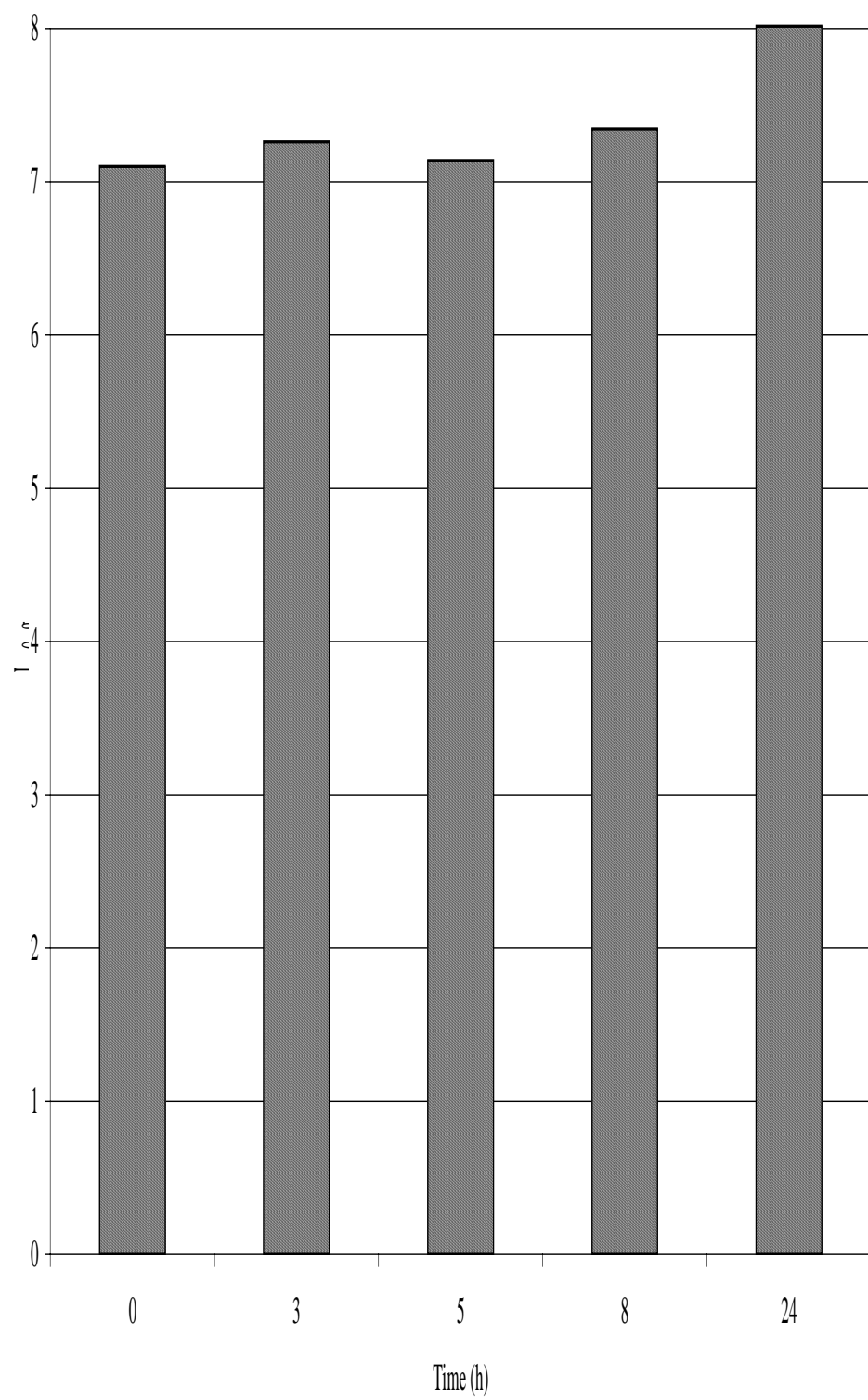
Figure 15: Survival of *Salmonella* Poona in homemade unpasteurized cantaloupe juice stored at 20ºC and plated on bismuth sulfite agar plates (n = 3).

Time (h)

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