RESISTANCE PROPERTIES AND CONTROL OF *Alicyclobacillus acidoterrestris*

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ABSTRACT

RESISTANCE PROPERTIES AND CONTROL OF *Alicyclobacillus acidoterrestris*

Spoilage caused by *Alicyclobacillus acidoterrestris* is a significant problem for the fruit juice industry. Since *A. acidoterrestris* can withstand to high temperatures and acidic environments, it has been suggested as the target organism for the design of thermal processes of fruit juices. On the other hand, little is known about the effect of the sporulation media on the wet–heat resistance characteristics. Moreover, the control of vegetative cell growth and spore germination in fruit juices by natural antimicrobials needs to be studied. Therefore, the objectives of this study were to determine the effects of sporulation media on the thermal inactivation kinetics of *A. acidoterrestris* DSM 3922 spores in the reconstituted apple juice using different sporulation media; to test the antimicrobial activities of natural plant extracts (grape seed and pomegranate fruit extract), to investigate the growth characteristics in pomegranate juice and pomegranate–apple blend juices (10–80%, v/v); and finally to observe the structural changes using scanning electron and atomic force microscopy. The results of this study clearly showed that spores had different structural, physicochemical and wet–heat resistance properties depending on the sporulation media. Especially, higher wet–heat resistance was obtained from spores produced on mineral containing media. In addition, the high heat resistance of spores was correlated to their higher dipicolinic acid content and Ca^{2+} ions in the core. Nevertheless, the required dose to inhibit spore germination in the apple juice with natural extracts and in pomegranate and blend juices (40% and 80%) was dependent on the type of antimicrobial, its concentration and sporulation media. Also, the growth of vegetative cells was inhibited in the apple juice with grape seed and pomegranate extracts; and in pomegranate and blend juices (40% and 80%). Then, the experimental inactivation data were fitted satisfactorily using the Weibull model. Microscopic studies suggested that the phenolic compounds in these natural extracts and juices might cause the leakage of cellular components from the cells and prevent the development of the spores into vegetative forms. The results obtained in this study will provide contributions for the design of thermal processes and to prevent the spoilage caused by *A. acidoterrestris* in the fruit juice industry.

ÖZET

Alicyclobacillus acidoterrestris'in KONTROLÜ VE DİRENÇ ÖZELLİKLERİ

Alicyclobacillus acidoterrestris'in neden olduğu bozulmalar, meyve suyu endüstrisi için önemli bir sorun oluşturmaktadır. Yüksek sıcaklık ve asidik ortamlarda dirençli olması nedeniyle *A. acidoterrestris*, meyve suyuna uygulanan ısıl işlemlerin tasarımı için hedef organizma olarak seçilmiştir. Bununla birlikte, sporulasyon ortamının yaş ısıl direnç karakteristikleri üzerine fazla bilgi bulunmamaktadır. Ayrıca, meyve sularında vejetatif hücre gelişimi ve sporların çimlenmesinin kontrolü için doğal antimikrobiyallerin kullanımının araştırılması gerekmektedir. Bu çalışmanın amacı farklı sporulasyon ortamları kullanılarak üretilen *A. acidoterrestris* DSM 3922 sporlarının, elma suyunda ısıl inaktivasyon kinetiği üzerine sporulasyon ortamının etkisinin belirlenmesi; doğal bitki ekstraktlarının (üzüm çekirdeği ve nar meyve ekstraktı) antimikrobiyal aktivitilerinin test edilmesi; nar ve nar–elma karışım sularında (%10–80, v/v) büyüme karakteristiklerinin araştırılması; ve son olarak da taramalı elektron ve atomik kuvvet mikroskobisi kullanılarak yapısal değişimlerin araştırılmasıdır. Bu çalışmanın sonuçları sporulasyon ortamına bağlı olarak sporların farklı yapısal, fizikokimyasal ve yaş–ısı direnç özelliklerine sahip olduğunu açıkça göstermiştir. Özellikle, mineral içeren ortamda üretilen sporların daha yüksek yaş ısı direncine sahip olduğu gözlenmiştir. Ayrıca, yüksek ısı direnci ile yüksek dipikolinik asit içeriği ve çekirdekteki Ca^{2+} iyonları miktarı arasında bir ilişki bulunmaktadır. Buna karşın, doğal ekstrakt içeren elma suyunda, nar suyunda ve karışım meyve sularında (%40–80) sporların çimlenmesini önlemek için gerekli olan dozun, kullanılan antimikrobiyale, konsantrasyonuna ve sporulasyon ortamına bağlı olarak değişiklik gösterdiği gözlenmiştir. Ayrıca, vejetatif hücrelerin büyümesi, üzüm çekirdeği ve nar ekstraktları içeren elma suyunda inhibe edilmiştir. Daha sonra, deneysel inaktivasyon verileri Weibull modeli kullanılarak modellenmiştir. Doğal ekstraklar ve meyve sularında bulunan fenolik maddelerin hücresel bileşenlerinin salınımına ve sporların vejetatif forma geçmesine engel olabileceği, mikroskobik çalışmalar ile belirlenmiştir. Bu çalışmada elde edilen sonuçların, meyve suyu endüstrisinde ısıl işlemlerin tasarımı ve *A. acidoterrestris* kaynaklı bozulmaların önlenmesi için katkı sağlayacağı düşünülmektedir.

Dedicated to my grandmother,
Nazife ÇELENK

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CHAPTER 1

INTRODUCTION

Spoilage of fruit juices occurs primarily due to the growth of yeasts, molds and lactic acid bacteria. Endospore forming bacteria were not of concern in the fruit juice spoilage because the majority of endospore formers can not survive in the acidic environment after spore germination. Therefore, fruit juices are only subjected to thermal pasteurisation to inactivate the spoilage and foodborne pathogens and products are then stored at refrigerated or ambient temperatures (Smit et al., 2011).

However, heat resistance studies indicated the ability of *Alicyclobacillus acidoterrestris* to survive pasteurisation applied to fruit juice and other acidic fruit products. Due to acidophilic nature, their endospores can germinate and increase in products to cell concentrations high enough to produce taint compounds leading to spoilage (Gocmen et al., 2005; Orr et al., 2000; Pettipher et al., 1997; Vieira et al., 2002; Wisotzkey et al., 1992).

The presence of *A. acidoterrestris* spores in fruit juices and fruit juice concentrates is a significant problem for the juice industry (Henczka et al., 2013). Spoilage of fruit juices caused by Alicyclobacilli is extremely difficult to be detected at early stages. In fact, this can be very detrimental for manufacturers because spoilage often results in product withdrawal from the market with consequent economic losses and company image damages. Therefore, spoilage is regarded as an industry–wide issue that requires effective control measures to be developed (Gobbi et al., 2010).

A survey by the European Fruit Juice Association (AIN) in 2005 indicated that 45% of the 68 participants from the fruit processing industry had experienced problems related to *Alicyclobacillus* in the three years preceding the survey, with 33% of these experiencing more than three incidents. Of those that had encountered spoilage problems, 35% of the incident were reported as being intermediately to majorly severe. The survey also revealed that *Alicyclobacillus* is a significant problem in processed fruit products, raw materials (beverage bases) and fruit juice concentrates (Howard, 2006).

Since *A. acidoterrestris* has been suggested as the target organism to be used in the design of adequate pasteurization processes of acidic food products (Silva et al.,

1999), the effects of different environmental factors on the resistance properties should be determined for fruit juice processing technologies to ensure the safety and prolong the shelf–life of the products. Effective control measures should be applied to fruit juices without affecting the quality. Elimination and control of this bacteria from the processing environment is challenging, and little information is available on how manufacturing practices and processing treatments affect *Alicyclobacillus* during concentrate production (Merle and Montville, 2014).

Sporulation conditions such as sporulation temperature and sporulation media affect the resistance properties of spores. Especially, the composition of the sporulation media and mineral concentrations in spores are important for wet–heat resistance. On the other hand, the mechanism of this effect is still unclear and no correlation has been found between the mineral composition and sporulation media (Carlin, 2011). Also, the effect of sporulation media on the chemical and antimicrobial resistance characteristics has not been investigated yet.

Problems occurred primarily in apple raw materials and the final products (Witthuhn et al., 2013). Thus, it is considered to represent important target microorganisms in the quality control of apple juices (Henczka et al., 2013). Therefore, reconstituted apple juice was used in this study. In order to better understand the resistance properties of *A. acidoterrestris*, the objectives of the present dissertation are:

(1) to investigate the spore characteristics (mineral composition, dipicolinic acid content, surface properties and guaiacol production ability) produced on different sporulation media,

(2) to investigate the effects of sporulation media on wet–heat, antimicrobial and chemical resistance,

(3) to determine the effects of food–grade antimicrobials such as grape seed and pomegranate fruit extract on vegetative cells and spore germination/outgrowth,

(4) to investigate the survival characteristics of cells and spores from different sporulation media in pomegranate and pomegranate–apple blend juices,

(5) to model cell inactivation data using survival models,

(6) to investigate the ultrastructural changes after wet–heat and antimicrobial treatments using scanning electron and atomic force microscopy.

CHAPTER 2

LITERATURE REVIEW

2.1. Bacterial Endospores

When *Bacillus* and *Clostridium* spp. encounter environmental stresses such as nutrient depletion, they form a dormant structure termed as endospore. The endospores have been focused on since their discovery in the late 19th century. These endospores are among the most resistant forms of living organisms (Carlin, 2011). Therefore, control of spores is one of the important problems in food preservation and the complete inactivation is often impossible without affecting food quality and structure.

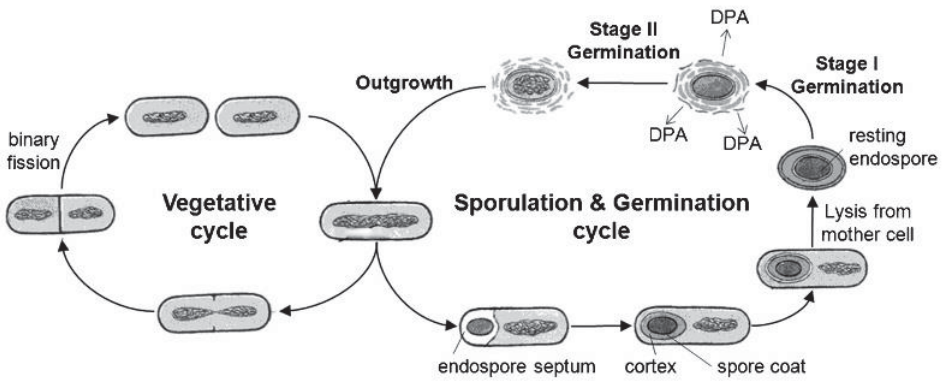

Figure 2.1. Life cycle of endospore forming bacterium
(Source: Ponce et al., 2008)

The process by which spores can leave the dormant state is referred to as germination (Fig.2.1). The germination is a biophysical and degradative process. During germination, the spore's inner membrane increases in fluidity and ion fluxes resume. Monovalent cations, potassium and sodium ions move across the spore membrane. Similarly, calcium ions and dipicolinic acid (DPA) are excreted. The peptidoglycan spore cortex is degraded, and the coat layers are partially degraded. ATP synthesis and oxidative metabolism also proceed. Moreover, DNA damage is repaired and the DNA–complexing small acid–soluble proteins (SASPs) are degraded by a specific protease into amino acids necessary for outgrowth (Moir et al., 2002). Outgrowth of spores

occurs due to the resumption of metabolic activity and cell growth (Driks, 2009). Therefore, spores are not metabolically active, and this provides the microorganism to survive over long periods under extreme environmental conditions (Reineke et al., 2013b).

2.1.1. Endospore Structure and Resistance

Spores can survive wet and dry heat, high hydrostatic pressure, desiccation, UV radiation, γ–radiation and antimicrobials that rapidly inactivate vegetative cells (Eijlander et al., 2011). The structure and chemical composition of the spore are important for their resistance (Paredes-Sabja et al., 2011) and differ from vegetative cells. Spore consists of the following layers: exosporium, spore coat, outer membrane, cortex, germ cell wall, inner membrane and central core (Fig.2.2).

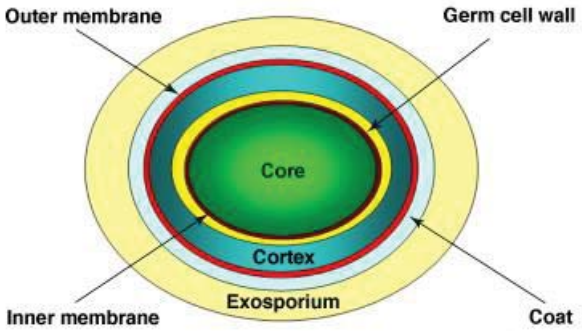

Figure 2.2. Structure of endospores
(Source: Setlow, 2007)

The core contains DNA, RNA and most enzymes. Low core water content, high levels of Ca–DPA (25% of core dry weight) and the saturation of spore DNA with α/β–type SASPs contribute to the resistance to physical and chemical agents (Paredes-Sabja et al., 2011). The inner membrane in which the lipids are largely immobile surrounds the core and it has low permeability to small molecules probably protecting the spore core. Outside of the inner membrane is the germ cell wall. It is composed of peptidoglycan and becomes the cell wall during spore outgrowth. Surrounding the germ cell wall is the cortex peptidoglycan. The outer membrane surrounded by the proteinaceous coat is essential for spore formation. The coat contains more than 50

spore–specific proteins, and protects the spore from chemicals and lytic enzymes (Paredes-Sabja et al., 2011).

DPA is a major chemical component found in the inner spore core (5–14% of spore dry weight) but not present in vegetative cells (Tabor et al., 1976). It is usually present in 1:1 molar ratio with Ca^{2+} (Fig.2.3). DPA release from spores occurs during the first minute of germination, spore activation by a lethal heat treatment or spore inactivation by wet–heat (Kort et al., 2005). DPA content in spores can be determined by boiling or autoclaving high levels of spores for 15 to 30 min, then centrifuging and measuring the DPA in the supernatant using chemicals or by measuring the optical density at 270 nm (Huang et al., 2007). Besides spectrophotometric methods (Janssen et al., 1958), ultraviolet spectroscopy (Scott and Ellar, 1978), Fourier transform infrared spectroscopy (Goodacre et al., 2000), liquid chromatography (Paulus, 1981), luminescence (Fell et al., 2001), electron monochromator mass polarography spectroscopy (Beverly et al., 2000), fluorescence (Hindle and Hall, 1999), and pyrolysis–gas chromatography/ion mobility electrophoresis spectroscopy (Goodacre et al., 2000) have been used for DPA analysis.

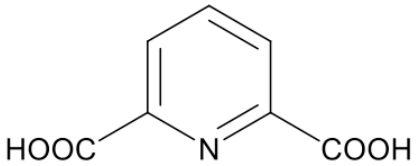

Figure 2.3. Structure of dipicolinic acid
(Source: Setlow, 2006)

DPA is synthesized in the mother cell and subsequently translocated into the forespore (Hintze and Nicholson, 2010). Ca–DPA is important in spore resistance to many environmental stresses and in spore stability. The Ca–DPA levels in spores can vary with species/strains as well as with sporulation conditions. One of its functions on the spore resistance is to lower the core water content, probably by replacing some core water. By this way, wet–heat resistance of spores can be increased by the protection of core proteins against inactivation or denaturation. Mutants of *Bacillus subtilis* spores lacking DPA were found to have a lower core wet density and to be sensitive to wet–heat (Huang et al., 2007; Paidhungat et al., 2000). Another function is to protect spore DNA against many damaging agents (Huang et al., 2007). On the other hand, DPA does

not play a role in the dry–heat resistance and also photosensitizes spores in aqueous suspensions to UV irradiation at 254 nm. Therefore, DPA–less spores are approximately 2.5–fold more resistant to UV–C than DPA–containing congenic spores. This compound also functions to maintain the spore dormancy. Spores with lower DPA are rather unstable and tend to germinate spontaneously (Paidhungat et al., 2000). It has also been suggested as a sensitive biomarker to rapidly detect *Bacillus* spores (Huang et al., 2007).

The resistance of spores is multifactorial and can not be explained by a single parameter (Sella et al., 2014). Table 2.1 shows the factors influencing the resistance of *Bacillus* spores against the applied treatments.

Table 2.1. Factors influencing the resistance of *Bacillus* spores to killing treatments (Source: Stella et al., 2014)

Treatment	**Major factors in resistance**	**Mechanism of spore killing**
Wet heat	• Core water content • α/β–type SASPs • Core mineralization • DPA	Damage of one or more essential proteins
Dry heat	• α/β–type SASPs • DNA repair	DNA damage
UV radiation	• DPA • α/β–type SASPs • Core water content • DNA repair	DNA damage
Ionizing radiation	• Components in the core • SASP • Core dehydration • DPA	DNA, RNA, proteins damage
Microwave radiation	• Core water content	Damage of one or more essential proteins Inner membrane damage DNA damage Coat disruption
Chemicals	• Coat • Inner membrane permeability • α/β–type SASPs • DNA repair	Depends on the chemicals: • DNA damage (nitrous acid, formaldehyde, alkylating agents such as ethylene oxide) • Inner membrane damage (ozone, hypochlorite, chlorine dioxide, ethanol, and strong acid) • Inactivation of spore cortex lytic enzymes (strong alkali)

Factors that contribute to spore resistance are spore coat, cortex peptidoglycans, α/β–type SASPs, DNA repair, core mineralization, spore maturation, core water content, inner membrane permeability, chemical state, sporulation conditions (sporulation medium, sporulation temperature) and other factors (supporting material,

recovery culture medium composition, osmolarity, pH and presence of germinants. The interactions between the environmental and intrinsic factors should be considered during the study of spore resistance (Fig.2.4) (Sella et al., 2014).

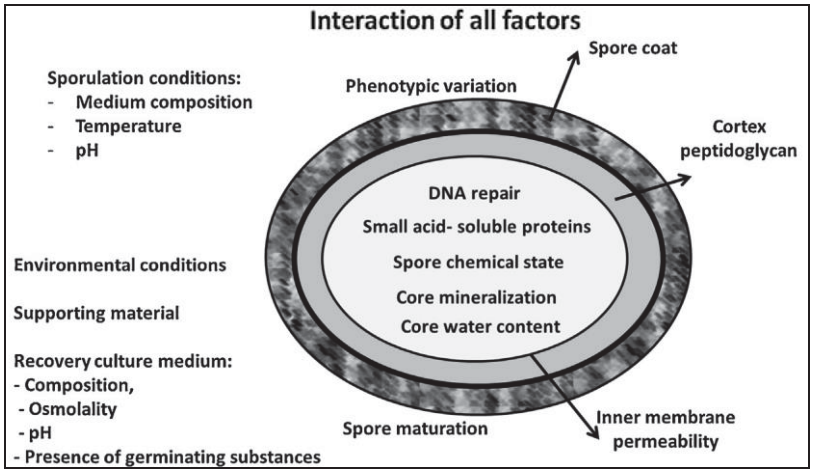

Figure 2.4. Main environmental and intrinsic factors contributing resistance (Source: Sella et al., 2014)

The exact mechanism of wet–heat is not known. The researchers proposed a model for the wet–heat inactivation of *Bacillus* spores as schematized in Fig.2.5. (Coleman et al., 2007; Coleman et al., 2010). As wet–heat treatment continues, damage to proteins at different rates occurs. If the loss of essential proteins is too much, the spores are dead. On the other hand, these dead spores still have DPA and the ability to initiate germination but can not progress in outgrowth because of the inactivation of some essential protein or proteins. When wet–heat continues, damage to one or more proteins in the inner membrane may also occur. This membrane ruptures leading to the rapid release of DPA and other small molecules. After that, water content in the core increases resulting in much more rapid heat inactivation of core proteins. Finally, denaturation of proteins leads to death of spores (Coleman et al., 2007).

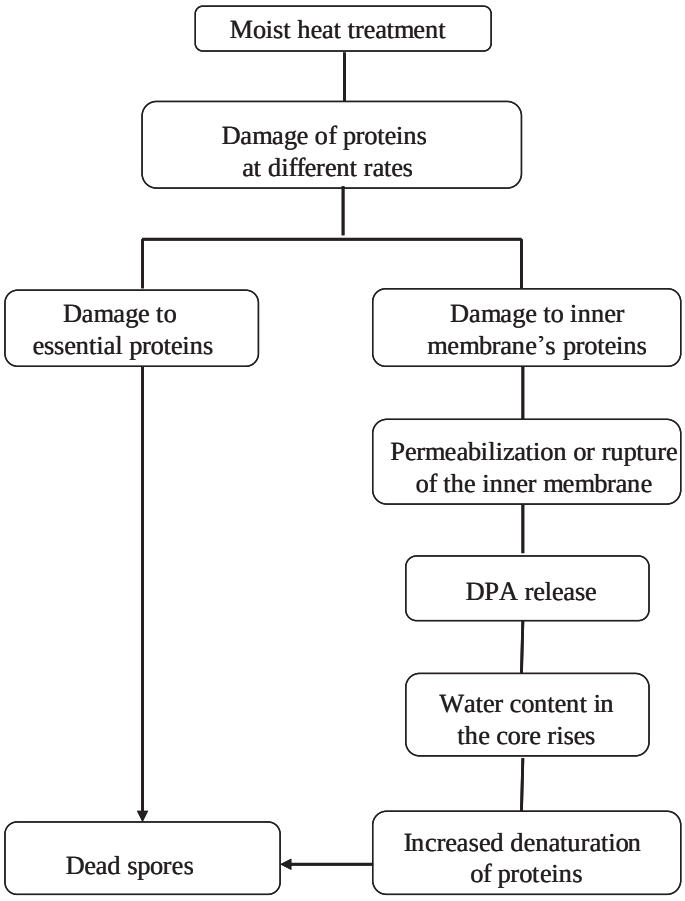

Figure 2.5. Mechanism for *Bacillus* wet–heat inactivation
(Source: Tiburski, 2013)

2.2. *Alicyclobacillus acidoterrestris*

In 1982, a new type of spoilage bacterium was first reported during the incidence of commercial pasteurised apple juice in Germany. The spoilage in these apple juices was flat–sour type with the production of offensive–smelling taint compounds and subsequently the microorganism responsible for this incident was identified as *Alicyclobacillus acidoterrestris* (Cerny et al., 1984; Wisotzkey et al., 1992). *A. acidoterrestris* is a thermoacidophilic, non–pathogenic, rod–shaped spore–forming bacterium with a central, subterminal, or terminal oval spore. It grows at pH values ranging from 2.5 to 6.0 and at temperature range of 25 to 60 °C (Silva and Gibbs, 2001). This organism is 2.9 to 4.3 μm in length and 0.6 to 0.8 μm in width. ω–alicyclic fatty acids are the major lipid components of *A. acidoterrestris* membranes and they are associated with the resistance of the organism to acidic conditions and high temperatures (Fig.2.6). Depending on the strain, ω–cyclohexane fatty acids comprise

15–91% of the total fatty acid content (Hippchen et al., 1981). It is supposed that there is a positive correlation between the growth temperature and fatty acid content. The ω–alicyclic fatty acids contribute the heat resistance because they stabilize the cell membranes and reduce the membrane permeability that leads to cell leakage and death (Jensen, 1999).

Figure 2.6. (a) ω–cyclohexyl fatty acids, (b) ω–cycloheptyl fatty acids
(Source: Goto, 2007a)

The growth properties of *A. acidoterrestris* depend on the juice type and the isolation source of the strains (Goto, 2007b). It has been detected in various spoiled commercial pasteurized fruit juices such as apple, tomato, white grape, grapefruit, orange and pineapple juices; but growth was not detected in red grape juices, Amazonian fruit Cupuacu, apple–cranberry, 10% fruit juices, pineapple juice and salsa (Silva and Gibbs, 2001). Inhibitory compounds present in these juices might possibly inhibit either spore germination or cell growth (Tokuda, 2007). For example, certain phenolic compounds in red grape juice were found to inhibit the spore germination and growth of *A. acidoterrestris* cells (Splittstoesser et al., 1994).

A relationship between the growth of *A. acidoterrestris* and incubation temperature was also established when spores were inoculated into different fruit juices. No detectable growth was observed in orange, apple or other non–carbonated fruit juices during storage at 4 °C. On the other hand, at 25, 35 and 45 °C, 10^2 to 10^5–fold increase in the cell counts was observed (Tokuda, 2007).

2.2.1. Sources of Contamination

The primary reservoir for *A. acidoterrestris* is soil that contaminates fruit during harvest or through windfall (Brown, 1995). Fruit in contact with the soil is more susceptible to contamination (Splittstoesser et al., 1998). Also, it has been suggested that fruit surfaces may be continuously contaminated with spores from the condensate wash water. After the extraction, juice could contain spores, and then contaminate the

evaporator. Rinsing the equipment surfaces and evaporators with condensate water containing spores may contaminate the juice entering into the evaporator or the final product. Therefore, fruit cleaning operations and condensate water systems should be improved to eliminate the presence of *A. acidoterrestris* spores in fruit juices. The water used to reconstitute concentrates to obtain full strength juice is another possible source of contamination (Palop et al., 2000; Wisse and Parish, 1998).

2.2.2. Spoilage by *Alicyclobacillus acidoterrestris*

Typical juice spoilage involves production of CO_2 and off–flavors by fermentative organisms resulting in bulging or exploding containers. Spoilage caused by Alicyclobacilli is different (Parish, 2006). Fruit juices are generally treated at temperatures of about 95 °C for 2 min (Komitopoulou et al., 1999). However, *A. acidoterrestris* spores have been shown to survive in such heat treatments and surviving spores can germinate and grow at pH<4 in fruit juices, leading to spoilage (Walker and Phillips, 2008a). Detection of *A. acidoterrestris* in fruit juices is difficult because the spoiled juice appears normal or has light sediment (Walker and Phillips, 2005). Since there is no CO_2 produced as an indicative of microbial growth, the powerful antiseptic and medicinal aromas may not be detected until consumers open the package. Therefore, spoilage is an important problem for manufacturer due to difficulties to eliminate *A. acidoterrestris* from the processing environment (Jensen, 1999). Guaiacol, 2,6–dibromophenol, and 2–methoxyphenol are the compounds responsible for off–flavor. Among these compounds, guaiacol is the major compound related to taint production. The possible precursors of guaiacol are ferulic acid, vanillin and vanillic acid. These compounds are ubiquitous in nature (Witthuhn et al., 2012). The guaiacol is formed from ferulic acid via vanillin (Fig.2.7) and can be detected by smell in fruit juices at 2 ppb (Bevilacqua et al., 2008c; Chang and Kang, 2004). *A. acidophilus*, *A. herbarius*, *A. hesperidium*, and *A. cycloheptanicus* can also produce guaiacol, however, *A. acidoterrestris* strains are primarily responsible for the spoilage and guaiacol production (Witthuhn et al., 2012). Ferulic acid is an aromatic phenolic compound present in fruits, vegetables, grains, beans, leaves, seeds, nuts, grasses, and flowers. This compound can be converted to vanillin and vanillic acid by many bacteria and fungi. Vanillin is also found in various fruit and fruit products such as strawberries, orange

juice, apple cider brandy, mango, wines and citrus juice (Witthuhn et al., 2012) and used as flavoring and aroma compound in the food industry. Gram–negative bacteria of the *Pseudomonas* genus, the genera *Amycolatopsis* and *Streptomyces*, Gram–positive bacteria such as *B. subtilis* and *Rhodococcus* spp., and the basidiomycete fungus *Pycnoporus cinnabarinus* have been found responsible for the conversion of ferulic acid into vanillin (Di Gioia et al., 2011; Witthuhn et al., 2012).

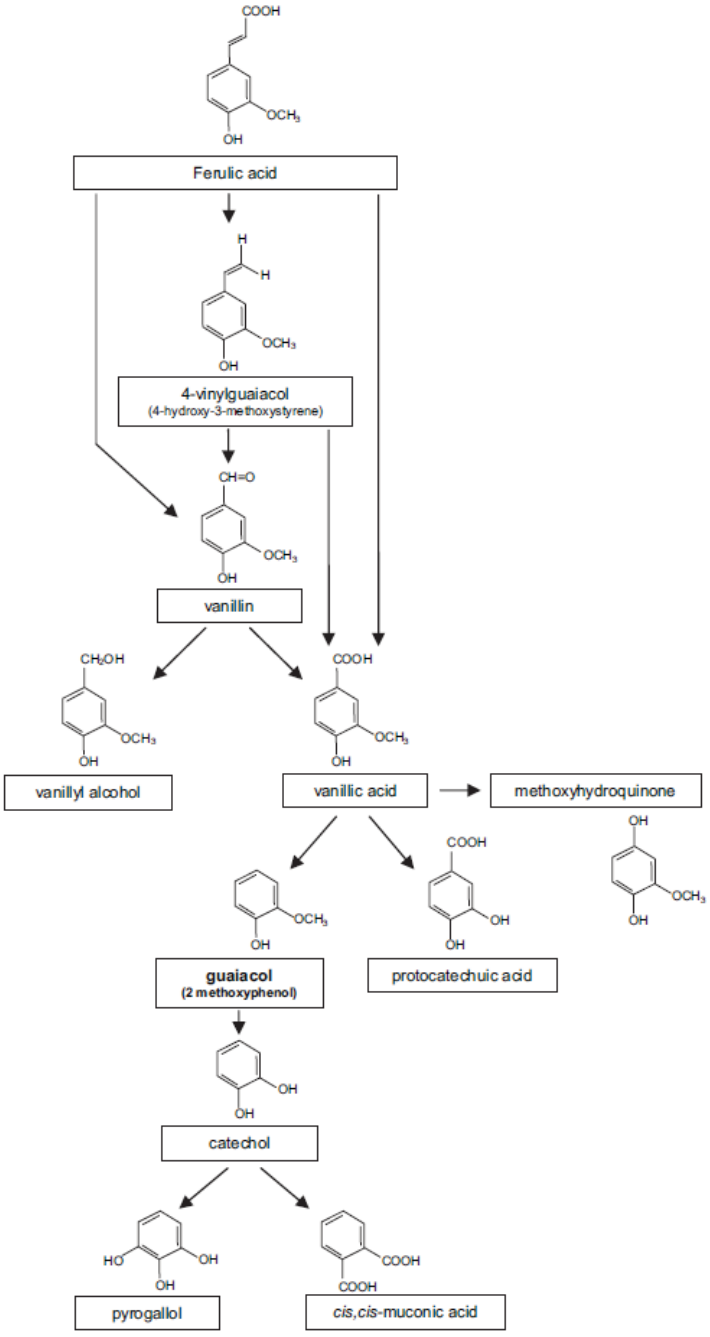

Figure 2.7. Microbial production pathways of guaiacol and other products by ferulic acid metabolism (Source: Smit et al., 2011)

Many soil bacilli can decarboxylate vanillic acid to produce guaiacol. Vanillic acid is naturally derived compound from the plant polymer lignin and can also be used in the beverages as an ingredient. Therefore, fruit juice processors are reformulating their products to remove vanillic acid from their products to prevent guaiacol production (Merle and Montville, 2014). Various factors are known to influence the guaiacol production such as cell concentration, incubation temperature, heat shock treatment and growth medium (Chang and Kang, 2004). In fact, the metabolic conversion of vanillic acid to guaiacol is faster than the conversion of vanillin to guaiacol (Witthuhn et al., 2012). The levels for detectable taint production in fruit juices are also generally reported as 10^4–10^5 CFU/mL (Komitopoulou et al., 1999).

The spoilage potential of juices by *A. acidoterrestris* is affected mainly by °Brix and then by storage temperature, and juice type. Therefore, single strength juice is a favorable environment for germination and growth leading to spoilage under certain conditions (Walls and Chuyate, 2000). Typically spoilage occurs in the spring or summer seasons, and most commonly in apple and orange juices. Since the pasteurization can not provide the complete inactivation of *A. acidoterrestris* spores, the final product should be kept at temperatures below 25 °C to prevent their growth, and the spoilage risk. When pasteurized fruit juices are distributed under refrigeration, subsequent chilling will cause a significant rise in costs (Bahçeci et al., 2003).

2.2.3. Control of *Alicyclobacillus acidoterrestris*

Complete inactivation of *A. acidoterrestris* in fruit juices can be obtained by increasing the intensity of thermal treatments. However, an increase in the treatment time or temperature is not possible because of irreversibly damage to their nutritive and sensorial properties (Bevilacqua et al., 2009).

Recently, new methods have been proposed in order to reduce the negative effects of thermal treatments (Bevilacqua et al., 2008c). These methods can be classified as high hydrostatic pressure, high pressure homogenization, supercritical carbon dioxide, ultrasonic wave, microwave, ohmic heating, high pressure CO_2, high pressure pasteurisation and short wave ultraviolet light (UV–C) as summarized in Table 2.2.

The chemical compounds have also been tested for the control of *A. acidoterrestris* spores in aqueous suspensions, on fruit and contact surfaces (Table 2.3).

Disinfectants to be used in the food industry should be safe, have no adverse effect on surface materials, be stable during storage and over a wide range of pH and temperatures, be resistant to environmental factors and have a broad spectrum of activity, be environmentally friendly and cost efficient (Møretrø et al., 2012). However, chemical disinfectants are less effective for the elimination of *A. acidoterrestris* spores from apple surfaces compared to their effectiveness in aqueous suspensions. Since the apple surfaces are hydrophobic, it protects vegetative cells and spores from contact with disinfectants. The use of detergent or solvent and more vigorous washing or rubbing of the apples would perhaps enhance the release of spores from the surface and increase the contact with disinfectants (Orr and Beuchat, 2000).

Nowadays, Western countries desire fewer synthetic additives and more friendly compounds (Burt, 2004). Therefore, bacteriocins, fatty acids, monoglycerides and essential oils could be considered promising bioactive compounds and their use might be proposed to control and/or inhibit Alicyclobacilli spore germination (Bevilacqua et al., 2008c). Several natural antimicrobials have been tested against *A. acidoterrestris* (Table 2.4). However, few studies have explained the mode of action of these antimicrobials on *A. acidoterrestris* cells and spores. For example, nisin does not inactivate spores or inhibit their germination, but prevents the post germination swelling, avoiding the formation of the vegetative form and the microbial growth (Delves-Broughton, 1990; Komitopoulou et al., 1999). It acts synergistically with heat to inactivate microorganisms and can be added directly into juices or incorporated in polymers that provide controlled release during storage (Merle and Montville, 2014). The use of nisin in foods is limited by its relatively high cost and by decreased antimicrobial activity in complex food matrices (Merle and Montville, 2014). Lysozyme acts by cleaving the glycoside bond between C_1 of N–acetylmuramic acid and C_4 of acetylglucosine in the bacterial cell wall. It is active against Gram–positive bacteria whereas it is ineffective against Gram–negative bacteria, due to the presence of a lipopolysaccharide layer in the outer membrane (Corbo et al., 2009). Lysozyme prevents the spore germination of *A. acidoterrestris* (Conte et al., 2006). Citral and limonene are aliphatic monoterpene compounds that are obtained from *Citrus* spp. They are used frequently to give flavour to soft drinks and other products. These essential oil components have Generally Recognized as Safe (GRAS) status by the FDA and their use is permitted in the E.U. (Bevilacqua et al., 2010b; Tiwari et al., 2009). Their specific mode of action is not well known but it is supposed that the effect is similar to

the action of phenolic antimicrobials (Tiwari et al., 2009). At low concentrations, they affect the enzymatic activity, generally associated with energy production. At high concentrations, they lead to protein denaturation. They can also affect the cytoplasmic membrane permeability, allowing the loss of macromolecules from inside the cell, disturbing the cytoplasmic membrane function such as electron transport, nutrient uptake, protein and nucleotide synthesis, and enzymatic activity and also interacting with the trans–membrane proteins, causing structural deformations (Huertas et al., 2014). The ultrastructural changes in vegetative cells and spores of DSMZ 2498 after enterocin AS–48 treatments were observed by transmission electron microscopy (TEM) (Grande et al., 2005). TEM images indicated the damage to the cell structure, bacterial lysis and disorganization of endospore structure. In a recent study, SEM images of *A. acidoterrestris* vegetative cells treated with bificin C6165 indicated pore formation and subsequently cell lysis (Pei et al., 2014).

Table 2.2. Technologies used for the control of *A. acidoterrestris*

Technology	Result	Reference
High hydrostatic pressure	• Inactivation of vegetative cells • Not effective on spores • Induction of germination in tomato sauce, and then inactivation of germinated spores	(Shearer et al., 2000) (Alpas et al., 2003) (Buzrul et al., 2005)
High pressure homogenization	• 1–2 log cell reductions at highest pressures • Not effective on spores	(Bevilacqua et al., 2007a)
Supercritical CO_2	• Complete inactivation of spores in apple juice above 65 °C, 100 bar for 40 min and 70 °C, 80 bar for 30 min	(Bae et al., 2009)
Ultrasound	• Not effective on spores in apple juice • Changes in sugar content, acidity, haze and juice browning	(Yuan et al., 2009)
Ohmic heating	• 5 log spore reductions in orange juice at 30 V/cm	(Baysal and Icier, 2010)
Short time microwave	• 2–fold reductions at 90% (6 min), 80% (7 min), and 100% power (5 min) in a cream of asparagus	(Giuliani et al., 2010)
Ultrasound	• 4.56 log spore reductions at 600 W/30 min in apple juice	(Wang et al., 2010a)
Microwave/Ultrasonic wave	• UW more effective than MW to inactivate vegetative cells in laboratory medium	(Wang et al., 2010b)
High pressure CO_2	• 4 log spore reductions in apple cream at 30 °C /10 MPa • No change in sensorial and rheological properties • Slight reduction in the vitamin C content	(Casas et al., 2012)
High pressure pasteurisation	• Inactivation of spores at 45 °C /600 MPa or at 65 °C /200 MPa in orange juice	(Silva et al., 2012)
Short wave UV–C	• 5.5 and 2 log spore reductions in white grape and apple juices, respectively	(Baysal et al., 2013)

Table 2.3. Chemicals used for the control of *A. acidoterrestris* spores

Chemical	Amount	Use	Treatment	Reduction	Reference
Chlorine	200 ppm	Aqueous suspensions	10 min	• 2.2 log CFU/mL	(Orr and Beuchat, 2000)
Chlorine	1000 ppm	Apple		• >5 log CFU/mL	
Acidified chlorite	500 ppm			• 0.4 log CFU/mL	
Acidified chlorite	1200 ppm			• <1 log CFU/mL	
H_2O_2	4%			• >5 log CFU/mL	
ClO_2	500 ppm			• <1 log CFU/mL	
Aqueous ClO_2	40 ppm	Aqueous suspensions	5 min	• >4 log	(Lee et al., 2004)
	80 ppm	Apple		• Undetectable levels	
	120 ppm			• Undetectable levels	
Gaseous ClO_2	High release sachets	Apple	1 h	• Undetectable levels	(Lee et al., 2006)
	Medium release sachets		1 h	• Undetectable levels	
	Low release sachets		1 h	• 2.7 log CFU/mL	
			2 h	• 3.7 log CFU/mL	
			3 h	• 4.5 log CFU/mL	
Sodium benzoate	0.1–0.5 ppm low inoculum	Apple juice	37 °C	• Growth inhibition	(Walker and Phillips, 2008b)
Potassium sorbate	0.5–1.5 ppm high inoculum				
Clorox[†]	2000 ppm	Food contact surface	90 °C /2 min	• 2.55 log CFU/cm^2	(Podolak et al., 2009)
Vortexx[‡‡]	2600 ppm			• 2.32 log CFU/cm^2	
Chlorous acid	268 ppm	Laboratory medium	5 min	• 1.6 log CFU/mL	(Lee et al., 2010)
			10 min	• 4.3 log CFU/mL	
			15 min	• 7.0 log CFU/mL	
Chlorous acid	268 ppm	Apple	10 min	• 5 log CFU/mL	(Lee et al., 2010)
Ozone (gaseous)	2.8 ppm	Apple juice	4 ° C /40 min	• 2.2 log CFU/mL	(Torlak, 2014)
	5.3 ppm			• 2.8 log CFU/mL	

[†] Clorox: sodium hypochlorite

[‡] Vortexx: hydrogen peroxide, peroxyacetic acid, and octanoic acid

Table 2.4. Natural antimicrobials used for the control of *A. acidoterrestri*s spores

Antimicrobial	Result	Reference
Nisin	• Inhibition of growth in apple, grapefruit, and orange juices by 5 IU/mL at 25 °C • Decrease $D_{80°C}$ value in apple juice from 41 min to 24 min • Inhibition of growth in the apple juice at the 100 IU/mL at 44 °C	(Komitopoulou et al., 1999)
Nisin	• Inhibition of spore outgrowth by 25–50 IU/mL in both orange and fruit–mixed fruits • Higher concentrations not effective because of the competitive effects of phenols in apple juice • Decrease $D_{90°C}$ by 24% in orange and 29% in apple drink • Higher levels required to inhibit the vegetative cells	(Yamazaki et al., 2000)
Nisin	• Inhibition of multiplication alone, and in combination with sodium benzoate or potassium sorbate	(Walker and Phillips, 2008b)
Bovicin H5	• Bactericidal against vegetative cells in mango pulp • Sporicidal against endospores • Decrease *D*–values (80–95 °C) by 77% to 95%	(De Carvalho et al., 2008)
Bificin C6165	• Inactivation of vegetative cells • Higher inhibition at lower pH (3.5) and 45 °C • Undetectable spore levels within 24 h in grape, peach and orange juices • No antimicrobial activity in commercial apple juice • Decrease $D_{90°C}$ values in juices • Suitable for encapsulation with Ca–alginate gel as a promising method	(Pei et al., 2014)
Lysozyme	• Higher sensitivity of spores (below the detection level in the solution) • Maximum activity in saline solution • Protective effect of laboratory medium	(Bevilacqua et al., 2007b)

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Table 2.4. (Cont.)

Antimicrobial	Result	Reference
Cinnamaldehyde	• Enhanced antimicrobial activity at low pH in laboratory medium • Prolongation of the lag phase at low doses • Decreased germination at high doses	(Bevilacqua et al., 2008a)
Cinnamaldehyde	• Inhibition of spore germination at 90 °C in tomato juice	(Bevilacqua et al., 2010c)
Cinnamaldehyde Eugenol	• Inhibition of spore germination in the laboratory medium and apple juice • Action of eugenol as a strengthening factor to reduce cinnamaldehyde amount • Higher prolongation of the lag phase by eugenol than cinnamaldehyde alone • Lower amounts required to inhibit in the apple juice than laboratory medium	(Bevilacqua et al., 2010a)
Cinnamaldehyde Eugenol Limonene	• Cinnamaldehyde most effective compound for the inhibition of spore germination • Growth inhibition with 500 ppm eugenol for 13 days • No activity of limonene on the inhibition of spore outgrowth in the laboratory medium	(Bevilacqua et al., 2008b)
Nisin Citral Limonene	• No decrease in *D*–value when added into the heating medium • >2 log reductions when nisin alone added into the plating medium • Strong synergistic effect between nisin and citral on the recovery medium • No inhibitory effect of limonene in both heating and recovery media	(Huertas et al., 2014)
Saponin extract	• 2.34 log spore reductions with commercial saponin /heat in concentrated juice • Spore inactivation with higher doses of purified extract in concentrated and reconstituted juice	(Alberice et al., 2012)
Citrus extracts	• The MICs of biocitro and lemon extract: 160–500 ppm • Enhanced antimicrobial action by thermal treatment in laboratory media and apple juice • No bioactivity loss before and after thermal treatments	*(*Bevilacqua et al., 2013)

2.2.4. Heat Resistance of *Alicyclobacillus acidoterrestris*

Since *A. acidoterrestris* has been involved in several spoilage incidents, most studies have focused on the determination of *A. acidoterrestris* heat resistance characteristics rather than other *Alicyclobacillu*s species (Smit et al., 2011). The reported *D* and *z*– values in fruit juices have been summarized in Table 2.5. It is clear that the thermal pasteurization (86–96 °C for approximately 2 min) does not eliminate *A. acidoterrestris* spores in fruit juices (Silva and Gibbs, 2004; Terano et al., 2005).

Table 2.5. Heat resistance of *A. acidoterrestris* in fruit juices and concentrates (Source: Smit et al., 2011)

Heating medium	**pH**	**SS (°Brix)**	**Strain**	**T (°C)**	***D*-value (min)**	***z*-value (°C)**
Apple drink (clear)	nr	nr[†]	AB–5	90	20.8	nr
Apple juice	3.20	nr	nr	90	15.00	nr
Apple juice	3.50	11.40	VF	85	56±14.00	7.7
				90	23±7.50	
				95	2.8±0.70	
Apple juice	3.51	nr	Z CRA 7182	80	41.15±0.24	12.2
				90	7.38±0.85	
				95	2.3±0.03	
Apple juice	3.68	12.20	DSM 2498	90	11.1±1.60	8.5
				93	4.2±0.70	
				96	2.1±0.20	
				100	0.7±000	
Apple nectar without AA[‡]	14.00	2.97	DSM 2498	90	14.4±0.8	9.2
				93	6.7±0.6	
				96	3.3±0.3	
				100	1.2±000	
Apple nectar with AA (250 mg/L)	14.00	2.95	DSM 2498	90	14.1±0.5	8.8
				93	6.4±0.5	
				96	3.1±0.3	
				100	1.0±000	
Berry juice	nr	nr	nr	81.8	11.00	7.20
				91.1	3.80	
				95	1.00	
Blackcurcant concentrate	2.50	58.50	NCIMB 13137	91	24.10±2.70	nr
Blackcurcant light concentrate	2.50	26.10	NCIMB 13137	91	3.84±0.49	nr
Concord grape juice	3.50	16.00	WAC	85	53.00	6.90
				90	11.00	
				95	1.90	
		30.00	WAC	85	76.00	6.60
				90	18.00	
				95	2.30	
		65.00	WAC	85	276.00	7.40
				90	127.00	
				95	12.00	

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Table 2.5. (Cont.)

Heating medium	pH	SS (°Brix)	Strain	T (°C)	*D*-value (min)	*z*-value (°C)
Cupuaçu extract	3.60	11.30	NCIMB	85	17.50±1.10	9.00
			13137	91	5.35±0.57	
				95	2.82±0.27	
				97	0.57±0.034	
Grape juice	3.30	15.80	WAC	85	57.00±13.00	7.20
				90	16.00±4.10	
				95	2.40±0.90	
Grapefruit juice	3.42	nr	Z CRA	80	37.87±0.20	11.60
			7182	90	5.95±0.32	
				95	1.85±0.05	
Orange juice	4.10	5.30	nr	95	5.30	9.50
Orange juice	3.15	9.00	46	85	60.80	7.20
				90	10.00	
				95	2.50	
			70	85	67.30	11.30
				90	15.60	
				95	8.70	
			145	85	94.50	7.20
				90	20.60	
				95	3.80	
			DSM 2498	85	50.00	7.90
				90	16.90	
				95	2.70	
Orange juice	3.50	11.70	NCIMB	85	65.60±5.50	7.80
			13137	91	11.90±0.60	
Orange juice	3.90	nr	Z CRA	80	54.30±0.42	12.90
			7182	90	10.30±0.30	
				95	3.59±0.04	
Orange drink	nr	nr	AB–5	90	23.10	nr
Mango pulp	4.00	nr	DSM 2498	80	40.00±1.50	21.27
				85	25.00±0.10	
				90	11.66±1.80	
				95	8.33±2.00	

†nr: not reported
‡AA=ascorbic acid

Heat resistance in fruit products are higher than those obtained in buffers at the same heating temperature and pH. This could be due to the food constituents that increase the heat resistance (Bahçeci and Acar, 2007b).

2.2.5. Factors Affecting Heat Resistance

There are a number of environmental factors that affect the heat resistance of *A. acidoterrestris* spores such as heating temperature, pH, soluble solid content, divalent cations, strain difference, and sporulation temperature.

2.2.5.1. Temperature

Heating temperature has the greatest impact on *D*–values (Table 2.5). A nonlinear decrease in *D*–values is observed with an increase in temperature (Bahçeci and Acar, 2007b; Maldonado et al., 2008; Silva et al., 1999). The effect of temperature is greater than that of pH and slight changes in temperature may have a considerable effect on *D*–values (Bahçeci and Acar, 2007b; Silva et al., 1999). Temperature also affects the impact of other parameters such as pH and soluble solid because their effects are more pronounced at lower temperatures (Komitopoulou et al., 1999; Silva et al., 1999).

2.2.5.2. pH

Linear decrease in *D*–values is observed with a decrease in pH (Table 2.5). This effect seems to be more pronounced at lower temperatures of 80–90 °C (Komitopoulou et al., 1999; Silva et al., 1999). On the other hand, Murakami et al. (1998) indicated that pH did not have a significant influence on heat resistance, as there were no significant differences between *D*–values of *A. acidoterrestris* AB–1 spores in Mcllvaine buffer at pH values ranging from 3.00 to 8.00 at a specified temperature. Also, temperature and specific juice properties play a significant role than pH as *A. acidoterrestris* still had a lower heat resistance in grapefruit juice (pH 4.0) than in orange juice (pH 3.9) (Komitopoulou et al., 1999; Silva et al., 1999). In addition, the type of acid used to acidify the heating medium does not influence the heat resistance because the *D*–values obtained in a model fruit juice system acidified with different organic acids (malic, tartaric or citric acids) did not significantly differ from the others obtained in the studied temperature range from 91 °C to 100 °C (Pontius et al., 1998).

2.2.5.3. Soluble Solid Content

There is a linear relationship between total soluble solids (SS) and *D*–values. Increasing SS content results in higher heat resistance (Silva et al., 1999). Therefore, destruction of endospores would be more difficult in fruit juice concentrate than in

single strength juice (Splittstoesser et al., 1998). The effect of SS became less pronounced as temperatures increased from 85 to 97 °C but no effect was observed at 97 °C (Silva et al., 1999). These researchers also suggested that water activity, rather than total SS, should be measured. Because different sugars at the same concentrations produce different water activities and could have different effects on *D*–values (Silva et al., 1999).

2.2.5.5. Divalent Cations

Mineralisation of endospores with divalent cations, such as calcium or manganase, contributes to the stabilisation of endospores against heat (Bender and Marquis, 1985). Calcium chelates DPA forming Ca–DPA, which further stabilises endospores and contributes to heat resistance. *A. acidoterrestris* endospores bind Ca^{2+} and Mn^{2+} more strongly at low pH compared to other *Bacillus* species and are also keep Ca–DPA levels constant. Thus, stabilisation of Ca–DPA and the ability to strongly bind divalent cations contribute to the heat resistance (Yamazaki et al., 1997).

2.2.5.4. Strain Difference

Heat resistance in *A. acidoterrestris* is strain specific (Table 2.5). In the related literature, three *A. acidoterrestris* strains were heat–treated in a model fruit juice acidified with malic acid to pH 3.7 (Pontius et al., 1998). Among these strains, VF and WAC had approximately the same heat resistance, but the other strain (IP) was less heat resistant. In Mcllvaine buffer (pH 4.0), strain AB–1 (Murakami et al., 1998) was approximately twice heat resistant than DSM 2498 (Bahçeci and Acar, 2007b). In another study, DSM 2498 and 46 had similar *D*–values, strains 145 and 79 were found more heat resistant in orange juice (pH 3.15, 9.00 °Brix) (Eiroa et al., 1999).

2.2.5.6. Sporulation Temperature

Increasing sporulation temperature increases the heat resistance (Smit et al., 2011).

2.3. Plant Extracts

The safety and shelf life of foods can be improved by novel technologies to prevent or delay microbial growth. Packing in controlled atmosphere, activated films, non–thermal treatments, irradiation, modified atmosphere packaging are among these technologies. On the other hand, most of these technologies may cause the loss of organoleptic properties in foods and thereby reduce consumer acceptability. Because of these reasons, the consumer demands are increasingly focusing on minimally processed food products, with less use of synthetic chemicals and without compromising food safety. Synthetic antimicrobials are approved in many countries, but nowadays the use of natural preservatives has been popular due to the adverse effects of these chemicals. Therefore, safe, effective and acceptable natural preservatives need to be discovered (Negi, 2012).

Natural antimicrobials should have at minimum the following characteristics (Davidson et al., 2013):

- Effective at low concentrations in its natural form,
- Economical at used levels,
- Cause no change in sensorial properties of the product,
- Inhibit a wide array of pathogenic and spoilage organisms,
- Nontoxic.

In most cases, natural antimicrobials show very high activity in laboratory media. After incorporation into a food system, they show little to no inhibitory activity against target microorganisms when used at similar concentrations (Davidson et al., 2013). The food matrix components such as proteins, lipids, complex carbohydrates, simple sugars and cations can reduce the antimicrobial activity. The type of food is important to incorporate these antimicrobials. The challenges related with application of these antimicrobials are as follows ranging from least to most difficult: carbohydrate–based beverages, bakery products, fruits, vegetables, dairy, meat, poultry and seafood products (Davidson et al., 2013). Carbohydrate–based beverages such as fruit juices and soft drinks are most suitable food matrices for the incorporation of natural antimicrobials. They have relatively low pH, protein and lipid contents that are very low to nonexistent. They provide a homogenous environment for the dispersion of the antimicrobials. The interaction only occurs with simple and complex carbohydrates.

Also, there is a positive relationship between the juice pH and antimicrobial concentration required for the inhibition and inactivation (Davidson et al., 2013).

The use of plant extracts as antimicrobial agents can be of great significance in food preservation. Natural antimicrobials such as plant extracts as pure compounds or standardized extracts provide many advantages to control the microbial growth because of their chemical diversity (Negi, 2012). Many of these plant extracts are in GRAS status. Their antimicrobial activity is most likely related with the adsorption of polyphenols to bacterial membranes with membrane disruption and subsequent leakage of cellular contents and the hydroperoxide generation from polyphenols (Akagawa et al., 2003; Ikigai et al., 1993; Otake et al., 1991).

2.3.1. Grape Seed Extract

Grape contains a large amount of phenolic compounds. Especially, the seed contains 60–70% of the total phenolic content of the fruit, comprising of monomeric phenolic compounds such as (+)–catechins, (-)–epicatechin and (-)–epicatechin–3–o– and dimeric, trimeric and tetrameric procyanidins (Shrestha et al., 2012). The increasing order of the antimicrobial activity in grape was flesh, whole fruit grape extracts, fermented pomace, skin, leaves and seeds (Xia et al., 2010). Grape seed extract (GSE) is a by–product derived from the grape seeds (*Vitis vinifera*) (from grape juice and wine processing) that is extracted, dried and purified to produce a polyphenolic compound rich extract (Lau and King, 2003). GSE is sold commercially as dietary supplement and listed on the "Everything Added to Food in the United States (EAFUS)". This extract also has GRAS status approved by FDA. Standardized GSEs contain 74 to 78% oligomeric proanthocyanidins and less than approximately 6% of free flavanol monomers on a dry weight basis (Burdock, 2005). Figure 2.8 represents the structures of the polyphenolic compounds found in GSE (Guendez et al., 2005). Proanthocyanidins in the form of monomeric phenolic compounds, such as catechin, epicatechin and epicatechin–3–O–gallate, and in dimeric, trimeric and tetrameric procyanidin forms are rich in GSE. These phenolics can combine with gallic acid to form gallate esters and ultimately glycosides. The red color and astringency taste of GSE can be attributed to polyphenol rich compounds especially proanthocyanidins

which may affect the color and sensory characteristics of the product when used at higher concentrations (Weber et al., 2007).

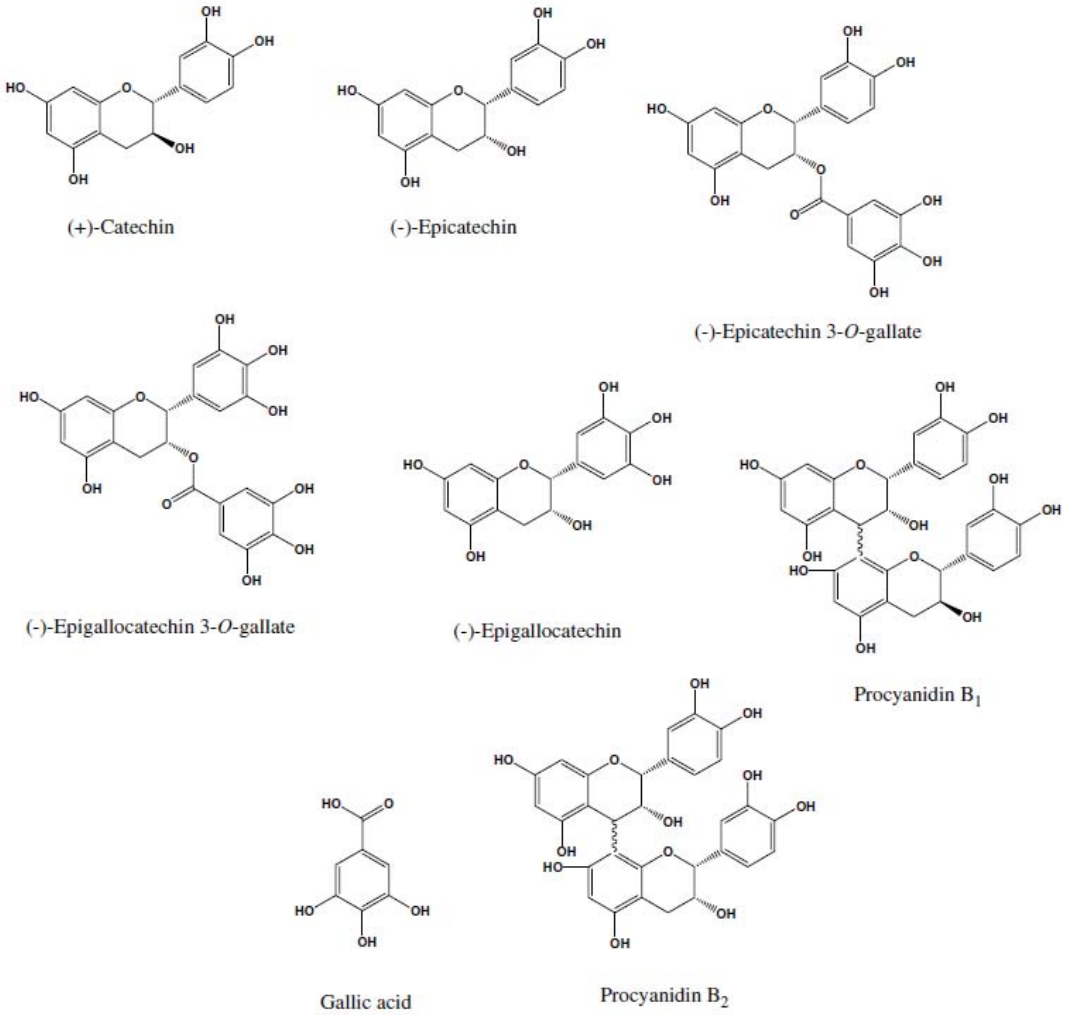

Figure 2.8. Chemical structures of the polyphenolic compounds in GSE (Source: Guendez et al., 2005)

The antimicrobial activity of GSE can be attributed to the general mode of action of the phenolics (Perumalla and Hettiarachchy, 2011). The phenolic compounds potentially disturb the function of bacterial cell membranes which causes bacterial growth retardation and multiplication. Moreover, these compounds are responsible for the adhesion binding, protein and cell wall binding, enzyme inactivation, and intercalation into the cell wall and/or DNA during inactivation of pathogens (Davidson and Taylor, 2007). Antimicrobial properties of GSE have been evaluated against foodborne pathogens (*L. monocytogenes*, *Salmonella* Typhimurium, *S. aureus*, *B. cereus*, *Enterobacter sakazakii*, *E. coli* O157:H7, and *Aeromonas hydrophila*) both *in*

vitro and to a limited extent in foods (Ahn et al., 2004; Anastasiadi et al., 2009; Kim et al., 2004; Rhodes et al., 2006; Sivarooban et al., 2007). The antimicrobial properties of GSE against Gram–positive (*Bacillus* and *Staphylococcus*) and Gram–negative bacteria (*P. aeruginosa* and *E. coli*) were also studied (Jayaprakasha et al., 2003). GSE (1%, w/w) also demonstrated antimicrobial activities against Gram–negative bacteria (*E. coli* O157:H7 and *S.* Typhimurium) in cooked ground beef treatments (Ahn et al., 2007).

GSE has also been reported to exhibit anti–inflammatory, cardioprotective, chemopreventive, anticarcinogenic, and antioxidant activities (Mahmoud, 2013).

2.3.2. Pomegranate Fruit Extract

Pomegranate (*Punica granatum L.*) is one of the important fruits grown in Turkey, Iran, USA, Middle East, Mediterranean and Arabic countries (Maskan, 2006). Pomegranate and pomegranate juice are products containing high antioxidant activity related to their phenolic content (Vázquez-Araújo et al., 2011). The high antioxidant activity of pomegranate is located mainly in the rind, because of its higher punicalagins and ellagic acid contents (Gil et al., 2000). The richly colored grains in pomegranate contain considerable amount of acids, sugars, vitamins (A and E), polysaccharides, minerals and phenolic compounds such as catechins, ellagic tannins and anthocyanins (3–glucosides and 3,5–diglucosides of delphinidin, cyanidin, and pelargonidin) and give a delicious and nutritional juice (López-Rubira et al., 2005). Pomegranate juice contains much more antioxidant compounds than other fruit juices and beverages (Gil et al., 2000). Ellagitannins are the compounds having higher antioxidant activity in pomegranate juice. They are classified as punicalagin (Fig.2.9a), ellagic acid (Fig.2.9b), punicalin, and anthocyanins responsible for its red–purple colour (Boggia et al., 2013). Among them, punicalagins are responsible for over 50% of the antioxidant activity of the pomegranate juice (Gil et al., 2000). Punicalagin is water soluble. Therefore, commercial pomegranate juice obtained by pressing the fruit contains this antioxidant in significant amounts (Patel et al., 2008).

Interest in pomegranate juice and its products has increased in recent years because of beneficial their health effects. Therefore, it is found in the first line of functional juice market (Vegara et al., 2013). A glass of pomegranate juice contains about 40% of the Recommended Daily Allowance (RDA) of Vitamin C (Du et al.,

1975). The health benefits of pomegranate juice are related to its anticarcinogenic, antioxidant, antiviral and anti–atherogenic activities (Braga et al., 2005; Malik and Mukhtar, 2006). These benefits have been attributed to the presence of phytochemicals predominantly polyphenols, including primarily hydrolysable ellagitannins, anthocyanins and other polyphenols (Gil et al., 2000).

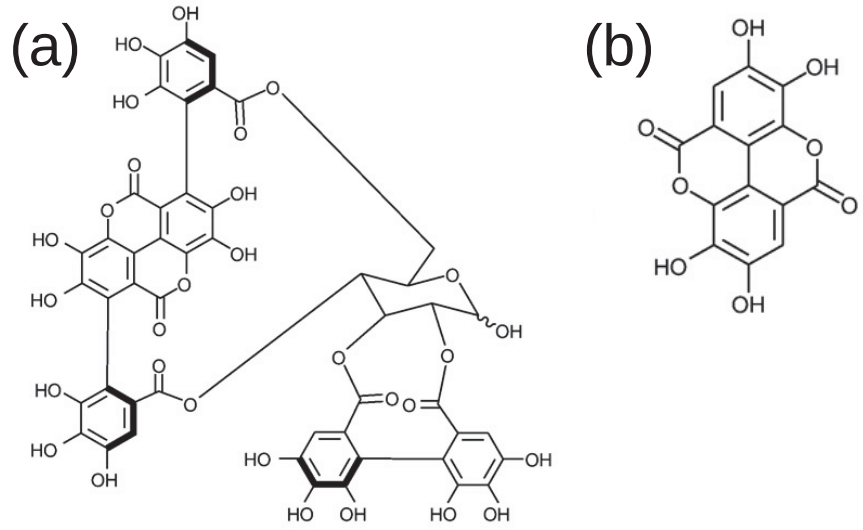

Figure 2.9. Chemical structures of (a) punicalagins and (b) ellagic acid
(Source: Bialonska et al., 2010)

2.4. Kinetics of Microbial Inactivation

Changes in microbial populations in foods over time (microbial kinetics) are controlled by extrinsic (storage conditions) and intrinsic factors (product characteristics). These factors are termed as environmental parameters (Thomas and Paw, 2003). Mathematical models are developed and analyzed to describe microbial behavior (inactivation, growth and survival) as a function of these environmental factors (temperature, pH, and antimicrobial concentrations) (Janssen et al., 2008). In order to determine optimum process conditions and controls to achieve desired results, the effect of process conditions on the microbial kinetics needs to be characterized and modeled mathematically (Alfredo and Arthur, 2009).

Kinetic parameters and models are essential to develop food preservation processes for ensuring safety. Primary models describe the response of microorganism to a single set of conditions over time. Furthermore, the models that describe the effect of environmental conditions such as physical, chemical and biotic features on the

primary model parameters are termed as secondary models (Thomas and Paw, 2003). The aim of the primary model for microbial growth is to describe the process kinetics using few parameters as possible and also to accurately define the stages of growth curve. When the increase in population density (log CFU/mL) is plotted against time, the resulting growth curve usually has four phases. These are lag, exponential (log), stationary, and death (decline) phases. The other primary models are survival models (Robin and Xuewen, 2003). Microbial inactivation is modeled mathematically using survival curves. These curves are obtained by plotting the $\log_{10}$ of the survival fraction vs. treatment time (t) applied (Fig.2.10).

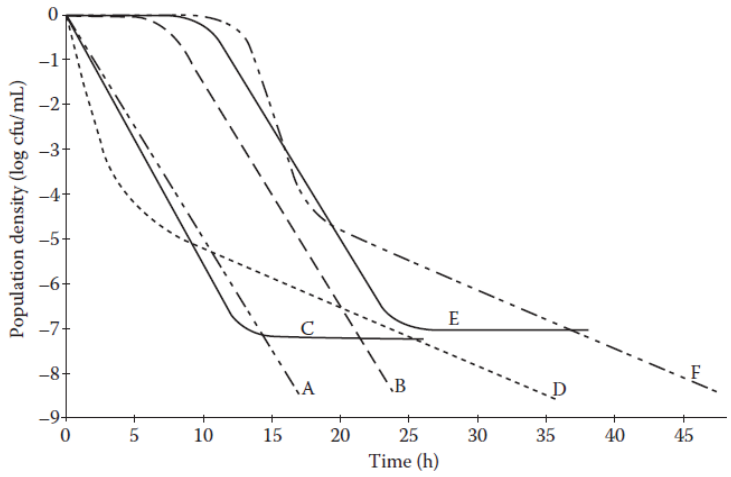

Figure 2.10. Shapes of (A) log–linear (B) survival with shoulder (C) survival with tail (D) biphasic (E) survival with shoulder and tail (F) biphasic with shoulder survival curves (Source: Barron, 2012)

Traditionally, microbial inactivation has been considered to follow first–order kinetics (linear curves). In the first–order kinetics, it is assumed that all cells and spores have identical resistance to a lethal treatment and each microorganism has the same inactivation rate (van Boekel, 2002). However, inactivation curves may not always be linear. There are two classes of nonlinear curves those with a shoulder (lag) before inactivation and those exhibiting tailing. These two behaviors may be present together as observed in biphasic inactivation (Robin and Xuewen, 2003). The common explanation for shoulders and tails in the survival curves is the presence of subpopulations having different resistances against applied treatments. Each one has with its own first–order inactivation kinetics (Stella et al., 2009). As an example, activation of dormant spores and the presence of subpopulations with different

resistance give rise to the shoulders and tails appearing as deviations from log–linearity in some survivor curves of spore–forming bacteria (Alfredo and Arthur, 2009).

In order to improve the use of inactivation models by the food industry and food microbiologists, a user–friendly interface, Geeraerd and van Impe Inactivation Model Fitting Tool was recently developed (Geeraerd et al., 2005). In this programme, nine different microbial survival models are available for user to evaluate the specific experimental data of the microbial population with time. The software covers all survivor curve shapes observed until now in the literature for vegetative cells (Stella et al., 2009). These models are log–linear curves; log–linear curves with shoulder; log–linear curves with tail; log–linear curves with both shoulder and tail; concave curves; convex curves; convex/concave curves followed by tailing; biphasic curves and biphasic curves with shoulder (Stella et al., 2009).

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Microorganism

A. acidoterrestris DSM 3922 isolated from garden soil (Hippchen et al., 1981). was kindly provided by Karl Poralla (Deutsche Sammlung von Mikroorganismem und Zellkulturen's collection, Braunschweig, Germany). This culture was grown on *Bacillus acidoterrestris* Agar (BATA; Merck, Darmstadt, Germany) adjusted to pH 4.0 with filter sterilized 1 N H_2SO_4 for 2 days at 43 °C, then stored in 20% (v/v) glycerol–*Bacillus acidoterrestris* broth (BATB, Döhler, Germany) at –80 °C.

3.1.2. Spore Production

A. acidoterrestris cells were pre–cultured at 43 °C for 4 h in 10 mL BATB to achieve a cell density of approximately 10^6–10^7 CFU/mL. Then, 100 µL culture was spread onto BATA (Merck), *Bacillus acidocaldarius* agar (BAA) (Darland and Brock, 1971), potato dextrose agar (PDA, BD Difco), and malt extract agar (MEA, Oxoid). Additionally, 1 mL of the culture was inoculated into 100 mL BATB and incubated with shaking at 120 rpm. Composition of the sporulation media is shown in Table 3.1. All inoculated plates and broth were incubated at 43 °C. Sporulation was determined by direct observation of free, fully refractile spores under the phase–contrast microscope (Olympus CX31, Japan) and the images were obtained using an image analyzing camera and software (Olympus DP–2 BSW, Japan). After reaching more than 85–90% of sporulation, spores were harvested by depositing 1–2 mL of cold sterile deionized water onto the surface of plates and dislodged from the agar surface by gently rubbing with a sterile swab. Spore suspensions were centrifuged at 4000 rpm for 20 min (4 °C). The supernatant was discarded and the pellet was resuspended in sterile deionized

water. Suspension was centrifuged again at 4000 rpm for 10 min (4 °C). This step was repeated three times. The final pellet was resuspended in sterile deionized water and mixed thoroughly. The spore suspensions were stored in 1.8–mL eppendorf tubes at –20 °C for further use. To determine spore count, spore suspensions were heat activated at 80 °C for 10 min, cooled on ice, and then serially diluted in Maximum Recovery Diluent (MRD, Oxoid). Ten–fold dilutions were surface–plated on PDA (pH 3.5). After incubation at 43 °C for 48 h, spore counts were determined and expressed as CFU/mL (Murray et al., 2007).

Table 3.1. Composition of the sporulation media

Sporulation media	**Composition**
PDA	Potato extract 4.0 g/L; dextrose 20.0 g/L and agar–agar 15.0 g/L. Acidified to pH 3.50±0.1 through a sterile solution of 10% (w/v) tartaric acid.
MEA	Malt extract 30 g/L, mycological peptone 5.0 g/L and agar–agar 15.0 g/L. Acidified to pH 3.50±0.1 through a sterile solution of 10% (w/v) tartaric acid.
BATA	Yeast extract 2.0 g/L, D(+)glucose 5.0 g/L, $CaCl_2 \cdot 2H_2O$ 0.25 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, $(NH_4)_2SO_4$ 0.2 g/L, KH_2PO_4 3.0 g/L, agar–agar 18 g/L and 1 mL trace element solution (included in the medium). Trace minerals solution contains $CaCl_2 \cdot 2H_2O$ 0.66 g/L, $ZnSO_4 \cdot 7H_2O$ 0.18 g/L, $CuSO_4$ 0.16 g/L, $MnSO_4 \cdot 7H_2O$ 0.15 g/L, $CoCl_2$ 0.18 g/L, H_3BO_3 0.1 g/L, $Na_2MoO_4 \cdot 2\ H_2O$ 0.3 g/L. Acidified to pH 4.0±0.2 through a sterile solution of 1 N H_2SO_4.
BAA	Yeast extract 1.0 g/L, $(NH_4)_2SO_4$ 0.2 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, $CaCl_2 \cdot 2H_2O$ 0.25 g/L, KH_2PO_4 3.0 g/L, glucose 5 g/L, agar–agar 15 g/L, and 1 mL trace element solution (1:1000, v/v). Trace element solution contains $FeSO_4 \cdot 7H_2O$ (0.28 g/L), $MgCl_2 \cdot 4H_2O$ (1.25 g/L), $ZnSO_4 \cdot 7H_2O$ (0.48 g/L). Acidified to 4.0±0.2 through a sterile solution of 1 N H_2SO_4.
BATB	Same with BATA but does not contain agar.

3.1.3. Juice Samples

Concentrated apple juice (70.3 °Brix) and pomegranate juice (65 °Brix) were provided by ASYA Fruit Juice and Food Ind. Inc. (Isparta, Turkey) and reconstituted to 11.3 and 14.26 °Brix by a refractometer (Mettler Toledo, USA). Measurements were performed at 20 °C. Diluted juice samples were tested for the presence of *Alicyclobacillus* spp. by membrane filtration method. Briefly, the membrane (0.45 μm pore size, Sartorious, Germany) that had been used to filter the reconstituted juice was transferred aseptically onto BATA. Then, the membranes on the plates were incubated

at 43 °C for up to seven days. The pH of the reconstituted juice samples was measured (Hanna instruments, Hungary). The pomegranate–apple blend juices were prepared by mixing the juice samples aseptically at the ratio of 80%, 40%, 20% and 10% (v/v). The °Brix and pH values of the blend juices were also measured (Table 3.2).

Color intensity and total color density of the juice samples were measured spectrophotometrically using a UV/Vis spectrophotometer (Cary 100 Bio, Varian Inc., CA, USA). Color intensity was determined at 520 nm. At this wavelength, monomeric anthocyanins that give the characteristic red color of juices have maximum absorption (Vegara et al., 2013). Total color intensity (TCD) was expressed as total absorbance values of the brown compounds, which show maximum absorbance values at 420 and 533 nm (Vegara et al., 2013). Before measurements, juice samples were diluted with deionized water to obtain the absorbance at 533 nm below 1.0. TCD was calculated by Eq 3.1, where DF is the dilution factor.

$$TCD = \left[\left(Abs_{420} + A_{533}\right) - 2\left(Abs_{700}\right)\right].DF \tag{3.1}$$

The pH (3.36–3.96) and °Brix values (11.30–14.26) of juice samples do not appear to be limiting on *A.acidoterrestris* DSM 3922 (Table 3.2), since its survival and growth at these values have been demonstrated (Tokuda, 2007). Color measurements indicated that monomeric anthocyanins and color density were higher in pomegranate and blend juices than apple juice (Table 3.2).

Table 3.2. Analytical characteristics and color measurements of juice samples

Juice	**pH**	**°Brix**	**CI**[†]	**TCD**[†]
Pomegranate juice	3.36 (0.0)[‡]	14.26 (0.02)	1.749 (0.023)	6.035 (0.173)
80% pomegranate–apple juice blend	3.42 (0.0)	13.66 (0.05)	1.059 (0.004)	4.625 (0.036)
40% pomegranate–apple juice blend	3.60 (0.0)	12.55 (0.01)	0.591 (0.009)	2.674 (0.092)
20% pomegranate–apple juice blend	3.74 (0.0)	12.00 (0.03)	0.350 (0.001)	1.706 (0.003)
10% pomegranate–apple juice blend	3.82 (0.0)	11.63 (0.01)	0.228 (0.000)	1.118 (0.002)
Apple juice	3.96 (0.0)	11.30 (0.10)	0.080 (0.001)	0.428 (0.007)

[†] CI, color intensity; TCD, total color density
[‡]All data were the means±standard deviation from three experiments, n=3

3.1.4. Grape Seed and Pomegranate Fruit Extracts

Commercial liquid extract derived from the grape seed was purchased from a local health food store. The chemical composition of grape seed extract was revealed by

Yavuzdurmaz (2013) using HPLC. Epicatechin (9827.07 ppm), gallic acid (700.93), quercetin–3–galactoside (536.52 ppm), rutin (277.25 ppm) and epicatechin gallate (249.22 ppm) were identified as the major components of grape seed extract (Table 3.3).

Table 3.3. Chemical composition of the grape seed extract
(Source: Yavuzdurmaz, 2013)

Compound	Amount (ppm)	Compound	Amount (ppm)
Epicatechin	9827.07	Catechin gallate	44.14
Gallic acid	700.93	Caffeic acid	28.87
Quercetin–3–galactoside	536.52	Vanillic acid	25.17
Rutin	277.25	Syringic acid	22.53
Epicatechin gallate	249.21	p–coumaric acid	17.31
Quercetin	74.13	Vanillin	16.09
Ferulic acid	65.07	Resveratrol	9.290

Dry powder of food grade pomegranate fruit extract (POMELLA) made from whole pomegranate extract was kindly provided by Blake Ebersole (Verdure Sciences, Noblesville, USA). Table 3.4 indicates the chemical analysis of pomegranate extract provided by the manufacturer.

Table 3.4. Chemical analysis of pomegranate fruit extract

Chemical Analysis	Test Method	Amount
Assay for actives		
Punicalagins	HPLC	30.49%
Ellagic acid	HPLC	3.25%
Polyphenols	UV–vis	58.75%
Gallic acid	HPLC	0.42%

3.2. Total Phenol Content

The total phenol content of the extract and juice samples was determined using the Folin–Coicalteu (FC) method (Al-Zoreky, 2009) with some minor modifications. Briefly, 0.2 mL of diluted grape seed extract (1/10 and 1/20, v/v), pomegranate fruit extract (1/100 and 1/200, v/v), apple juice (1/5 and 1/10, v/v), pomegranate juice (1/100, v/v) and 80%, 40%, 20% and 10% (v/v) pomegranate–apple blend juices (1/10–1/100) was mixed with 1 mL of 10–fold diluted FC reagent and then mixed by vortexing. After 3 min incubation at room temperature, 0.8 mL of Na_2CO_3 (7.5%, w/v)

was added, vortexed again and then left in the dark. After 1 h, the absorbance of the samples was measured at 765 nm using a UV/Vis spectrophotometer (Cary 100 Bio, Varian Inc., CA, USA). A calibration curve was prepared with gallic acid (0–0.09 mg/mL). The results were expressed in gallic acid equivalents (GAE) in mg/mL grape seed extract, mg/mg pomegranate extract and mg/L juice. The analysis was performed in triplicate measurements.

3.3. Antioxidant Activity

3.3.1. FRAP Method

Ferric reducing antioxidant power was determined based on the protocol developed by Bi et al. (2013). The FRAP reagent was freshly prepared by mixing the stock solutions of 300 mM sodium acetate buffer (pH 3.6), 10 mM 2,4,6–tripyridyl–s–triazine (TPTZ, dissolved in 40 mM HCl), 20 mM ferric chloride ($FeCl_3$. $6H_2O$) in 10:1:1 ratio, shaken by vortexing and kept in the dark for 10 min at 37 °C. Then, 0.1 mL diluted grape seed extract (1/1000 and 1/2000), pomegranate fruit extract (1/200 and 1/400), apple juice (1/5 and 1/10), pomegranate juice (1/100, 1/200, and 1/400) and blend juices (1/10–1/400) was mixed with 4 mL FRAP reagent. After 10 min, the absorbance values were determined at 593 nm. The FRAP solution was used as a blank. The results were expressed as Trolox equivalents (TE) using a calibration curve in the range of 0–0.1 mM. All assays were carried out in triplicate.

3.3.2. DPPH Method

Free radical scavenging activity was determined using a stable 2,2–diphenly–2–picrylhydrazyl radical (DPPH) based on the protocol developed by Bi et al. (2013). Instead of methanol, ethanol was used in the assays. Briefly, 4 mL DPPH solution (0.14 mM in ethanol) was added into 0.1 mL of juice samples or diluted extracts (1% pomegranate extract, w/v; 1% grape seed extract, v/v). After that, the mixture was shaken by vortexing and incubated in the dark for 45 min at room temperature. The mixture of 0.1 mL of ethanol and 4 mL of DPPH solution was used as control. After

incubation, the absorbance of samples and controls were read at 517 nm. Free radical scavenging activity was calculated as % inhibition of DPPH using the Eq. 3.2 (Katalinić et al., 2010).

$$(\%\ \ Inhibition\ \ of\ DPPH\ ) = \left[\left(OD_{control} - OD_{sample}\right)/OD_{control}\right].100 \quad (3.2)$$

Lower absorbance values indicate higher free radical scavenging activity of samples (Katalinić et al., 2010).

3.4. Wet–Heat Treatment

3.4.1. Thermal History of Apple Juice

The required time to reach the desirable temperature was recorded and referred as come–up–time (CUT). During this time, only the fruit juice was heated without any spores. This time was monitored since it could affect the results of the wet–heat inactivation kinetics. After CUT, N_o was considered as the initial count for modeling.

3.4.2. Wet–Heat Treatment

A temperature–controlled water bath (P–Selecta Precisdig, Spain) was used for the wet–heat treatment of spores. The water bath was adjusted to the intended temperature (85, 87.5 °C or 90 °C). After heat–shock at 80 °C for 10 min in the water bath and then cooling on ice, the spore stock suspension (10^6–10^7 CFU/mL) was centrifuged at 14800 rpm for 5 min. The pellet was resuspended in 1 mL of apple juice and the suspension was transferred to a screw–cap test tube (160 × 100 mm glass tube with an inside diameter of 16 mm). Tube containing inoculated apple juice was then placed in the water bath. During the thermal treatments, a K–type thermocouple (Hanna instruments, Hungary) was placed in another test tube containing the same volume of apple juice as a control. When the control was reached to the target temperature, the other heated tube containing spore suspension was removed from the water bath immediately to determine N_0 for modeling. After each sampling time, viable spore counts were determined by serially diluting aliquots of juice in MRD and then plating

on PDA in duplicates (Baysal and Icier, 2010). A minimum of seven time intervals were used: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 min at 85 °C; 0, 5, 10, 15, 20, 25, 30, 40, 50, and 60 min at 87.5 °C and 0, 5, 10, 15, 20, 25, and 30 min at 90 °C, respectively.

3.4.3. Determination of Heat Resistance Parameters

Survival curves were obtained by plotting the logarithm of the survival fraction (N/N_0) versus the treatment time (min). As the number of logarithm of the survival fraction $S(t)$ decreases linearly over treatment time (Schaffner and Labuza, 1997), the log–linear model (Bigelow and Esty, 1920) from GlnaFiT Excel add–in software was used to fit the experimental data (Geeraerd et al., 2005) by the following equation:

$$log\ N/N_0 = -k_{max}.t/ln10 \tag{3.3}$$

where N is the spore count (CFU/mL) after exposure to the thermal treatment for a specific time t (min), N_0 is initial spore count (CFU/mL) measured immediately after come–up–time. D (decimal reduction time) is the time required to destroy 90% of the spores (min) and can be calculated as the reciprocal of the rate constant (k_{max}) using the Eq. 3. 4;

$$D = ln10/k_{max} \tag{3.4}$$

By replacing Eq. 3.4 into the Eq. 3.3, Eq. 3.5 can be obtained by the following equation;

$$log\ N/N_0 = -t/D \tag{3.5}$$

All thermal resistance determinations were repeated at three times on separate days. For each data set, D–value was calculated using corresponding rate constant and the results were expressed as the mean±standard deviation from three experiments. The z–value is the change of temperature (°C) required for 1–log cycle change in D–values and was calculated using the Eq. 3.6.

$$z = (T_2 - T_1)/(\log D_1 - \log D_2) \tag{3.6}$$

3.4.4. Dipicolinic Acid Analysis

The DPA content of the spore suspensions during wet–heat was measured as previously described (Rotman and Fields, 1968). The untreated samples were autoclaved at 121 °C for 20 min to determine the total DPA content (Janssen et al., 1958). First, treated and autoclaved suspensions were centrifuged at 14800 rpm for 15 min. After centrifugation, the supernatant (800 µL) was mixed with 200 µL solution containing 1% (w/v) $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ and 0.1% (w/v) L–cystein in 0.05 M sodium acetate buffer (pH 4.0) (Planchon et al., 2011). The absorbance of the sample was measured spectrophotometrically at 440 nm. The dry weight of each spore suspension was determined by completely drying the remaining spore pellet. A standard calibration curve was used to calculate the amount of DPA in supernatants. Finally, the DPA contents were expressed as µmole DPA/mg spore dry weight and the release (%) was calculated based on the relative proportion of DPA content of wet–heat treated spores to their total DPA content.

3.4.5. Soluble Protein and Nucleic Acid Leakage

Heat–treated samples were taken at various heating intervals and centrifuged at 4 °C (14800 rpm for 10 min) immediately. The supernatant was examined for leakage of nucleic acid and protein by measuring the absorbance values at 260 and 280 nm, with UV spectrophotometer (Cary 100 Bio, Varian Inc., CA, USA) (Zhang et al., 2012).

3.4.6. Inductively Coupled Plasma Mass Spectrometry

The concentrations of Mg^{2+}, Ca^{2+}, Mn^{2+} and Fe^{2+} minerals in the spore suspensions were assayed using inductively coupled plasma spectroscopy (Agilent 7500 ce Series, Tokyo, Japan) (Igura et al., 2003). After acid digestion in HNO_3 solution (2% v/v), spore samples were centrifuged and the supernatant was filtered with 0.22 µm membrane filter. The remaining spore pellet was dried, and then weighed to express mineral concentration per mg spore dry weight. For ICP–MS, the resultant supernatant was diluted with 2% HNO_3 solution (1:10 v/v). The spike standard was prepared

between 0–1000 ppm and added (20 ppb) into each sample. During measurements, germanium was used as an internal standard to calculate the recovery and monitor the sensitivity range of the ICP–MS. It was added at the concentration of 10 ppb into each sample. Two measurements were done for each sample.

3.4.7. Morphological Changes After Wet–Heat Treatment

Ultrastructural changes in *A. acidoterrestris* spores were determined by SEM and AFM imaging. Firstly, untreated and treated spores were centrifuged (14000 rpm for 10 min). Next, the bacterial pellet was suspended in sterile deionized water and the suspension was centrifuged again. This washing step was repeated three times. After that, the pellet was resuspended in sterile deionized water. Finally, the resultant suspension (10 μL) was fixed onto clean glass slides and air–dried overnight for SEM. For SEM imaging, samples were coated with gold at a vacuum of 0.09 mbar, for 90 s, at 15 kV, in argon gas at a power of 15 mA and examined with a scanning electron microscope (Phillips XL–30S FEG).

For AFM analysis, a 2–3 μL of the final suspension was placed on glass slides and dried in air. Samples were examined within 24 h and were prepared at the same time. AFM was carried out in tapping mode using nanoprobes cantilevers made of silicon (Digital Instruments, MMSPM Nanoscope IV). The AFM images were recorded by scanning an area of 5×5, 10×10, 15×15, and 20×20 μm.

3.4.8. Energy Dispersive X–Ray Analysis

Energy dispersive X–Ray analysis (EDX) is a method to analyze the elemental composition at the ultrastructural level. When electrons from external sources strike atoms in the material, energy in the form of an X–ray photon is emitted giving X–ray characteristics of the element. EDX is used to simultaneously observe the morphology and chemical composition of cells (Bassi et al., 2009).

Imaging and analysis by EDX were done with a scanning electron microscope (Phillips XL–30S FEG) that was equipped with an X–ray detector (Oxford Aztec X–Act detector). SEM–EDX was performed on untreated spores (Hintze and Nicholson, 2010). Spores (10^4–10^5 CFU/mL) in sterile deionized water were deposited onto glass slides,

then air–dried. Elemental analysis (the percentage of the detected elements with respect to one other) was carried out using an EDX micro–analyzer. Images were taken at 10,000 × magnification. Imaging of the specimens was performed with an accelerating voltage of 5.00 kV. Analysis was performed on a small diameter spot that was centered on each spore. Relative proportion of Na, Mg, Al, P, S, K, Ca, Mn and Cu (%) of the spores was investigated for single spore. Six different spectra were collected for each sample.

3.5. Antimicrobial Activity on Vegetative Cells

3.5.1. Antimicrobial Activity of Natural Extracts

A. acidoterrestris cells were grown overnight on PDA at 43 °C. The colonies were suspended in 10 mL MRD to obtain a bacterial density of McFarland 2.0 (10^7 log CFU/mL) by using a densitometer (Den–1, HVD Life Sciences, Austria). After centrifugation at 14800 rpm for 5 min, the pellet was resuspended in 10 mL apple juice. Sterile test tubes containing 9 mL apple juice were added with different concentrations of grape seed extract (0–4%, v/v) and pomegranate extract (0–4%, w/v). Then, the tubes were inoculated separately with 1 mL of the bacterial suspension. The initial cell population was determined for each concentration after inoculation. Next, the samples were incubated at 37 °C by shaking at 120 rpm. At regular time intervals, up to 336 h the viable cell counts were determined by spread plating onto the surface of PDA (pH 3.5) and incubating the plates at 43 °C for 48 h.

3.5.2. Antimicrobial Activity of Pomegranate and Blend Juices

A. acidoterrestris cells were grown overnight on PDA at 43 °C. The colonies were suspended in 10 mL MRD to obtain a bacterial density of McFarland 1.0 (10^6 CFU/mL) by using a densitometer (Den–1, HVD Life Sciences, Austria). After centrifugation, the pellet was resuspended in 10 mL pomegranate, apple and pomegranate–apple blend juices [80%, 40%, 20%, 10%, (v/v)]. The initial cell population was determined for each concentration and control. Next, the samples were

incubated at 37 °C by shaking at 120 rpm. At regular time intervals, viable cell counts were determined by spread plating onto the surface of PDA (pH 3.5). Finally, the plates were incubated at 43 °C for 48 h.

3.5.3. Modeling Cell Inactivation Data

Before modeling inactivation data, the plate counts were transformed to $\log_{10}$ values. Survival curves were obtained by plotting the logarithm of survivors against the treatment time (h). The Gearaerd and Van Impe Inactivation Model Fitting Tool (GInaFIT) (Geeraerd et al., 2005) was used for testing different types of microbial survival models such as the log–linear (Bigelow and Esty, 1920), the log–linear tail (Geeraerd et al., 2000) and the Weibull models (Mafart et al., 2002).

3.5.3.1. First–Order Kinetics

The 1st–order model assumes that all cells in a population have identical resistance to a lethal treatment and the number of logarithm of the survivors decreases linearly over treatment time. This model was also termed as log–linear equation and explained in Section 3.4.3. The log–linear equation is only appropriate for linear inactivation curves (where time is on the x–axis and log (N/N_0) is on the y–axis) (Schaffner and Labuza, 1997).

3.5.3.2. Weibull Model

The Weibull distribution is widely used to describe microbial inactivation. This model is based on the assumption that cells in population have different resistances and the resistance to stress follows a Weibull distribution (Bialka et al., 2008). The Weibull model was used with the Eq. 3.7 (Mafart et al., 2002);

$$logN_t = logN_0 (t/\delta)^{\beta} \tag{3.7}$$

where N_t represents the number of surviving population after time t (h); N_0 is the initial number of microorganisms (t=0); β is the shape parameter (dimensionless)

showing upward (β<1) and downward concavity (β>1). Upward concave curves are associated with the adaptation of the remaining cells to the applied stress. Downward concave indicates the increased damage of the remaining cells in applied stress. If β=1, the Weibull model reduces to a log–linear model. (van Boekel, 2002). Also, δ is the scale parameter (h) and corresponds to the time for the first decimal reduction (De Oliveira et al., 2013). This is distinguished from the conventional *D* value; the significance of the δ value is restricted to the first decimal reduction of surviving spores or cells from N_0 to $N_0/10$. Time to 4–log reduction (t_{4D}) was calculated by Eq. 3.8 (Levy et al., 2012).

$$t_{4D} = \delta(4)^{\frac{1}{\beta}} \tag{3.8}$$

3.5.3.3. Log–Linear and Tail

The Log–linear tail model was used with the Eq. 3.9 (Geeraerd et al., 2000);

$$logN_t = log\left(\left(10^{logN_o} - 10^{logN_{res}}\right).e^{(-k_{max}t)} + 10^{logN_{res}}\right) \tag{3.9}$$

Where N_{res} is the residual population density (log CFU/mL) that characterizes tailing of inactivation kinetics (Hereu et al., 2012) and k_{max} is the inactivation rate of the log–linear part of the curve (h^{-1}) (Izquier and Gómez-López, 2011).

3.5.4. Model Evaluation

Goodness of fit provides the agreement between the model predictions and the observed data (Hagquist and Stenbeck, 1998). The values of root mean square error (*RMSE*), coefficient of determination (R^2) and adjusted R^2 ($adj–R^2$) values were compared for model evaluation.

RMSE measures the average deviation between the observed and fitted data sets as shown in Eq. 3.10. A lower *RMSE* value indicates less residual variance and better fit of the data for the model (Huang et al., 2012).

$$RMSE = \sqrt{\frac{(log_{10} N_{fitted} - log_{10} N_{observed})^2}{n - p}} = \quad (3.10)$$

n is the number of observations, p is the number of parameters in the model, $N_{observed}$ is the observed population (CFU/mL) and N_{fitted} is the fitted population (CFU/mL).

R^2 indicates how close the data to the fitted model (Eq. 3.11). An R^2 value closer to one indicates a better fitting accuracy of the model (Huang et al., 2012).

$$R^2 = 1 - \left(\frac{SS_E}{SS_T} \right) \quad (3.11)$$

Where SS_E is the residual sum of squares and SS_T is the total sum of squares.

Adj–R^2 is used to determine how well the model fitted to the experimental data using Eq. 3.12.

$$Adj - R^2 = \left[1 - \frac{(n-1)\left(1 - \frac{SS_R}{SS_T}\right)}{(n-p)} \right] \quad (3.12)$$

Where SS_R is the sum of squares of regression.

Best fit of the data is obtained when *RMSE* is the smallest and R^2 value is close to 1. If two models have the same or similar *RMSE*, the simpler one can be chosen (Berney et al., 2006).

3.5.5. Morphological Changes in Cell Structure

Ultrastructural changes in *A. acidoterrestris* cells after antimicrobial treatments were determined by SEM and AFM imaging as described in Section 3.4.7.

3.6. Antimicrobial Activity on Spores Produced on Different Media

3.6.1. Guaiacol Production of Spores in Apple Juice

Peroxidase is an enzyme found in almost all plants, animals and various microorganisms. Its function is to catalyze the peroxidation reactions. In the peroxidase enzyme colourimetric assay (PECA), hydrogen peroxide (H_2O_2) and guaiacol are used as oxidizing and hydrogen donor substrates, respectively. As presented in Fig.3.1, four molecules of guaiacol are converted into tetraguaiacol in the presence of H_2O_2 and peroxidase (Bahçeci and Acar, 2007a; Goto, 2007a).

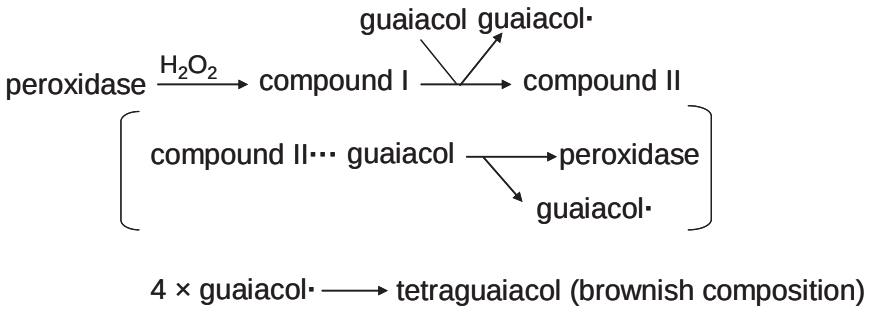

Figure 3.1. Principle of peroxidase assay
(Source: Goto, 2007a)

After heat activation at 80 °C for 10 min in a water bath and cooling on ice, the spore suspension was centrifuged at 14800 rpm for 5 min. After that, the pellet was dissolved in apple juice and inoculated into 20 mL apple juice containing a final concentration of 100 ppm vanillin or vanillic acid. Vanillin and vanillic acid were used as guaiacol precursors. Three different inoculum levels were chosen (low: 10^1 CFU/mL, medium: 10^3 CFU/mL, and high: 10^5 CFU/mL). After inoculation, the samples were divided into 1 mL portions and transferred into eppendorf tubes. Then, the tubes were incubated at 37 °C. Guaiacol concentration was determined at certain time intervals by the PECA. Briefly, 1 mL sample was centrifuged at 14800 rpm for 10 min. Then, the resultant supernatant (300 µL) was mixed with sequentially 2 mL 0.2 M potassium phosphate buffer (4.18 g/L K_2HPO_4 and 23.95 g/L KH_2PO_4, pH 6.0), 300 µL horseradish peroxidase (5 U dissolved in potassium phosphate buffer), and 300 µL 0.5% (w/v) H_2O_2 (Witthuhn et al., 2012). Apple juice samples supplemented with guaiacol precursors without spores were used as controls.

The absorbance of the samples was measured at 420 nm. A calibration curve was prepared in the range of 0–2 mM standard guaiacol in apple juice.

3.6.2. Antimicrobial Activity of Natural Extracts

First, the spore suspensions (10^6 spores/mL) were heat–shocked at 80 °C for 10 min and then cooled in ice–water bath. After centrifugation at 14800 rpm for 5 min, the pellet was dissolved in 10 mL apple juice containing different concentrations of grape seed extract (0–2%, v/v) and pomegranate extract (0–4%, w/v). Then, the initial spore population was determined for each concentration and control. The samples were incubated at 37 °C by shaking at 120 rpm. Inoculated apple juice without antimicrobials was used as control. The total population (cells and spores) was evaluated at regular time intervals during incubation. The samples were serially diluted in MRD; 10–fold dilutions were then spread onto the surface of PDA (pH 3.5), and inoculated plates were then incubated at 43 °C for 48 h.

3.6.3. Antimicrobial activity of Pomegranate and Blend Juices

First, the spore suspensions (10^6 spores/mL) were heat–shocked at 80 °C for 10 min and then cooled in ice–water bath. After centrifugation at 14800 rpm for 5 min, the pellet was dissolved in 10 mL pomegranate and blend juice samples. Then, the initial spore population was determined for each concentration and control. Similarly, the samples were incubated at 37 °C by shaking at 120 rpm. The total population (cells and spores) was evaluated at regular time intervals during incubation. The samples were serially diluted in MRD; 10–fold dilutions were then spread onto the surface of PDA (pH 3.5), and inoculated plates were incubated at 43 °C for 48 h.

3.6.4. Changes in Spore Structure After Antimicrobial Treatments

Ultrastructural changes in *A. acidoterrestris* spores after antimicrobial treatments were determined by SEM as described previously in Section 3.4.7.

3.6.5. Surface Hydrophobicity

The surface hydrophobicity was determined using the microbial adhesion to hydrocarbon (MATH) assay with minor modifications (Rosenberg et al., 1980; Seale et al., 2008). The cells or spores were suspended in 0.1 M KCl (pH 3.5) at an absorbance of 1.0 at 600 nm (OD_{before}). The suspension (2 mL) was mixed with 1 mL of hexadecane in a round bottom test tube, vortexed for 1 min, and then incubated at 37 °C for 10 min. After incubation, the tubes were vortexed again for 2 min and allowed to settle at 20 °C for 30 min to separate into the hydrophilic aqueous and hydrophobic hexadecane phases. Then, the absorbance of the aqueous phase was measured at 600 nm (OD_{after}). Finally, the percent hydrophobicity was determined according to the Eq. 3.13.

$$\%Hydrophobicity = \left[1 - OD_{aqueous} \,/ OD_{control}\right] .100 \tag{3.13}$$

Samples were classified based on the following criteria (Chae et al., 2006); strongly hydrophobic (>55%), moderately hydrophobic (30–54%), moderately hydrophilic (10–29%) and strongly hydrophilic (<10%).

3.7. Chemical Treatments

3.7.1. Hydrogen Peroxide

The resistance of spores to H_2O_2 was determined as previously described (Moeller et al., 2011) with minor modifications. Spore suspension in water (965 µL) was placed in 1.8 mL eppendorf tube and 100 µL suspensions were removed to determine the initial spore count. After this step, the remaining spore suspension was mixed with 135 µL concentrated H_2O_2 (30%) giving a final concentration of 4% (v/v). This suspension was incubated at room temperature with continuous gentle mixing. After 10 min, 100 µL samples were removed and immediately diluted 1:10 with a bovine liver catalase (100 µg/mL). Then, the samples were incubated at least 15 min for neutralization. Viable spore counts were determined by serially diluting the samples and plating on PDA. For 2% H_2O_2 treatment, similarly spore suspension (1033 µL) was placed in an eppendorf tube and 100 µL suspension was removed to determine the

initial spore count. After this step, the remaining spore suspension was mixed with 67 µL concentrated H_2O_2 (30%) giving a final concentration of 2% (v/v). After neutralization with bovine liver catalase, viable cell counts were determined as described above.

3.7.2. Sodium Hydroxide and Fumaric Acid

Resistance of spores to sodium hydroxide (NaOH) and fumaric acid was tested at room temperature. Briefly, 2 N NaOH (500 µL) was added into 500 µL spore suspension (10^5–10^6 CFU/mL). Therefore, the final concentration of the chemical was diluted two–fold. For fumaric acid treatment, the spore suspensions were centrifuged and then, the pellet was dissolved in fumaric acid (0.2%, w/v). Immediately thereafter, a 100–µL aliquot from the mixture was withdrawn. This was considered to be the zero–time (t_0). At different time intervals, 100–µL aliquots were withdrawn from the reaction tube, immediately neutralized in 0.1 M KPO_4 (pH 7.5) by vortexing for 1 min. Surviving spores were enumerated by plating serial dilutions of the chemically–treated suspensions onto PDA (pH 3.5). Then, the plates were incubated at 43 °C for 48 h. For each treatment, reduction ($\log_{10}$ CFU/mL) was determined (Planchon et al., 2011).

3.8. Statistical Analysis

Each experiment was performed in triplicate. The mean values and standard deviations were calculated by Excel (Microsoft Corp., USA). Data from three replicates were analyzed by one way analysis of variance (ANOVA) using Minitab 16.0 (Minitab Inc., UK). The Tukey–Kramer test was used to compare the means of treated groups. The confidence interval used to determine statistical significance was 95% (p<0.05).

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Wet–Heat Treatment

4.1.1. Phase Contrast Microscopy and Sporulation

Spore properties and germination efficiency are affected by the differences in environmental conditions such as sporulation temperature, chemical composition, and pH of the sporulation medium (Eijlander et al., 2011). Since there is no standardized medium for the production of *A. acidoterrestris* spores, different media (PDA, BATA, BAA and MEA) were tested for sporulation of *A. acidoterrestris*.

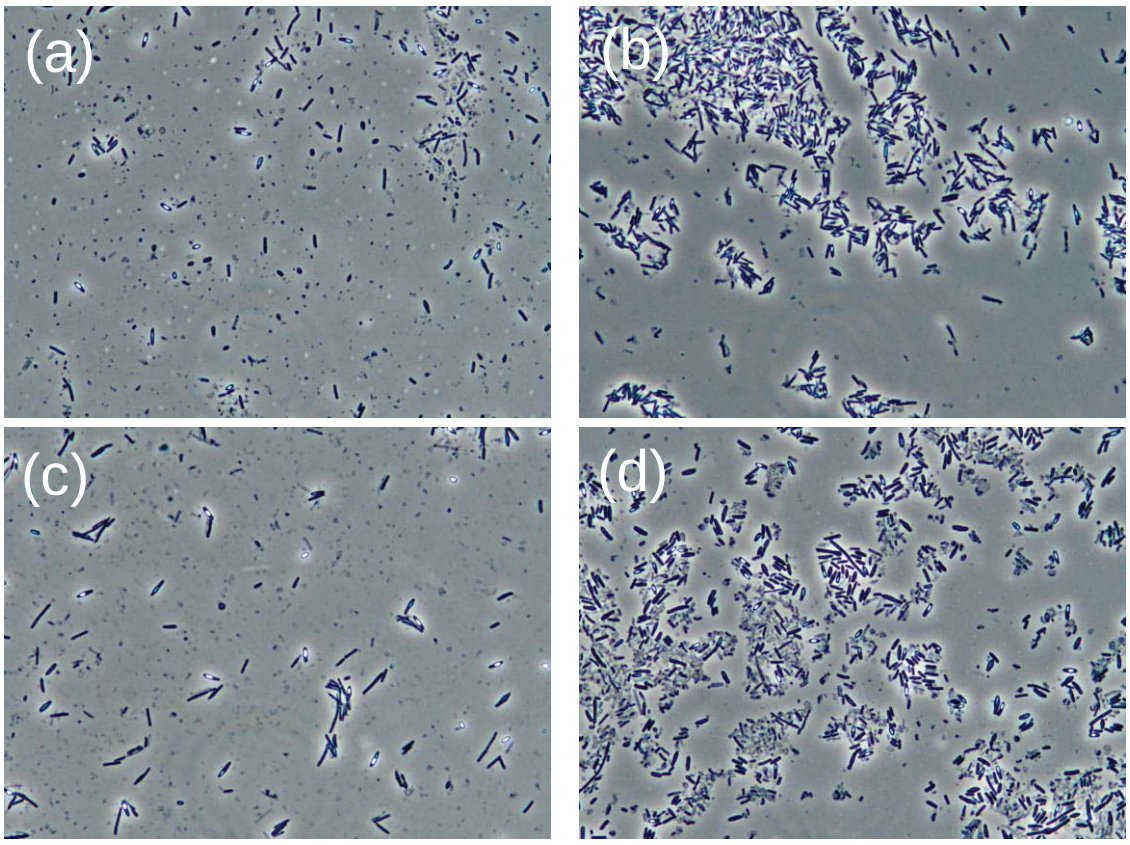

Figure 4.1. Phase contrast microscope images of sporulated *A. acidoterrestris* cells on (a) PDA (b) BATA (c) BAA (d) MEA

Sporulation efficiency was monitored by phase contrast microscopy (Fig.4.1). Phase contrast microscopy is used to measure the exact number of spores and vegetative cells present in the same population. Phase–bright spores can be easily distinguished from vegetative cells under phase contrast microscope (Burns and Minton, 2011). Non–germinated, intact spores are phase–bright, while germinated spores and vegetative cells are phase dark under phase contrast microscope (Fig.4.1). Spores produced on BATA, BAA and MEA agar were usually predominant (>95%) after 4 days incubation at 43 °C compared to spores harvested from PDA plates after 10 days incubation (>82%).

This technique was used instead of colony forming unit (CFU) counts to determine the sporulation efficiency. Because, the number of observed stress–resistant CFU is not directly comparable to the number of spores within the same population and CFU measurements should be combined with another technique such as phase contrast microscopy to accurately determine the spore numbers of bacterial cells (Burns and Minton, 2011).

In addition, the heating to an insufficiently high temperature deactivates spores instead of activation. This situation is termed as "heat induced dormancy". Theoretically, above critical temperatures, heat activation from sublethal heating results in more uniform growth, a higher percentage of spores germinating and, consequently, a higher count can be obtained (Turnbull et al., 2007).

The presence of dark poles around the spores produced on PDA and MEA is observed when the images are magnified (Fig.4.2a and d). The dark poles around spores under phase contrast microscope might be the indicator of the presence of exosporium (Buhr et al., 2008). On the other hand, spores from mineral containing media seem not to contain the dark poles (Fig.4.2b and c).

The exosporium is the outermost structure of spores and is composed of protein (43–52% of dry weight), lipids (15–18% of dry weight), carbohydrates (20–22% of dry weight) and minor components (approximately 4%) such as Ca, Mg and undetermined components. Although it has been suggested that the adherent, hydrophobic properties of the exosporium may be responsible for the pathogenicity of spores, its exact function is not known (Leggett et al., 2012). Similarly, it has been suggested that *B. anthracis* spores may have medium–dependent differences in exosporium structure or degree of glycosylation (Colburn et al., 2011). Therefore, we can conclude that the sporulation medium may affect the exosporium structure of *A. acidoterrestris* spores.

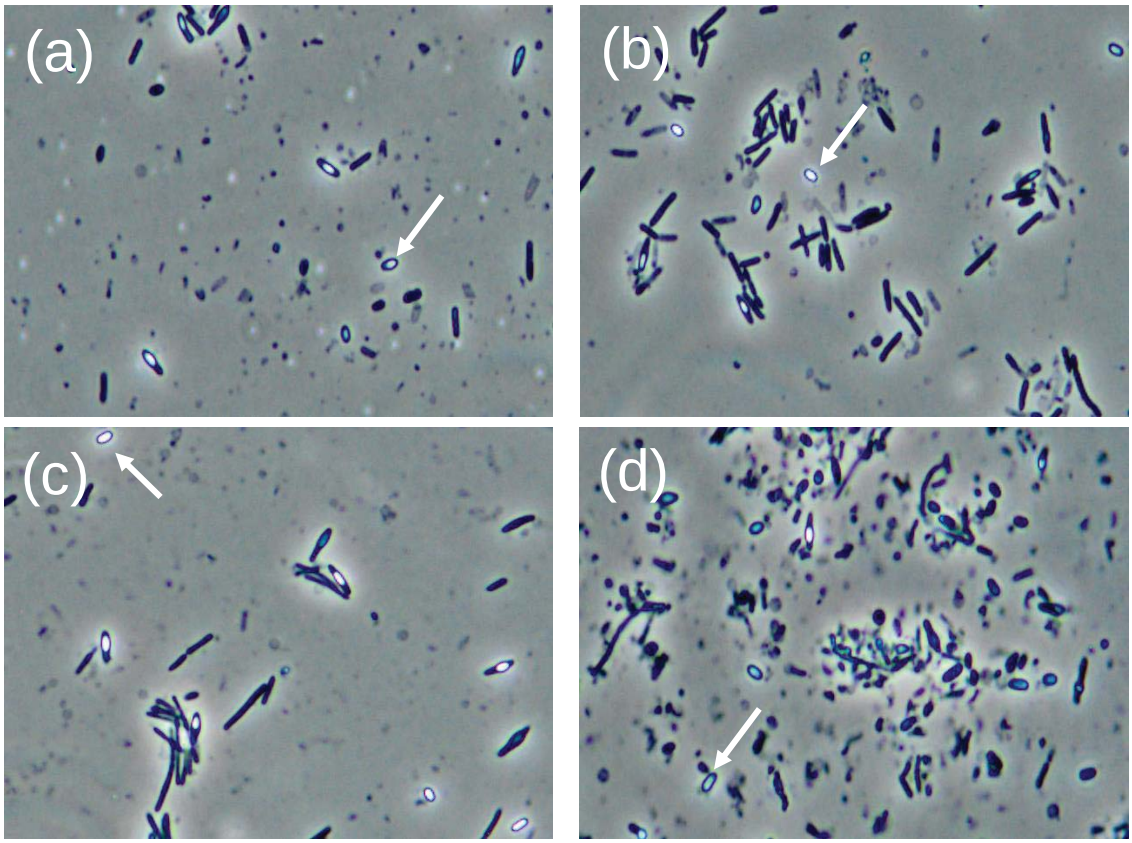

Figure 4.2. Phase contrast microscope images of *A. acidoterrestris* cells during growth on (a) PDA (b) BATA (c) BAA (d) MEA

4.1.2. Thermal History of Apple Juice

A K–type thermocouple was fitted to the center of the test tubes containing apple juice in order to obtain thermal history of apple juice during come–up–time (CUT) period. Thermal history of apple juice at selected temperatures was shown in Fig. 4.3. The thermal CUT was recorded when the target temperatures (85, 87.5 and 90 °C) were achieved.

Within the selected ranges, thermal treatment had CUT of approximately 56.7±2.9 s for 85 °C, 75.0±5.0 s for 87.5°C, and 136.7±5.8 s for 90 °C. Since the extent of inactivation during CUT was very small and the treatment times were relatively long, the inactivation during CUT was not considered for modeling. Therefore, the heated tubes were removed from the water bath immediately after the CUT to determine N_o (initial spore number at t=0).

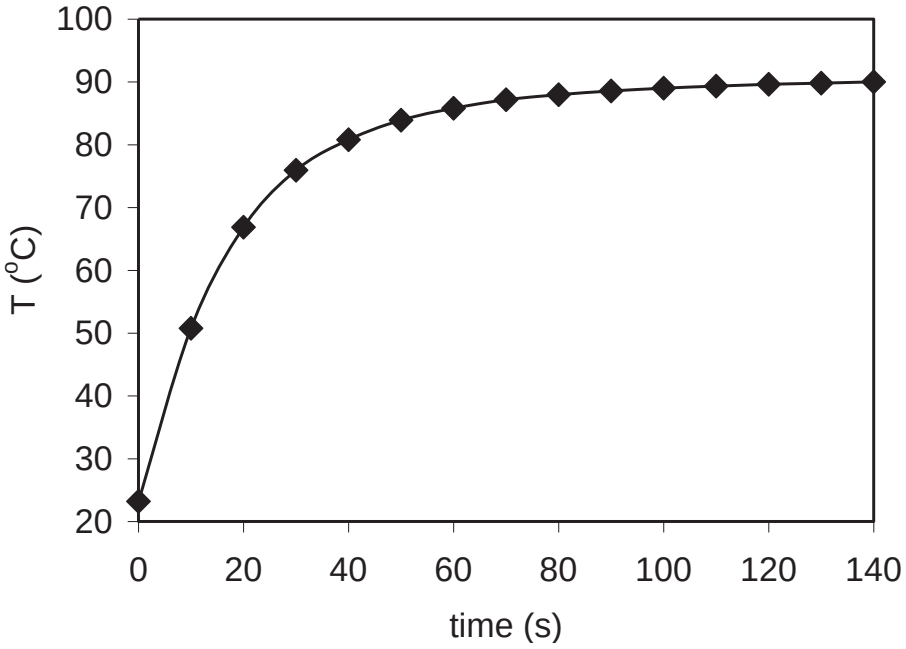

Figure 4.3. Thermal history of apple juice during come–up–time

4.1.3. Kinetics of Wet–Heat Inactivation in Apple Juice

Understanding the way in which microorganisms decline in response to heat is important for the engineering design of thermal inactivation processes used in the food, pharmaceutical, and bioprocess industries (Ursula and Barron, 2012). Since *A. acidoterrestris* has been suggested as the target to be used in the design of adequate pasteurization processes of acidic food products (Silva et al., 1999), the effects of environmental factors such as sporulation media on the wet–heat inactivation kinetics should be determined for fruit juice processing technologies to ensure the microbial safety and prolong the shelf–life of fruit juices.

Although thermal resistance parameters of *A. acidoterrestris* spores in various fruit juices have been reported previously, this is the first study investigating the effect of sporulation media on the wet–heat inactivation kinetics of *A. acidoterrestris*. In all survival curves, the logarithmic survival ratio, $S(t)$ decreased with time during wet–heat inactivation. If a plot of $logS(t)$ versus time is linear, it follows a first–order kinetics (van Boekel, 2008). Based on the survival curves (Fig. 4.4–4.6), *A. acidoterrestris* spores produced on different media exhibited log–linear inactivation (first–order kinetics) patterns during wet–heat treatments. Although novel models could be used to describe *A. acidoterrestris* spore inactivation kinetics, the first–order kinetics (Bigelow

model) was still being used to calculate *D* and *z*–values and to compare them with the previous published data (Silva et al., 2012).

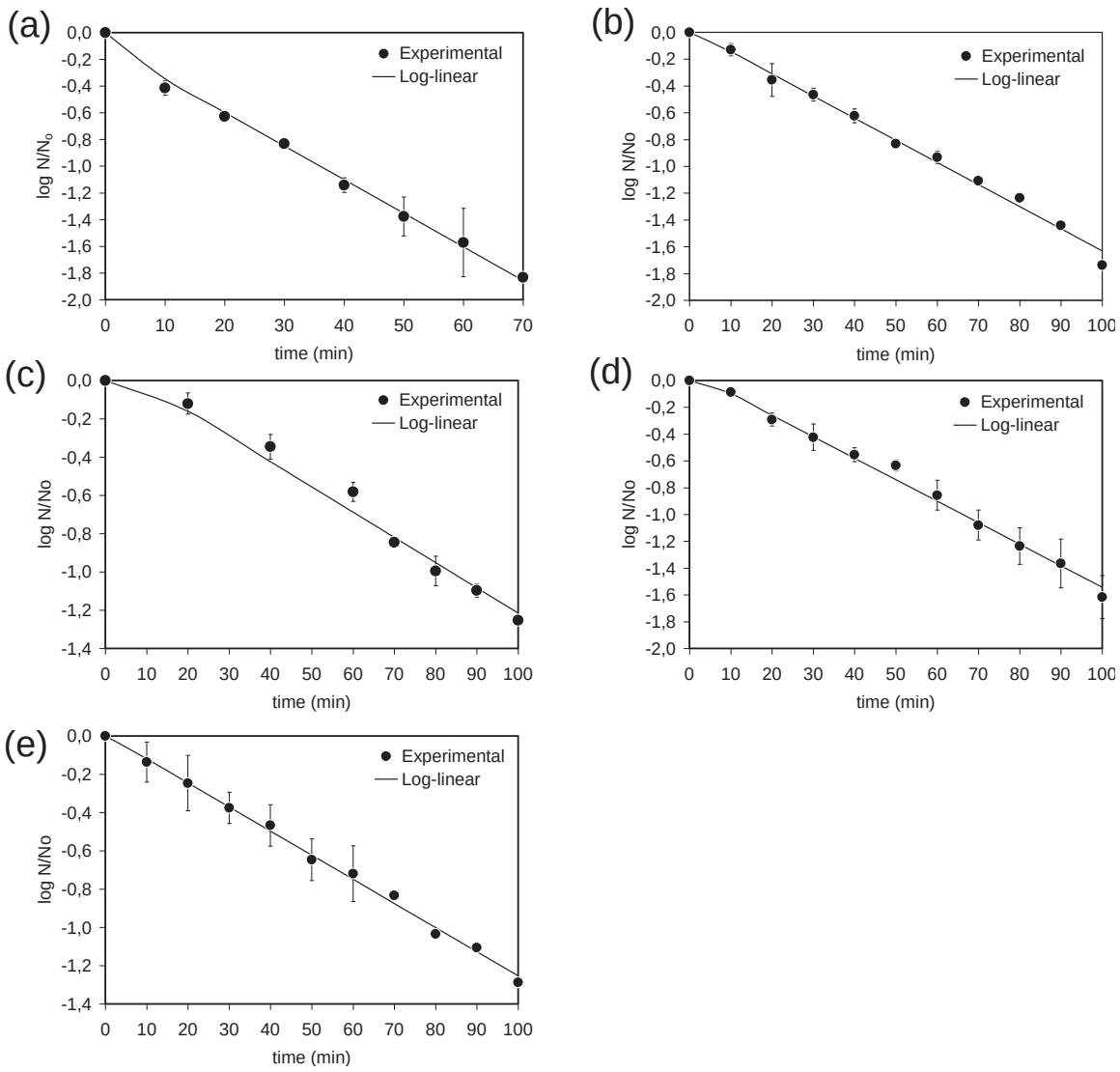

Figure 4.4. Inactivation kinetics of spores at 85 °C in apple juice fitted by the log–linear model (a) PDA (b) MEA (c) BATA (d) BATB (e) BAA

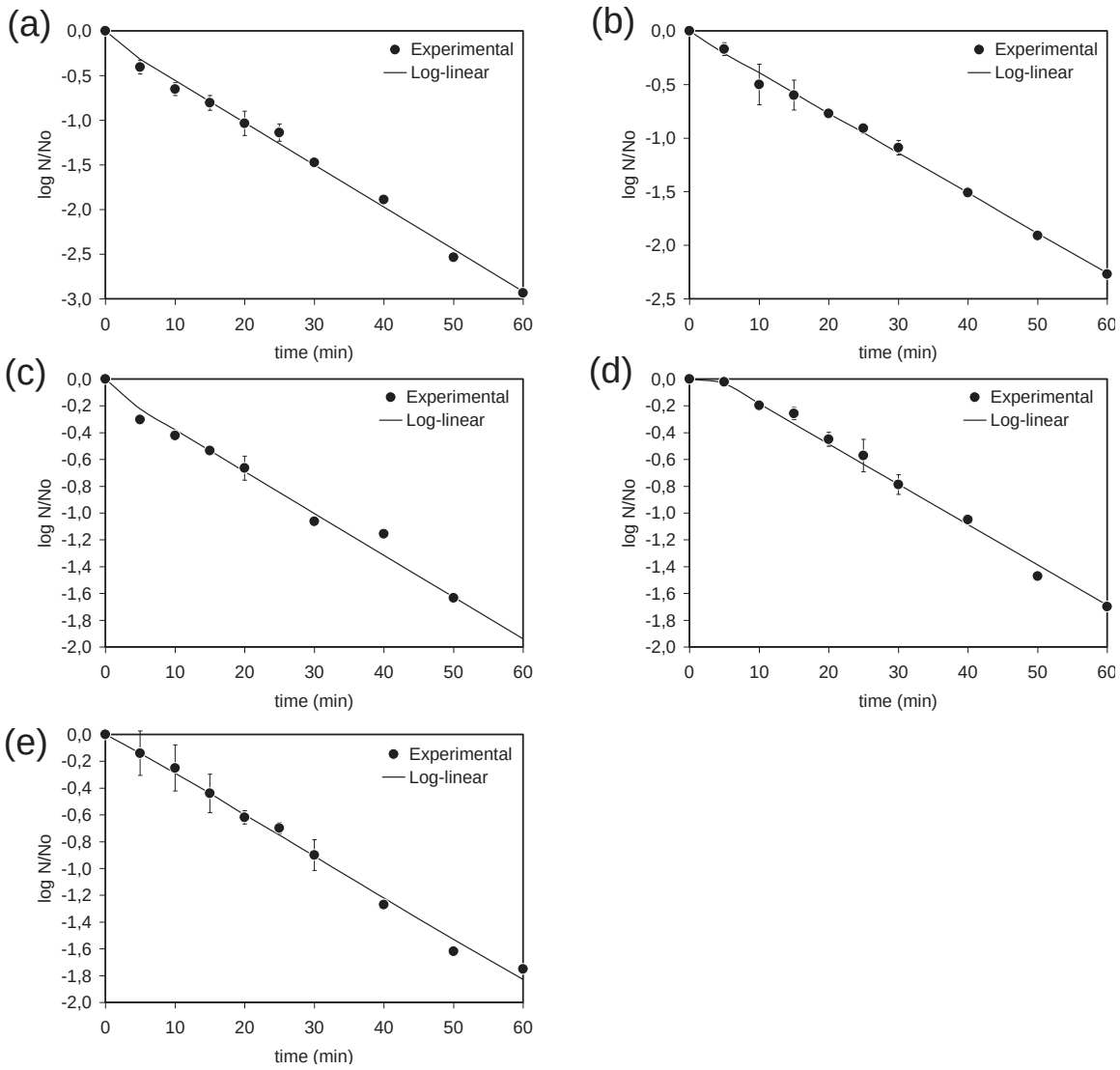

Figure 4.5. Inactivation kinetics of spores at 87.5 °C in apple juice fitted by the log–linear model (a) PDA (b) MEA (c) BATA (d) BATB (e) BAA

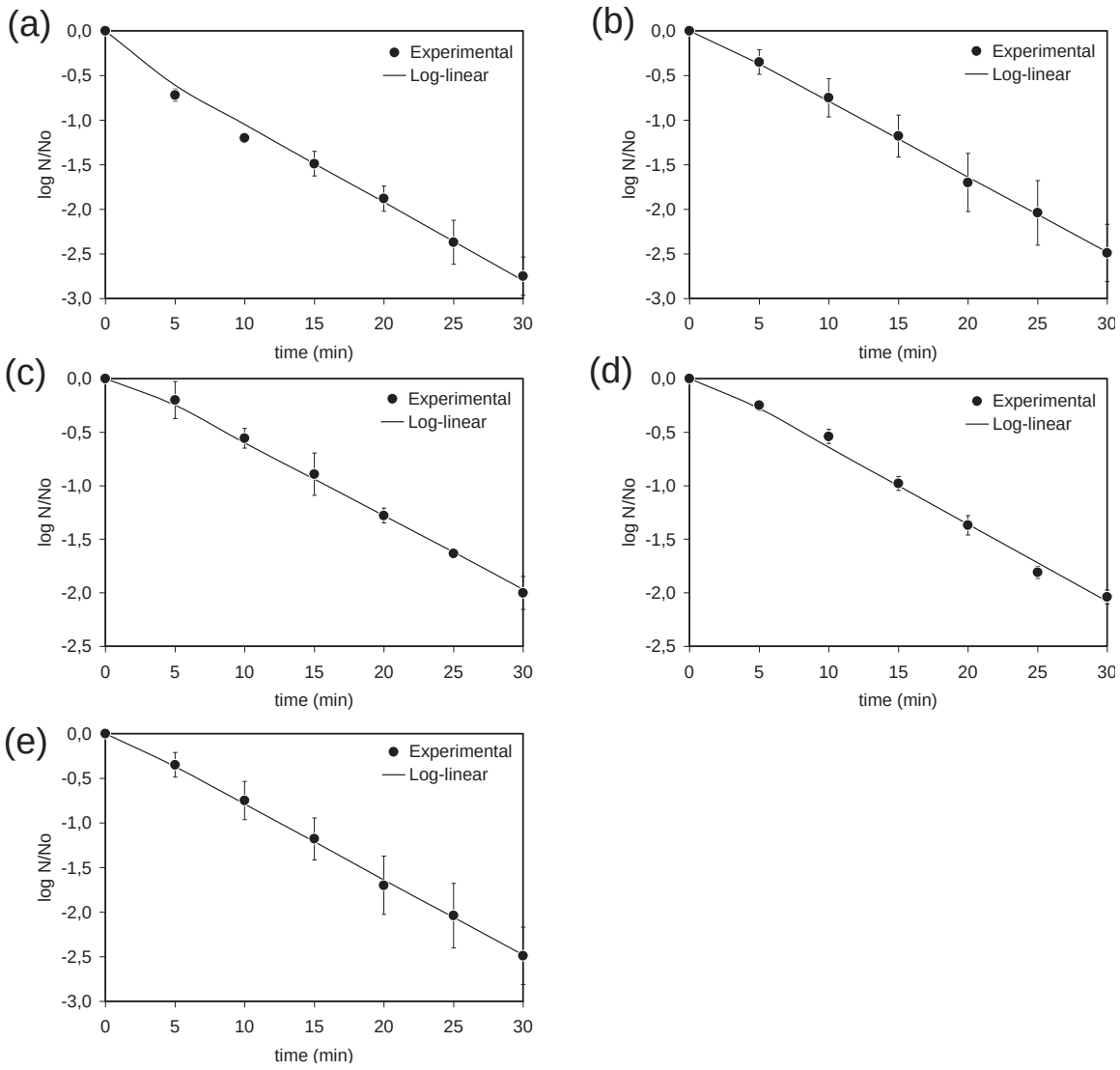

Figure 4.6. Inactivation kinetics of spores at 90 °C in apple juice fitted by the log–linear model (a) PDA (b) MEA (c) BATA (d) BATB (e) BAA

The statistical indices calculated by the GlnaFiT Excel Add–in programme were used for model comparison and given in Appendix A (Fig.A1–A3). The log–linear model provided good fit to the experimental data within the studied temperature range (Table 4.1). For the proposed model, R^2 values can be replaced by $Adj–R^2$. As seen in Table 4.1, higher $Adj–R^2$ values (0.974–0.997) were obtained.

Table 4.1. Statistical indices of the log–linear model for wet–heat inactivation

Sporulation media	Temperature (°C)	Statistical indices	
PDA	85.0	*RMSE*	0.056
		R^2	0.993
		R^2*–adj*	0.992
MEA	85.0	*RMSE*	0.050
		R^2	0.993
		R^2*–adj*	0.992
BATA	85.0	*RMSE*	0.075
		R^2	0.978
		R^2*–adj*	0.974
BAA	85.0	*RMSE*	0.028
		R^2	0.996
		R^2*–adj*	0.995
BATB	85.0	*RMSE*	0.053
		R^2	0.991
		R^2*–adj*	0.990
PDA	87.5	*RMSE*	0.083
		R^2	0.993
		R^2*–adj*	0.992
MEA	87.5	*RMSE*	0.082
		R^2	0.986
		R^2*–adj*	0.984
BATA	87.5	*RMSE*	0.047
		R^2	0.996
		R^2*–adj*	0.996
BAA	87.5	*RMSE*	0.055
		R^2	0.993
		R^2*–adj*	0.992
BATB	87.5	*RMSE*	0.065
		R^2	0.989
		R^2*–adj*	0.988
PDA	90.0	*RMSE*	0.118
		R^2	0.987
		R^2*–adj*	0.985
MEA	90.0	*RMSE*	0.067
		R^2	0.997
		R^2*–adj*	0.996
BATA	90.0	*RMSE*	0.056
		R^2	0.995
		R^2*–adj*	0.994
BAA	90.0	*RMSE*	0.048
		R^2	0.998
		R^2*–adj*	0.997
BATB	90.0	*RMSE*	0.074
		R^2	0.993
		R^2*–adj*	0.991

Table 4.2 summarizes the inactivation kinetic parameters of the log–linear model. The *D*–values for all spore suspensions decreased significantly as the heating temperature increased ($p<0.001$).

Table 4.2. Estimated parameters of the log–linear model

Sporulation media	$k_{max85°C}$ (1/min)	$k_{max87.5°C}$ (1/min)	$k_{max90°C}$ (1/min)	$D_{85°C}$ (min)	$D_{87.5°C}$ (min)	$D_{90°C}$ (min)
PDA	0.06±0.01	0.11±0.02	0.20±0.02	$41.7±7.6^{Ca†}$	$22.4±4.5^{Bb}$	$11.6±1.2^{ABc}$
MEA	0.04±0.0	0.09±0.01	0.23±0.01	$57.6±0.0^{BCa‡}$	$26.7±1.9^{ABb}$	$9.9±0.5^{Bc}$
BATA	0.03±0.0	0.07±0.0	0.16±0.01	$76.8±0.0^{Aa}$	$32.9±0.0^{Ab}$	$14.7±0.6^{Ac}$
BAA	0.03±0.0	0.07±0.01	0.20±0.07	$76.8±0.0^{Aa}$	$31.5±2.4^{Ab}$	$11.9±2.1^{ABc}$
BATB	0.04±0.01	0.07±0.0	0.16±0.01	$67.2±11.1^{ABa}$	$32.9±0.0^{Ab}$	$14.1±0.4^{Ac}$

*For each data set, the *D*–value was calculated using corresponding rate constant (k_{max}) and the results were expressed as the mean±standard deviation from three experiments
†Values with different capital letters in the same column are significantly different ($p<0.05$)
‡Values with different lower case in the same row are significantly different ($p<0.05$)

The results indicated that there were significant differences ($p<0.05$) in the wet–heat resistances among spores formed on different sporulation media. Especially, the spores produced on mineral–containing media (BATA, BAA and BATB) were found more resistant than those formed on non–mineral containing media (PDA and MEA). The $D_{85°C}$–values were 41.7, 57.6, 76.8, 76.8 and 67.2 min; $D_{87.5\ °C}$–values were 22.4, 26.7, 32.9, 31.5, 32.9 min; and $D_{90°C}$–values were 11.6, 9.9, 14.7, 11.9 and 14.1 min for spores produced on PDA, MEA, BATA, BAA and in BATB, respectively. There was a significant difference between the heat resistance of spores made in liquid (BATB) and on agar (BATA) at 85 °C ($p<0.05$). On the other hand, $D_{87.5°C}$ and $D_{90°C}$ values of spores formed in BATB and on BATA were not different significantly (Table 4.2). Thermal resistance parameters of *A. acidoterrestris* spores in apple juice have been determined by other researchers. Splittstoesser et al. (1994) found $D_{85°C}$ as 56.0±14 min for the strain VF. $D_{90°C}$ values in apple juice were found as 15.0 min (Cerny et al., 1984); 23.0±7.5 min (Splittstoesser et al., 1994), 7.8±0.85 min (Komitopoulou et al., 1999); and 11.1±1.6 min (Bahçeci and Acar, 2007b). In the related literature, the differences between the *D*–values were interpreted due to the differences in the test strains, inoculum levels, incubation temperatures, sporulation temperature, differences in nutrient composition and pH of the heating medium, water activity, presence or absence of divalent cations and antimicrobial compounds (Bahçeci and Acar, 2007b). Similarly, Mazas et al. (1995) determined the thermal resistance characteristics of *B. cereus* spores sporulated on different types of sporulation media (Nutrient agar

supplemented with Mn^{2+}, Fortified Nutrient agar, Angelotti Medium and Milk agar). They found clear differences between the *D*–values for spores produced on the tested four media. In another study, the effect of temperature and pH during sporulation on heat resistance of *B. weihenstephanensis* and *B. licheniformis* spores were studied (Baril et al., 2012). They found that a decrease in heat resistance either at low/high temperature or at acidic pH. The influence of sporulation environment is known to have a great impact on spore resistance and spore formation characteristics of *Bacillus* (Desriac et al., 2013). On the other hand, the effect of sporulation environment on spore resistance properties is not taken into account in heat process calculations (Carlin, 2011). Also, the effect of sporulation media on the wet–heat resistance of *A. acidoterrestris* has not been considered during the design of thermal process calculations in fruit juices or acidic food products. When the thermal resistances were compared using *D*–values, spores produced on mineral containing media exhibited greater resistance to the wet–heat. This may be due to the mineralization of endospores with divalent cations found in BATA, BAA and BATB. In fact, mineralization of endospores with divalent cations such as Ca^{2+} or Mn^{2+}, contributes to the stabilization of endospores against heat. Ca^{2+} also chelates DPA to form Ca–DPA, which then stabilizes endospores and contributes to heat resistance. *A. acidoterrestris* endospores bind Ca^{2+} and Mn^{2+} more strongly at low pH compared to *Bacillus* spp. Their endospores are also able to keep Ca–DPA levels constant. Thus, stabilization of Ca–DPA concentrations and the ability to strongly bind divalent cations contribute to the heat resistance of *A. acidoterrestris* spores (Yamazaki et al., 1997).

The sporulation conditions in liquid or solid medium, with various carbon or mineral sources have been shown to affect heat resistance of spores (Mah et al., 2008a). In the present study, it was found that the preparation in a liquid medium may yield spores with lower thermal resistance as those made on agar medium depending on the heating temperature. Similar to our findings, Rose et al. (2007) also found that *B. subtilis* spores made in liquid media had lower resistance to heat at 90 °C and several chemicals. Spores produced in liquid were also able to germinate more readily with several agents. They also observed significant variations in the composition of the spore inner membrane. However, they could not find differences in the levels of DPA, core water, SASP and individual coat proteins or the cross–linking of coat–protein. It has been suggested that this difference might arise from other factors such as the strength of peptidoglycan cortex of spores prepared in liquid or on agar (Popham et al., 1995).

The *z*–value is a valuable tool used to change commercial processing conditions decreasing either by the time to achieve product microbial safety and stability, or the temperature to improve product quality. Indeed, when heating time is decreased, the *z*–value is used to determine the new target processing temperature. Likewise, if a lower temperature is desired to improve product flavor, the *z*–value provides the increased time needed to achieve the same product safety and stability as with the previous process conditions (Parish, 2006). The estimated *z*–values were determined by plotting the log*D* values against heating temperatures and then taking the reciprocal of the slope from linear regression (Daryaei and Balasubramaniam, 2013). Figure 4.7 represents the plots of log*D* values against heating temperatures and corresponding correlation coefficients (R^2) for each spore suspension prepared from different sporulation media.

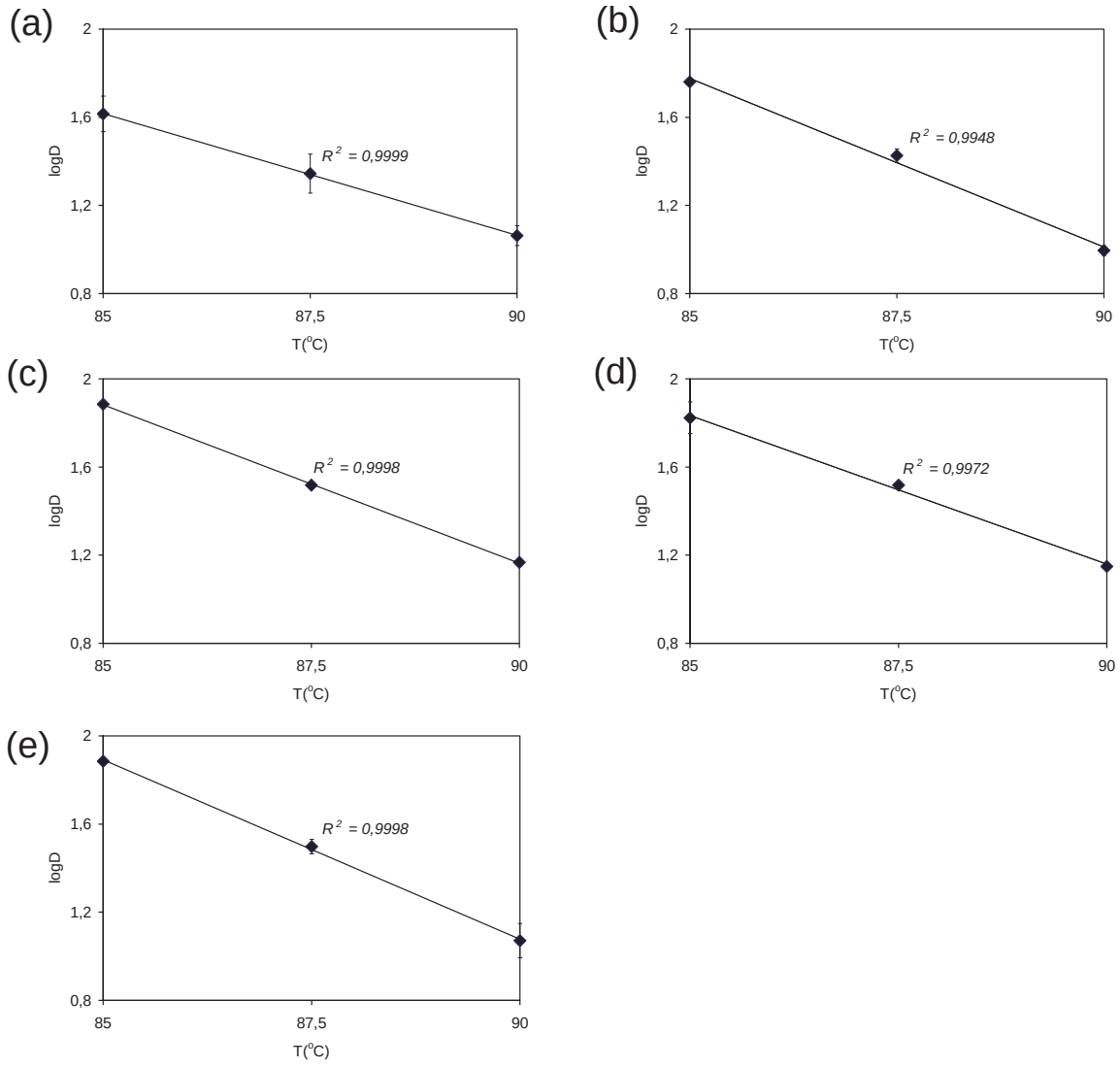

Figure 4.7. z–values of spores produced on (a) PDA (b) MEA (c) BATA (d) BATB (e) BAA in apple juice

As indicated in Table 4.3, the *z*–values were found to be 9.05±0.17 (R^2=0.999), 6.60±0.83 (R^2=0.995), 6.96±0.18 (R^2=0.999), 6.15±0.29 (R^2=0.999), and 7.46±0.69 °C (R^2=0.997) for spores from PDA, MEA, BATA, BAA and BATB media, respectively using the plots represented in Fig.4.7. The estimated *z*–values found in this study were in accordance with the previously reported values (6.4°C to 12.2 °C) for apple juice (Bahçeci and Acar, 2007b; Komitopoulou et al., 1999; Previdi et al., 1997; Splittstoesser et al., 1994). In the related literature, the *z*–value was found to be independent of sporulation media (Mazas et al., 1995), sporulation pH (Baril et al., 2012; Mazas et al., 1997), and sporulation temperature (Condon et al., 1992; Gonzales et al., 1999; Raso et al., 1995; Baril et al., 2012). On the other hand, the *z*–value of spores produced on PDA was significantly (p<0.05) different from the *z*–values of spores produced on BATA, BAA and MEA.

Table 4.3. Estimated z–values by the log–linear model

Sporulation media	***z* (°C)**	R^2
PDA	9.05±0.17^{A‡}	0.999
MEA	6.60±0.83^{B}	0.995
BATA	6.96±0.18^{B}	0.999
BAA	6.15±0.29^{B}	0.999
BATB	7.46±0.69^{B}	0.997

†Values were expressed as the mean±standard deviation from three experiments
‡Values with different capital letters in the same column are significantly different (p<0.05)

4.1.4. Dipicolinic Acid Analysis

When spores are steam autoclaved, principally all their DPA is released (Janssen et al., 1958). Therefore, the total DPA content of the spores produced on different media was determined after autoclaving at 121 °C for 20 min. DPA levels of the spores were calculated using the calibration curve in Appendix B (Fig.B1). It was found that the DPA contents of spores produced on mineral containing media was significantly higher than nonmineral containing media (p<0.005). The results indicated that spores produced on BATA (0.47±0.00 µmole) and BAA (0.49±0.01 µmole) have higher DPA than those of spores produced on PDA (0.21±0.00 µmole) and MEA (0.26±0.02 µmole) on the basis of the dry weight (Table 4.4). In fact, Ca–DPA levels in the spores can depend on species/strains and sporulation conditions (Huang et al., 2007). The obtained results in this study also emphasize the importance of the sporulation medium composition on the

DPA levels of *A. acidoterrestris* spores. Based on the colorimetric assay, DPA content of the spores was also expressed as spore dry weight and significantly varied among spores (3.5–8.2%) obtained from different media (Table 4.4). The amount of DPA release from heat–treated spores (85 °C for 60 min, 87.5 °C for 30 min and 90 °C for 15 min) was also determined (Table 4.4). After 60 min at 85 °C, the spores produced on PDA and MEA released most of their DPA, approximately 93% and 83%, respectively. Whereas, the spores formed on mineral containing media (BATA and BAA) released approximately 70% of their DPA. Futhermore, the DPA release of spores from nonmineral containing media after 87.5 °C treatment for 30 min was higher than that of spores from mineral containing media. The correlation coefficient (R^2) between the DPA release and corresponding $D_{85°C}$ and $D_{87.5°C}$–values was higher than 0.98 indicating the presence of a linear relationship. At 90 C°, the DPA release from spores produced on nonmineral containing media was higher but there was no clear correlation between the DPA release and $D_{90°C}$–values.

Table 4.4. DPA content and release during wet–heat at 85 °C, 87.5 °C and 90 °C

Sporulation media	DPA content		DPA release (%)		
	µmole/mg spore	% dry weight††	85 °C/60 min	87.5 °C/30 min	90 °C/15 min
PDA	0.21 ± 0.00^{A}	3.51 ± 0.00^{A}	$93.27\pm2.47^{Aab\dagger}$	87.35 ± 0.49^{Ab}	98.5 ± 0.28^{Aa}
BATA	0.47 ± 0.00^{B}	7.85 ± 0.00^{B}	$70.00\pm1.41^{Cab\ddagger}$	52.0 ± 1.41^{Bb}	75.65 ± 5.87^{Ba}
BAA	0.49 ± 0.01^{B}	8.19 ± 0.17^{B}	71.79 ± 2.43^{Ca}	60.5 ± 0.71^{Ca}	68.2 ± 4.53^{Ba}
MEA	0.26 ± 0.02^{C}	4.35 ± 0.34^{C}	82.65 ± 0.49^{Ba}	76.45 ± 0.78^{Db}	84.2 ± 1.13^{ABa}

*Data were expressed as the mean±standard deviation from three experiments
†Values with different capital letters in the same column are significantly different (p<0.05)
‡Values with different lower case in the same row are significantly different (p<0.05)
††% dry weight of DPA was expressed by the percentage of the amount of mg DPA to the total mg spore dry weight

DPA content is one of the most prominent factors to wet–heat (Paidhungat et al., 2000). The DPA contents of spores produced on non–mineral containing media (PDA and MEA) were lower than mineral containing media (BATA and BAA). Therefore, the increase in the DPA content of spores formed on mineral containing media could contribute to the increase in the wet–heat resistance. Indeed, the role of DPA in spore resistance is to lower the core water content, probably by replacing some core water. This process can increase wet–heat resistance by protecting core proteins from inactivation or denaturation (Baweja et al., 2008). However, the role of DPA release in spore killing by wet–heat treatment is not clear. The heat inactivation of spore does not

instantaneously result in the release of its DPA content. Some factors other than DPA release are involved in heat inactivation (Kort et al., 2005). In *B. stearothermophilus* spores, it has been suggested that DPA release occurs before spore killing but in *B. megaterium* and *B. subtilis* spores, DPA release follows spore killing rather than precedes it (Mallidis and Scholefield, 1985; Belliveau et al., 1992; Coleman et al., 2010). Based on the results of DPA release, it can be concluded that DPA release occurs before spore killing. Although most of the DPA were released from spores, the majority of the spores were still alive.

4.1.4. Inductively Coupled Plasma Mass Spectrometry

The ICP–MS results showed that Ca^{2+} concentrations in spores produced on mineral containing media (BATA and BAA) were approximately twofold higher than spores produced on nonmineral containing media (PDA and MEA). Ca^{+2} is important for the stabilization of spore heat resistance by chelating with DPA. It also increases the heat resistance (Yamazaki et al., 1997). On the other hand, we could not find any relationship between the Mg^{2+}, Mn^{2+} and Fe^{2+} levels and heat resistance (Table 4.5).

Table 4.5. ICP–MS analysis of spores grown in different sporulation media

Sporulation media	**Mg^{2+}**	**Ca^{2+}**	**Mn^{2+}**	**Fe^{2+}**
		µmole/mg spore		
PDA	$0.16\pm0.00^{B*}$	$0.24\pm0.02^{A\dagger}$	0.003 ± 0.00^{D}	0.03 ± 0.00^{A}
BATA	0.44 ± 0.05^{A}	0.56 ± 0.01^{B}	0.008 ± 0.00^{C}	0.03 ± 0.01^{A}
BAA	0.22 ± 0.00^{B}	0.51 ± 0.01^{B}	0.014 ± 0.00^{B}	0.01 ± 0.00^{A}
MEA	0.40 ± 0.04^{A}	0.26 ± 0.02^{A}	0.031 ± 0.00^{A}	0.01 ± 0.00^{A}

*Data were expressed as the mean±standard deviation from three experiments
†Values with different capital letters in the same column are significantly different ($p<0.05$)

In a previous study, the effect of sporulation medium supplemented with different cations ($CaCl_2$, $MgCl_2$, $BaCl_2$, $MnCl_2$, $SrCl_2$, $SnCl_2$, $CuCl_2$, $ZnCl_2$, $CoCl_2$) on the heat resistance of *A. acidoterrestris* AB–1 strain was determined (Yamazaki et al., 1997). They indicated that there was no difference in $D_{89\,^{\circ}C}$ and $D_{90\,^{\circ}C}$–values among the native (produced on media containing Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+} and Sr^{2+}), demineralized or remineralized spores. Yamazaki et al. (1997) found that the Ca contents (0.3–0.57 µmol/mg dry weight) in three different forms were greater than the previously reported DPA content of *A. acidoterrestris* (0.23 µmol/mg dry weight) (Shibata et al., 1993).

Finally, they suggested that the presence of constant amount of Ca–DPA and a stronger binding characteristic of divalent cations, especially Ca^{2+} and Mn^{2+}, is related to specific heat resistance of *A. acidoterrestris* spores. As seen in Table 4.5, the Ca^{2+} concentration was found 0.24, 0.56, 0.51 and 0.26 μmole per mg of spores produced on PDA, BATA, BAA and MEA, respectively. And, the DPA contents of these spores were 0.21, 0.47, 0.49 and 0.26 μmole/mg spore, respectively (Table 4.4). The Ca^{2+} concentrations correlated strongly with DPA contents, with an R^2 value of 0.96. There was also strong correlation between the Ca^{2+} concentrations and $D_{85°C}$ and $D_{87.5°C}$ values, with an R^2 value of 0.87 and 0.90, respectively. On the other hand, no correlation was found between the Ca^{2+} concentration and $D_{90°C}$–value (R^2 =0.62). As mentioned earlier, Yamazaki et al. (1997) indicated that there was no difference in $D_{89°C}$ and $D_{90°C}$–values among the native, demineralized or remineralized forms. There should be a relationship between the stronger binding characteristics of Ca^{2+} and DPA depending on the heating temperature. At 85 °C and 87.5 °C, the effect of Ca–DPA complex on heat resistance was more pronounced. At 90 °C, this complex had no effect on heat resistance.

4.1.5. Scanning Electron Microscopy of Wet–Heat Treated Spores

SEM micrographs were taken to determine the size of the untreated spores and to visualize damage to the spore external structure after wet–heat treatment at 85 °C and 90 °C. The spores of *A. acidoterrestris* were previously reported as 0.9–1.0 μm in width and 1.5–1.8 μm in length (Bevilacqua et al., 2008c). Fig.4.8 indicates the untreated spores from different sporulation media. In fact, the size of *A. acidoterrestris* spores produced on different media has not been determined so far. It was interesting to find that significant variations were obtained in the average width sizes depending on the sporulation media used (Table 4.6). On the other hand, the length of the spores produced on PDA, BATA and MEA were similar.

Table 4.6. The average values of width and length of untreated spores

Sporulation media	Width (μm)	Length (μm)
PDA	0.878±0.101 ^{A†}	1.450±0.126 ^{B}
BATA	0.818±0.078 ^{B}	1.446±0.119 ^{B}
BAA	0.837±0.122 ^{AB}	1.541±0.238 ^{A}
MEA	0.754±0.106 ^{C}	1.444±0.145 ^{B}

†Values with different capital letters in the same column are significantly different (p<0.05)

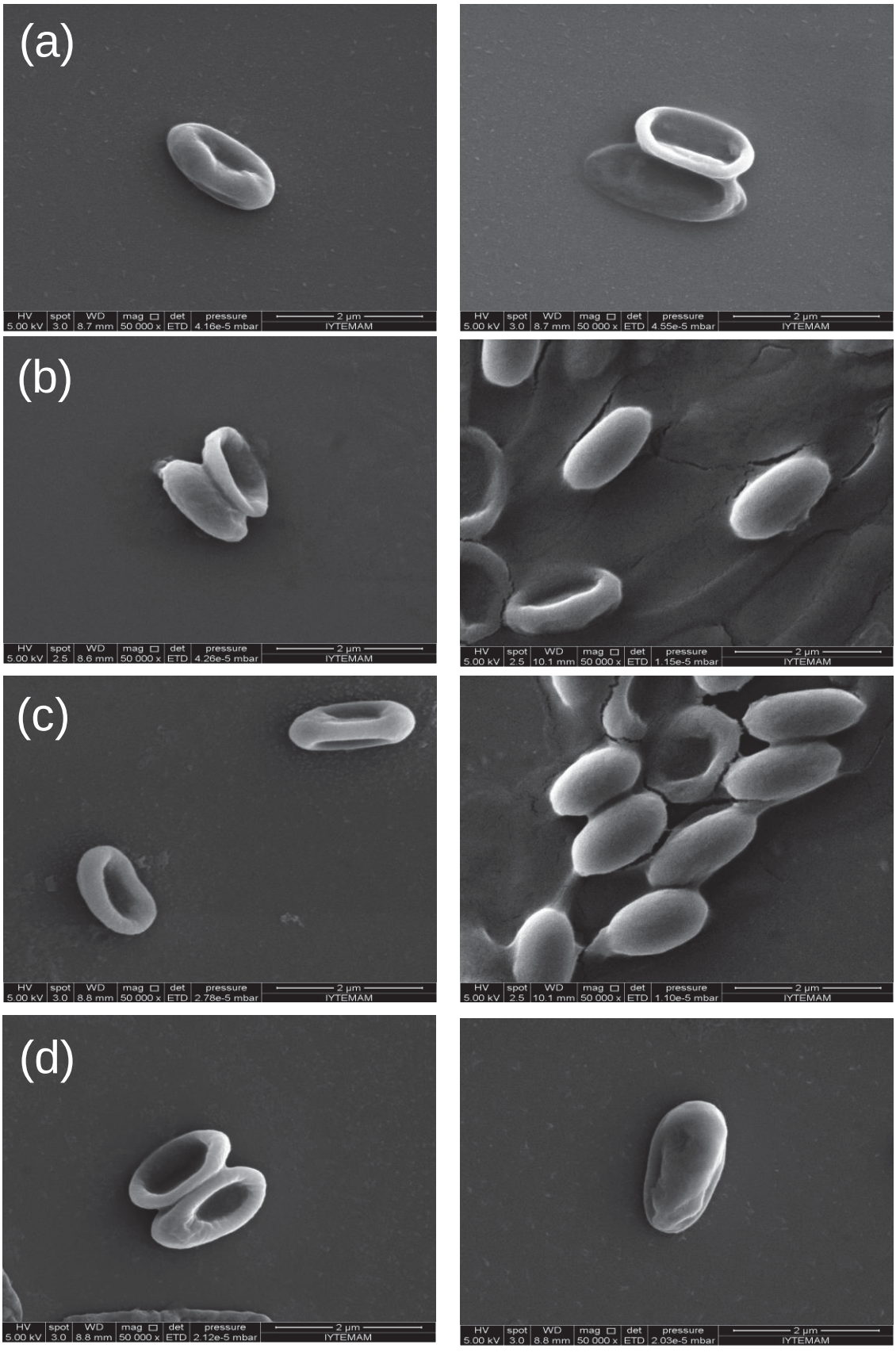

Figure 4.8. SEM images of untreated spores (a) PDA (b) BATA (c) BAA (d) MEA

Before analyzing the ultrastrustural changes on wet–heat treated spores, the morphology of steam autoclaved spores was determined by SEM imaging. It is known that steam autoclaving destroys both outer and inner structures (Fonzi et al., 1999). The changes on spore external structure due to moist heat and pressure after autoclaving were dependent on the sporulation media used (Fig.4.9).

SEM images of autoclaved spore suspensions indicated that some spores lost their internal volume and had wrinkled appearance most probably due to the release of intracellular components compared to untreated spores (Fig.4.8). Autoclaved spores were found to indicate a fully hydrated spore core surrounded by the cortex because of the thermal destruction of inner spore membrane (Reineke et al., 2013a). This is in turn related to full DPA release (Janssen et al., 1958) from spores and the inactivation of core enzymes (Setlow, 2000).

Since DPA and other small molecules constitute 5–15% of the dry weight of spores, the autoclaved spores collapse because of the release of these compounds. Therefore, the loss of internal volume results in wrinkled appearance (Perkins et al., 2004). The wrinkled appearance (Fig.4.9b) and loss of internal volume (Fig.4.9c) is more obvious among spores produced on mineral containing media (BATA and BAA). On the other hand, spores produced on PDA and MEA retain their structural integrity although these spores were completely inactivated by autoclaving (Fig.4.9a and 4.9d). The presence of cellular debris was also observed among autoclaved spores produced on BATA and BAA.

Since the DPA was found to constitute 7.85% and 8.19% dry weight of spores produced on BATA and BAA, respectively, the changes were more obvious after autoclaving of these spores (Table 4.5). Therefore, the loss of DPA from spores formed on mineral containing media after autoclaving was higher than that of spores produced on nonmineral containing media with lower DPA content (PDA, 3.51% and MEA, 4.35%). The presence of undifferentiated solids surrounding the spores following autoclaving was also observed among *B. subtilis* and *C. perfringens* spores (Perkins et al., 2004) and it was also suggested that the spores swell, split and release their contents upon autoclaving. In another study (Kim et al., 2009), it was reported that *B. licheniformis* spores exhibited a degraded outer coat and distorted inner coat after autoclaving. Also, the core of autoclaved spores appeared larger compared to untreated ones. On the other hand, they could not detect significant changes in the cortex structure; only one portion of the cortex was degraded.

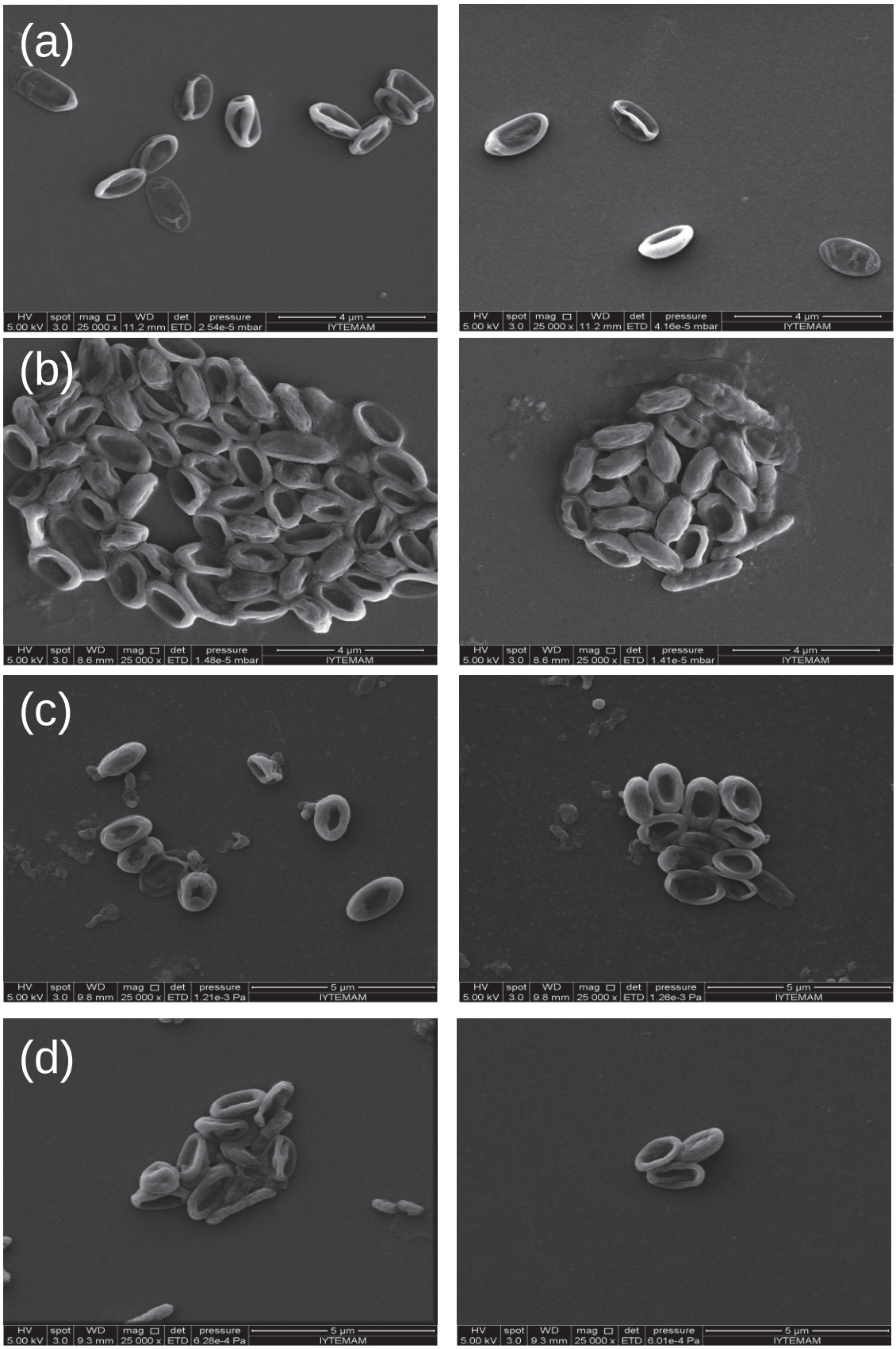

Figure 4.9. SEM images of autoclaved spores (a) PDA (b) BATA (c) BAA (d) MEA

SEM imaging was also used to visualize the spores after wet–heat treatment at 85 °C for 60 min. Although some of the spores produced on PDA (Fig. 4.10a) and MEA media (Fig. 4.10d) exhibit a high degree of roughness in the spore core most probably due to protein denaturation, a majority of the spores produced on mineral containing media do not indicate visible structural damage after treatment (Fig.4.10b and 4.20c)

The calculated $D_{85°C}$ values were 41.7 and 57.6 min for spores produced on PDA and MEA, respectively. Spores formed on PDA and MEA showed a reduction in spore counts of approximately 1.6 and 1.0 log after exposure to 85 °C for 60 min (Fig.4.4). The DPA release was also found as approximately 93% and 83% for spores formed on PDA and MEA (Table 4.4).

The presence of solid inner core among spores may occur because of the slow DPA release from heat–resistant spores produced on mineral containing media (Fig.4.10b and 4.10c). Approximately, 70 and 71.8% of DPA was released from spores produced on BATA and BAA after 85 °C for 60 min, respectively. Since the $D_{85°C}$ values for spores produced on BATA and BAA were approximately 76.8 min, the log reductions were approximately 0.6 and 0.7, respectively after 60 min treatment at 85 °C. Similarly, it has been reported that high levels of wet–heat killed spores among *Bacillus* spp. can retain DPA if treatment conditions are not too extreme (Coleman et al., 2007; Coleman et al., 2010).

Heat causes ribosome aggregation, loss of nutrients and ions, DNA strand breakage, and inactivation of essential enzymes (Maňas and Pagán, 2005). Coleman et al. (2007) reported that moist–heat treated spores often appeared as injured. Although they could be recovered on rich medium, in contrast to unheated spores, they were poorly recovered on medium supplemented with high salt or low nutrients. They suggested that some proteins can be damaged by moist–heat although this damage may only be conditionally lethal. In fact, the inactivation of spores by wet heat is found to be related to the ruptures of spore's inner membrane permeability barrier and damage to the core enzymes (Coleman et al., 2007). Significant protein denaturation can occur during wet–heat treatment and prior to Ca–DPA release (Setlow, 2006)

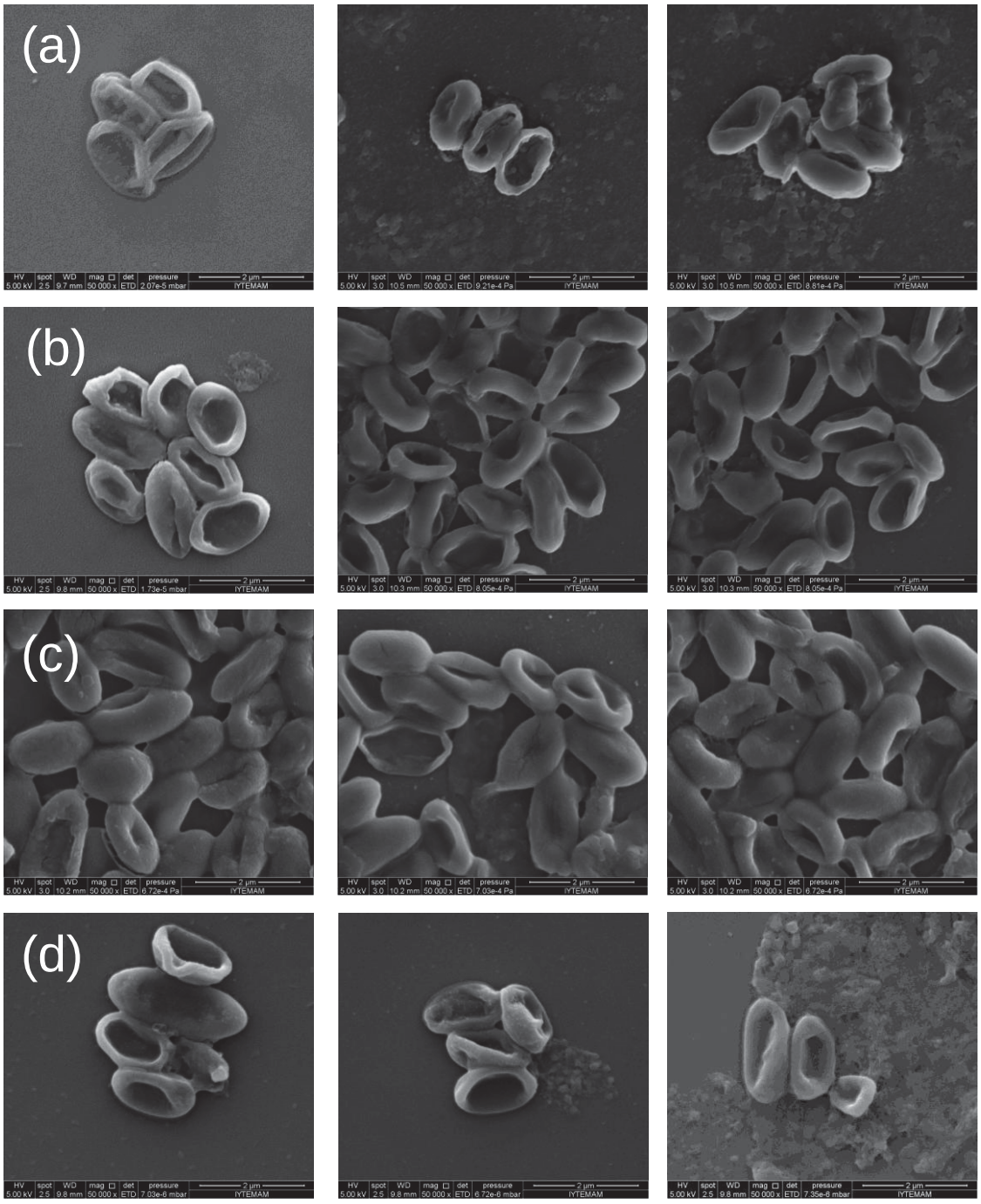

Figure 4.10. SEM images of spores after wet–heat treatment at 85 °C for 60 min grown on (a) PDA (b) BATA (c) BAA (d) MEA

When spores were wet–heat treated at different time intervals at 90 °C, they showed distinguishable structural differences (Fig.4.11–14). The surface of the spores after 30, 45 and 60 min of heating is similar to the autoclaved spores (Fig.4.9) and lose of internal volume most probably due to the release of cellular components from core is more obvious. Structural integrity also seems to be more damaged after 30, 45 and 60 min treatments. Fast heating is shown to destruct the endospore surfaces of *B. cereus*

than autoclaving. Because fast heating causes fast boiling of water and denatures protein within endospores leading to destruction of the spore components. In contrast, slow heating process during autoclaving gives the proteins quite enough time for gradual coagulation before the water inside and outside of the endospores reaches the boiling point thus protecting the shape of endospores (Mustafa et al., 2010).

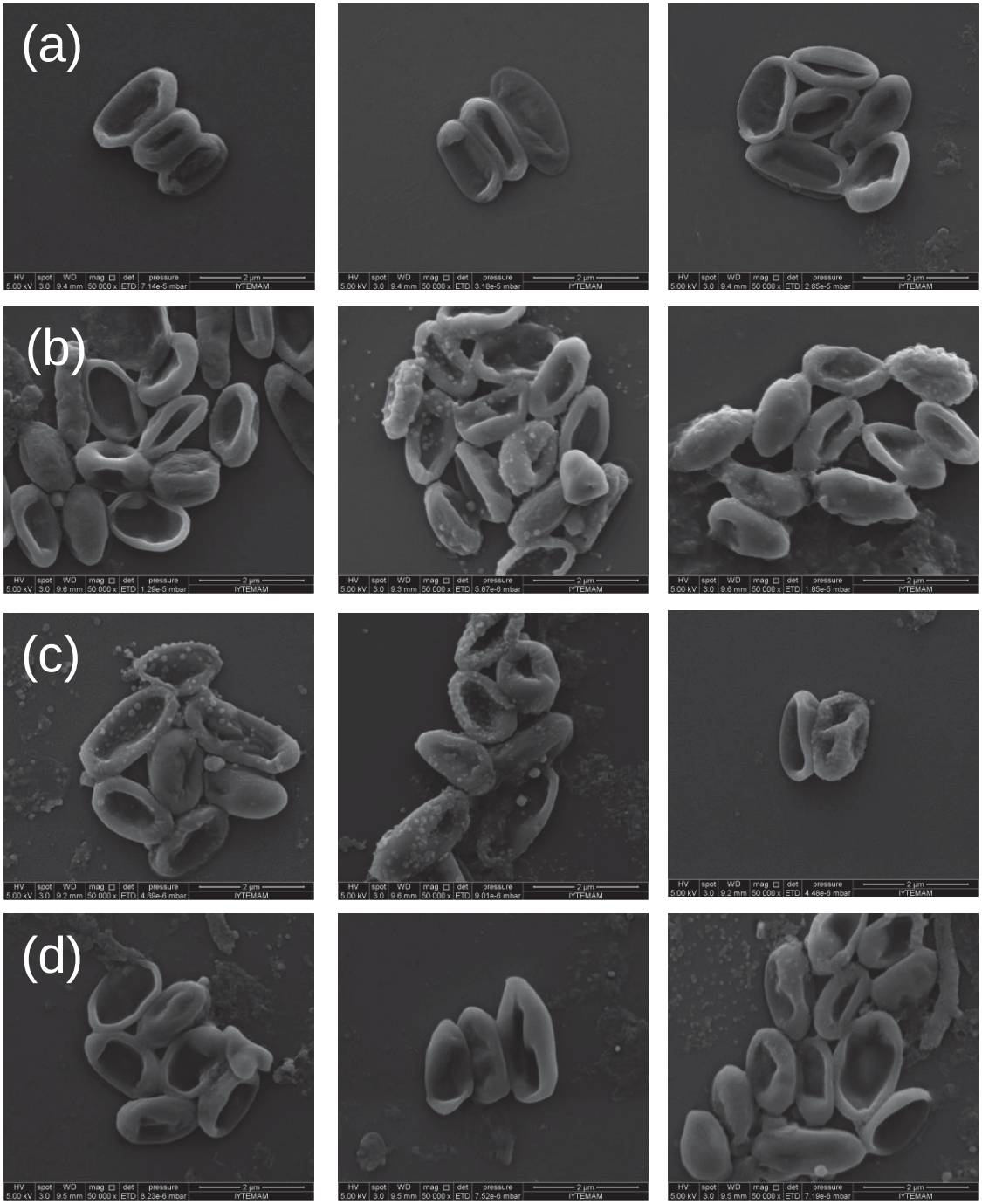

Figure 4.11. SEM images of spores grown on PDA after heat treatment at 90 °C for (a) 15 min (b) 30 min (c) 45 min (d) 60 min

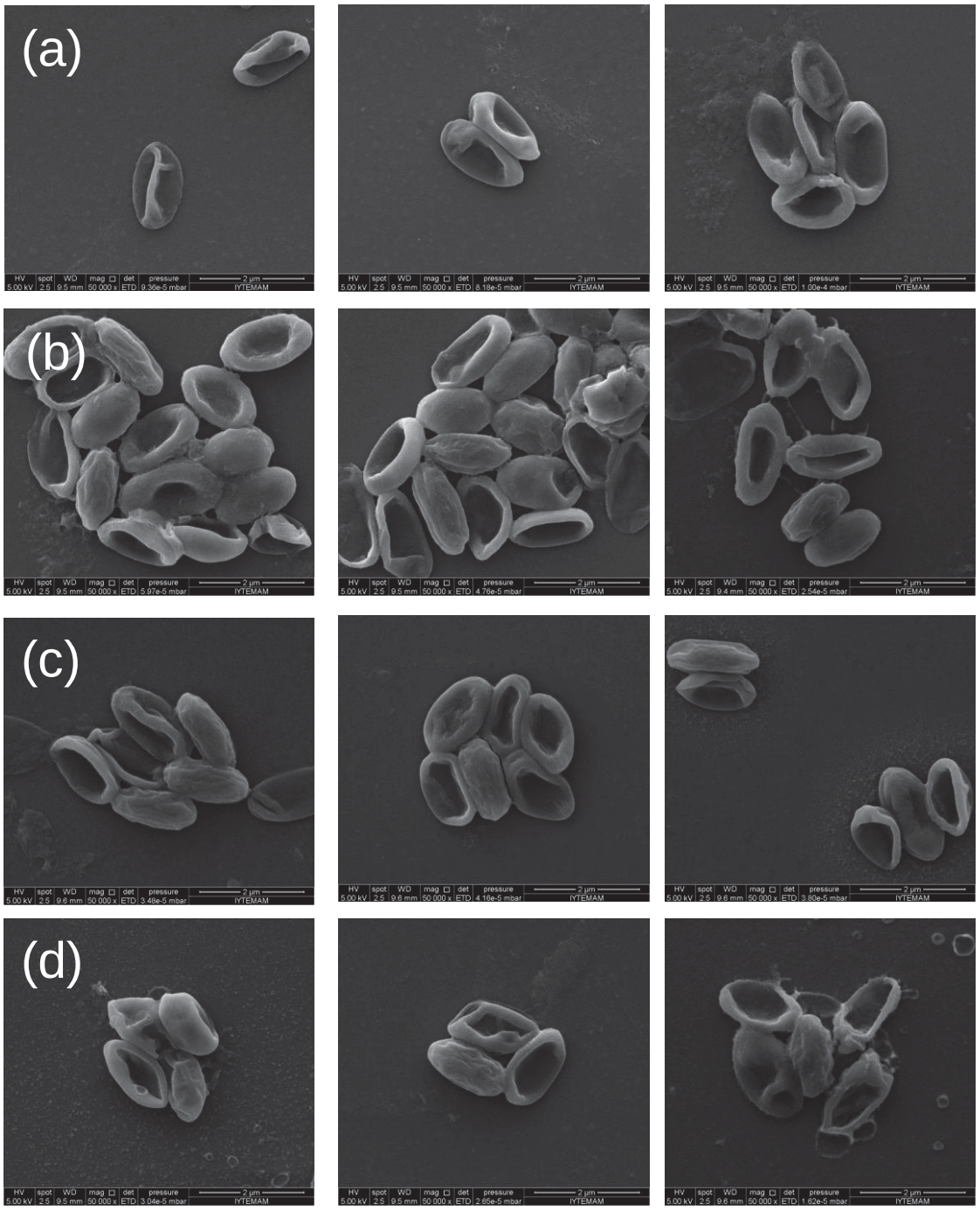

Figure 4.12. SEM images of spores grown on BATA after heat treatment at 90 °C for (a) 15 min (b) 30 min (c) 45 min (d) 60 min

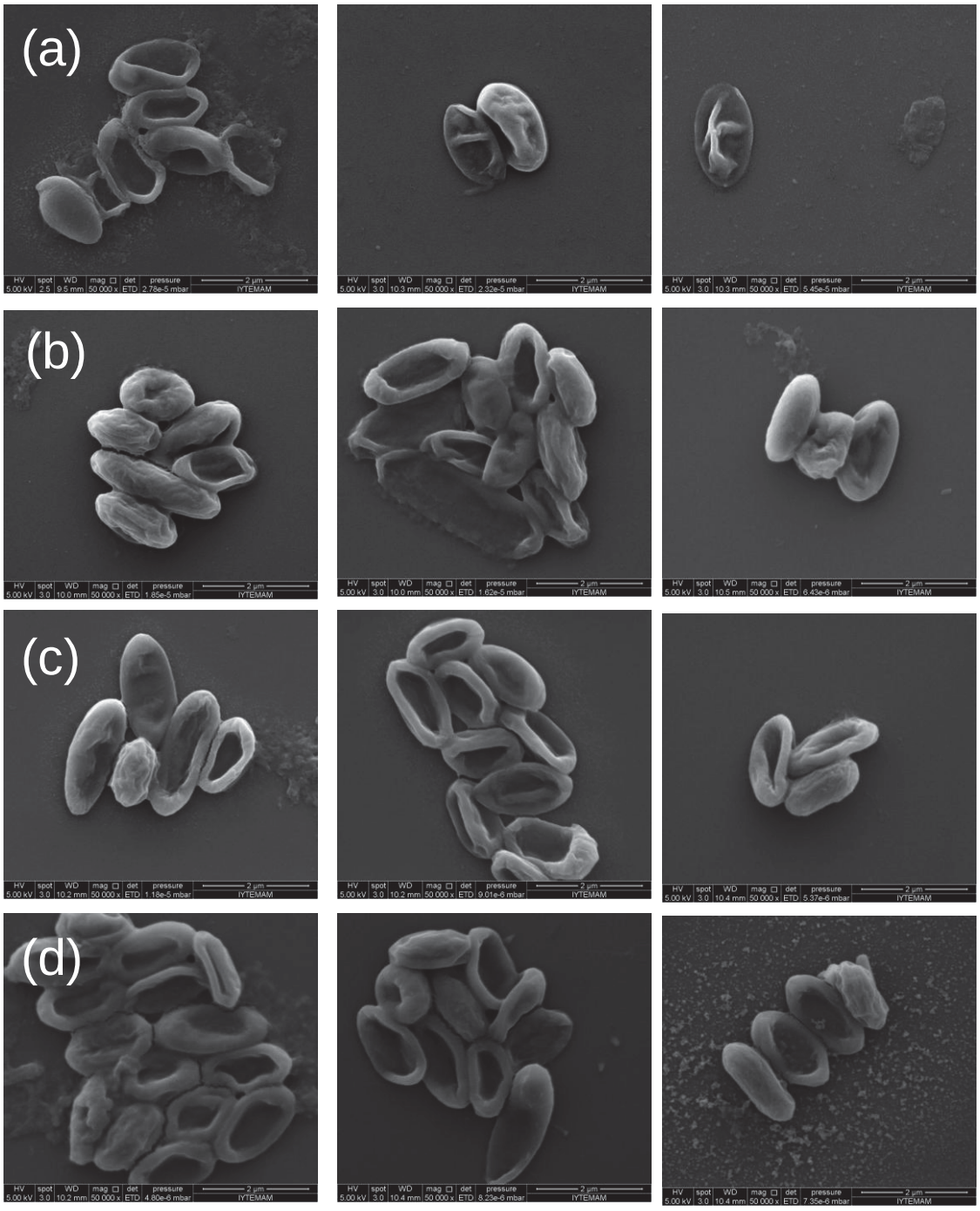

Figure 4.13. SEM images of spores grown on BAA after heat treatment at 90 °C for (a) 15 min (b) 30 min (c) 45 min (d) 60 min

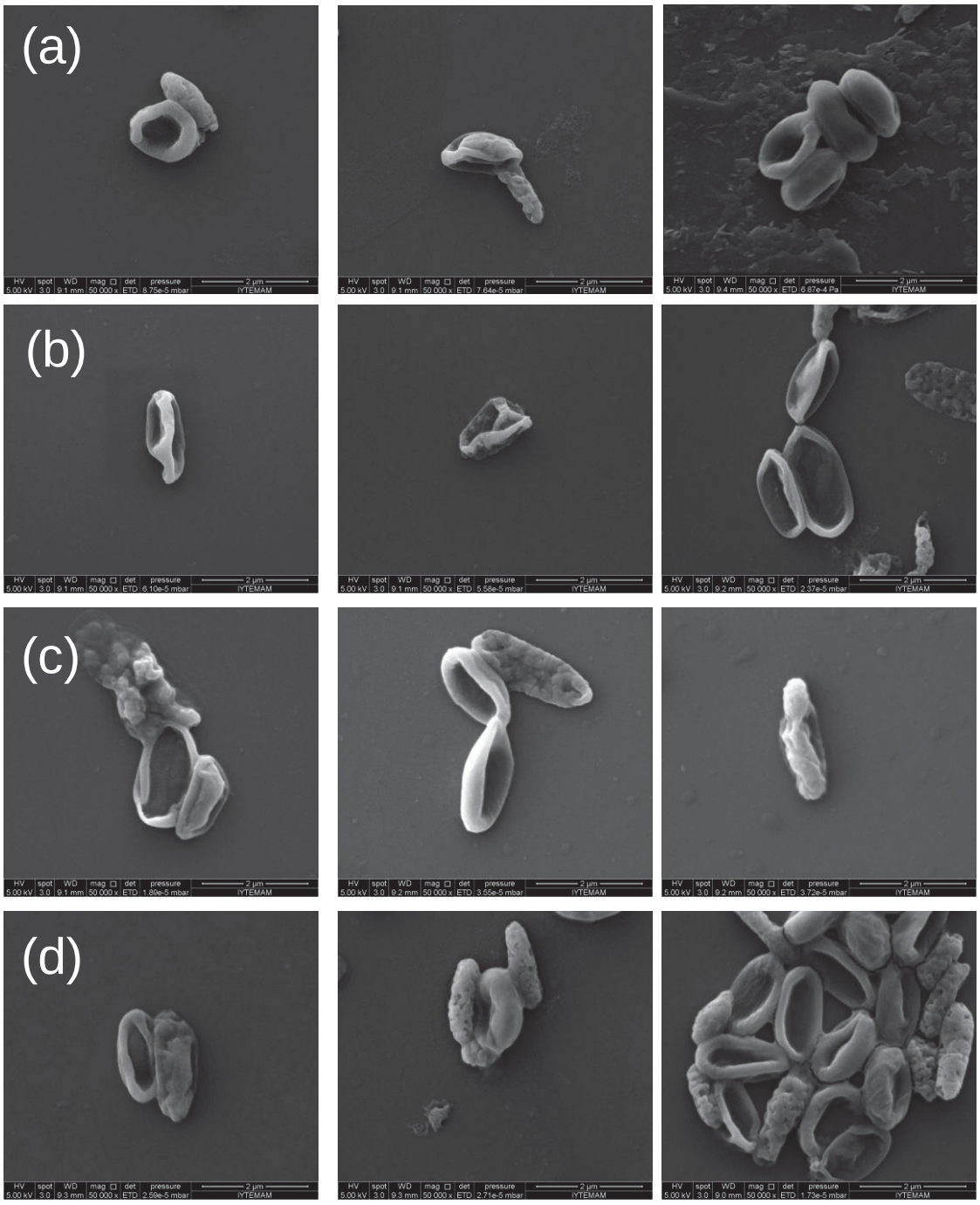

Figure 4.14. SEM images of spores grown on MEA after heat treatment at 90 °C for (a) 15 min (b) 30 min (c) 45 min (d) 60 min

4.1.6. Atomic Force Microscopy of Wet–Heat Treated Spores

For better understanding the mechanism of wet–heat, untreated and treated spores were imaged by AFM. This technique is more advantageous than SEM. Samples do not require fixation, conductive coating or to be imaged under vacuum conditions.

Samples can be imaged in pseudo–physiological conditions such as in a physiological buffer or in a growth medium. This technique has been applied to visualize the hydrated bacteria. On the other hand, imaging in liquid often show reduced topographical contrast compared to images of air–dried samples, and some features such as flagella can not be visualized (Fernandes et al., 2009). Tapping mode is generally preferred for microbiological samples because lateral shear forces are minimized. In contrast, contact mode can result in sweeping weakly adsorbed cells and molecules from the surface (Webb et al., 2011). Therefore, the spore surface morphology of treated and untreated spores was characterized by tapping mode using air–dried samples. Very few studies have used this technique for the morphological characterization of bacterial spores (Zolock et al., 2006). The spores adhered to the hydrophilic glass surface were imaged at the scan rate to 1 Hz or sometimes 0.5 Hz to prevent the dislodging of the spores from the surface. The spore concentration was also decreased to prevent aggregation during drying. To observe the detailed spore morphology, a single spore was monitored by a smaller scan area (5×5 μm). Based on the AFM images, spores were classified as rough surface spores on PDA and MEA and smooth surface spores on BATA and BAA. It has been reported that spores having rough surface indicate a greater resistance to heat than those with a smooth surface (Lindsay et al., 1990) but in the present study, the results were different from this previous finding. Untreated spores from PDA (Fig.4.15 a–d) and MEA (Fig.4.15 e–h) are planiform, elliptical rods having outer ridges and creases while spores produced on BATA (Fig.4.16 a–d) and BAA (Fig.4.16 e–h) had smooth outer surfaces. It has been proposed that these ridges are formed early in spore formation when the spore volume decreases. When the spore swells during germination, the ridges unfold (Chada et al., 2003). The presence of grooves, bumps or grain–like features on *Bacillus* spore surface has also been revealed by AFM (Zolock et al., 2006). Spores with ridges may have lost their exosporium during culturing or sampling whereas spores with smooth appearance might retain their exosporium (Saif Zaman et al., 2005). As mentioned earlier, the presence of dark poles around spores under phase contrast microscope might be the indicator of the presence of exosporium. Moreover, *B. atrophaeous* spores were lack of an exosporium and the core appeared to be small, the cortex and coat layers possessed defined ridges (Buhr et al., 2008) as observed for spores produced on mineral containing media. As mentioned earlier, *B. anthracis* spores may express medium–dependent differences in exosporium structure or glycosylation degree (Colburn et al., 2011).

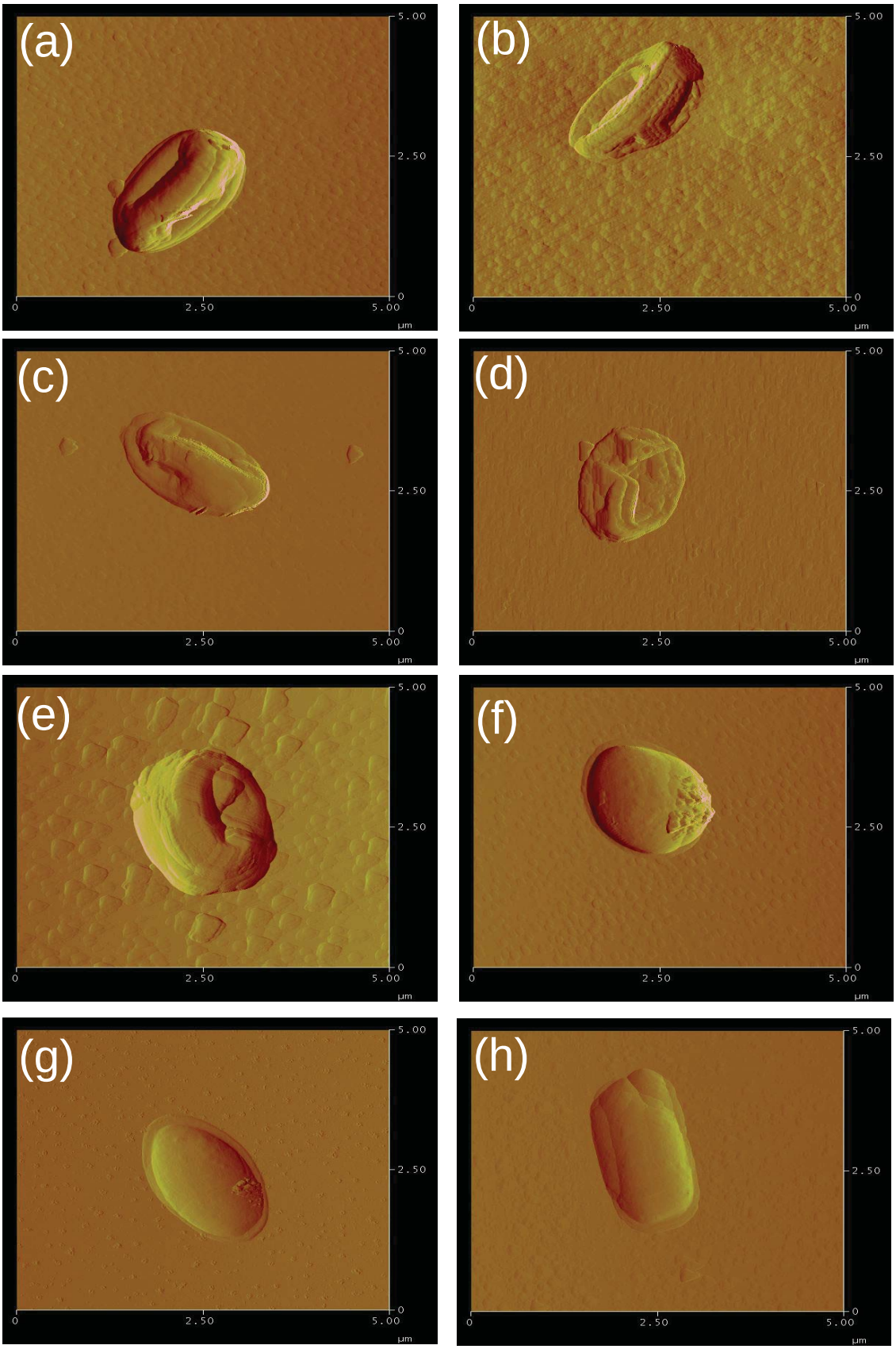

Figure 4.15. AFM images of spores produced on (a–d) PDA and (e–h) MEA

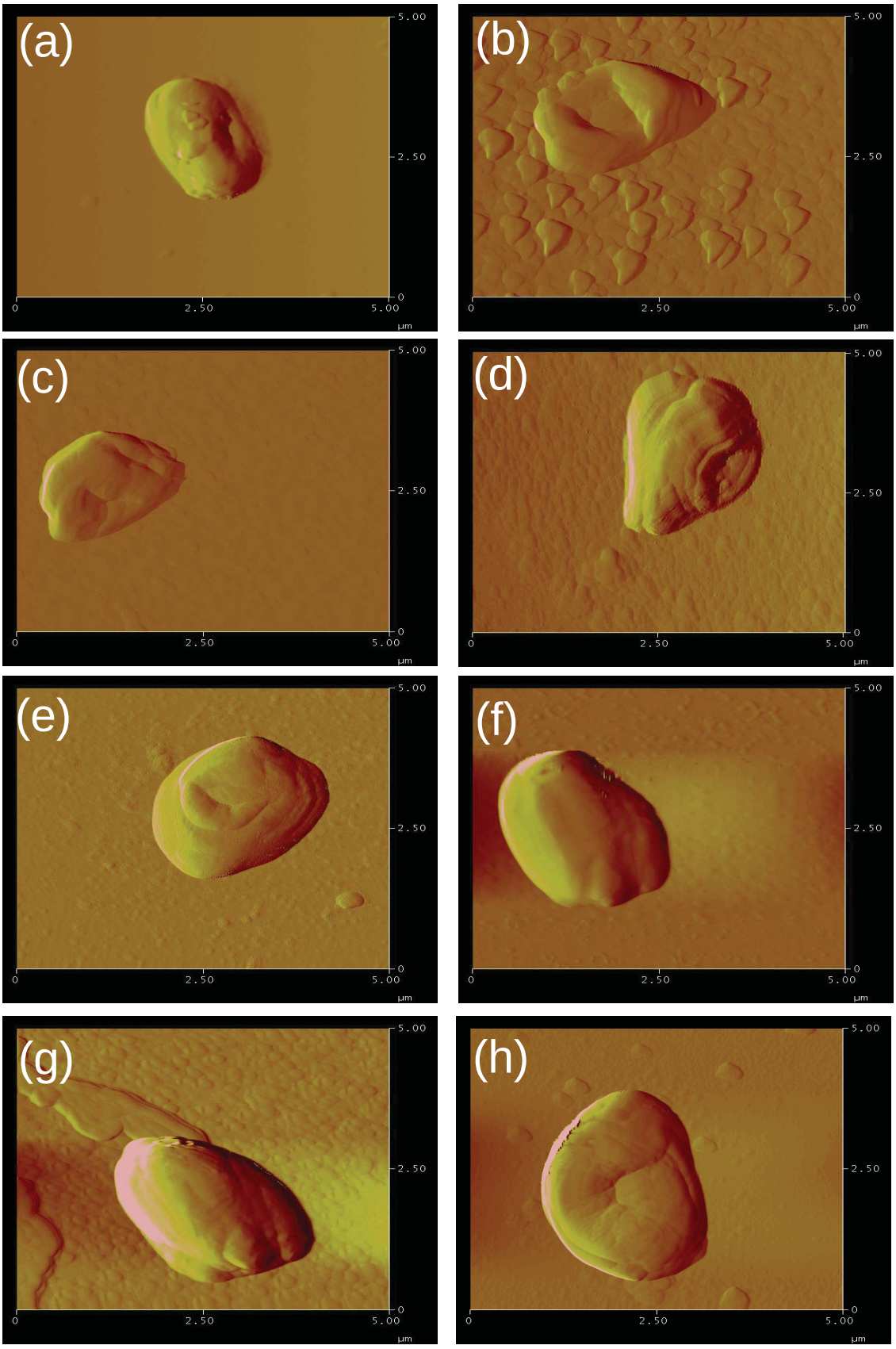

Figure 4.16. AFM images of spores grown on (a–d) BATA and (e–h) BAA

Changes in spore morphology after wet–heat treatment at 90 °C for 60 min were also imaged using AFM technique. The results were also similar to the SEM images. In fact, wet–heat treated spores are collapsed most probably due to the dehydration (Fig.4.17–4.20). Heat–sensitive spores were found to contain thinner coat layers and cortex regions than do heat–resistant *C. sporogenes* spores by transmission electron microscopy (TEM) (Mah et al., 2008b). They found that the spore coat's multilayered structure composed of highly cross–linked polypeptides may function (at least partially) to protect spores against damage by heat. Therefore, the analysis of spore components using TEM will be more useful to obtain detailed information on both outer and inner structural changes and their functions on heat resistance of spores.

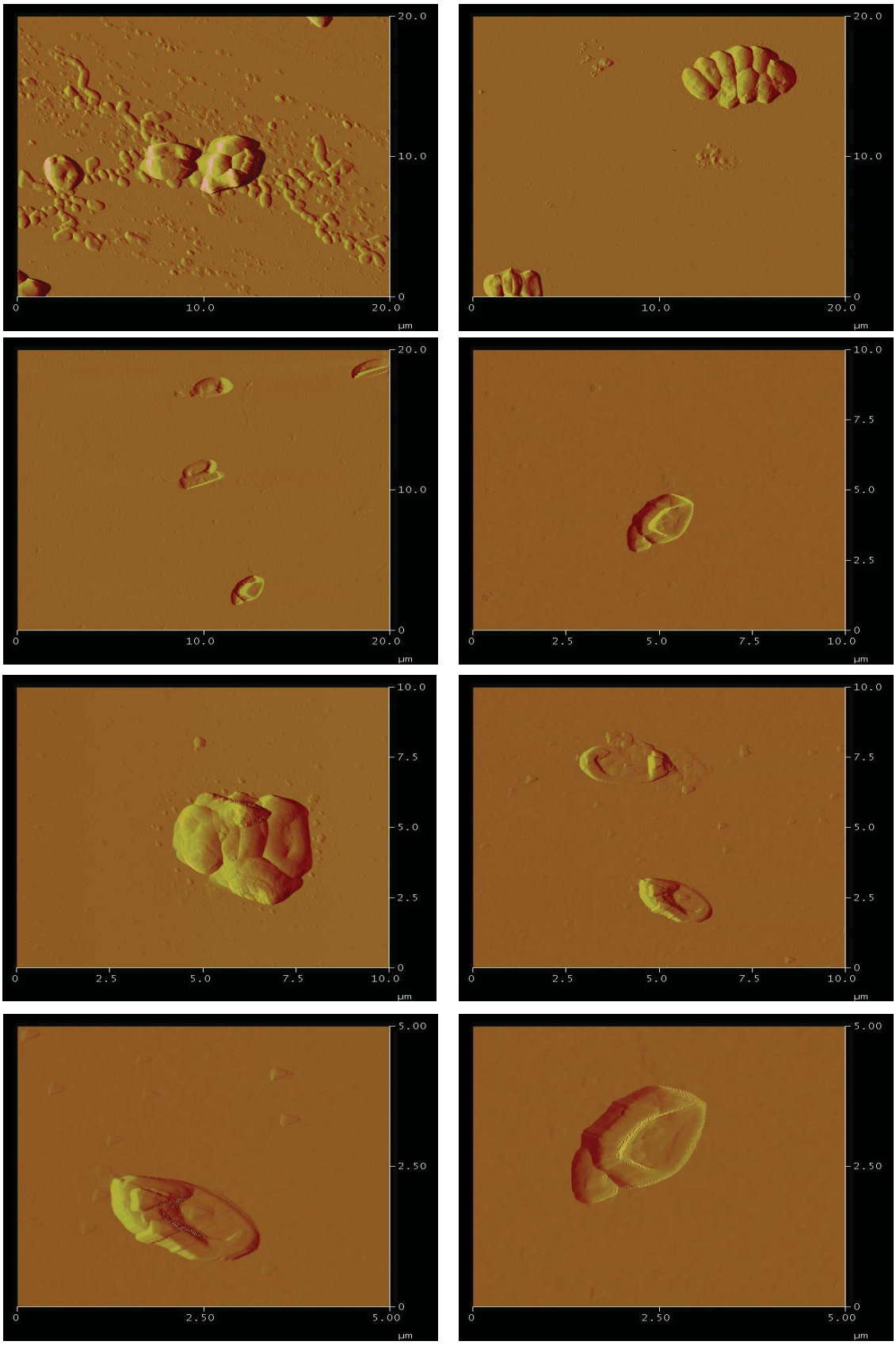

Figure 4.17. AFM images of wet–heated spores from PDA at 90 °C for 60 min

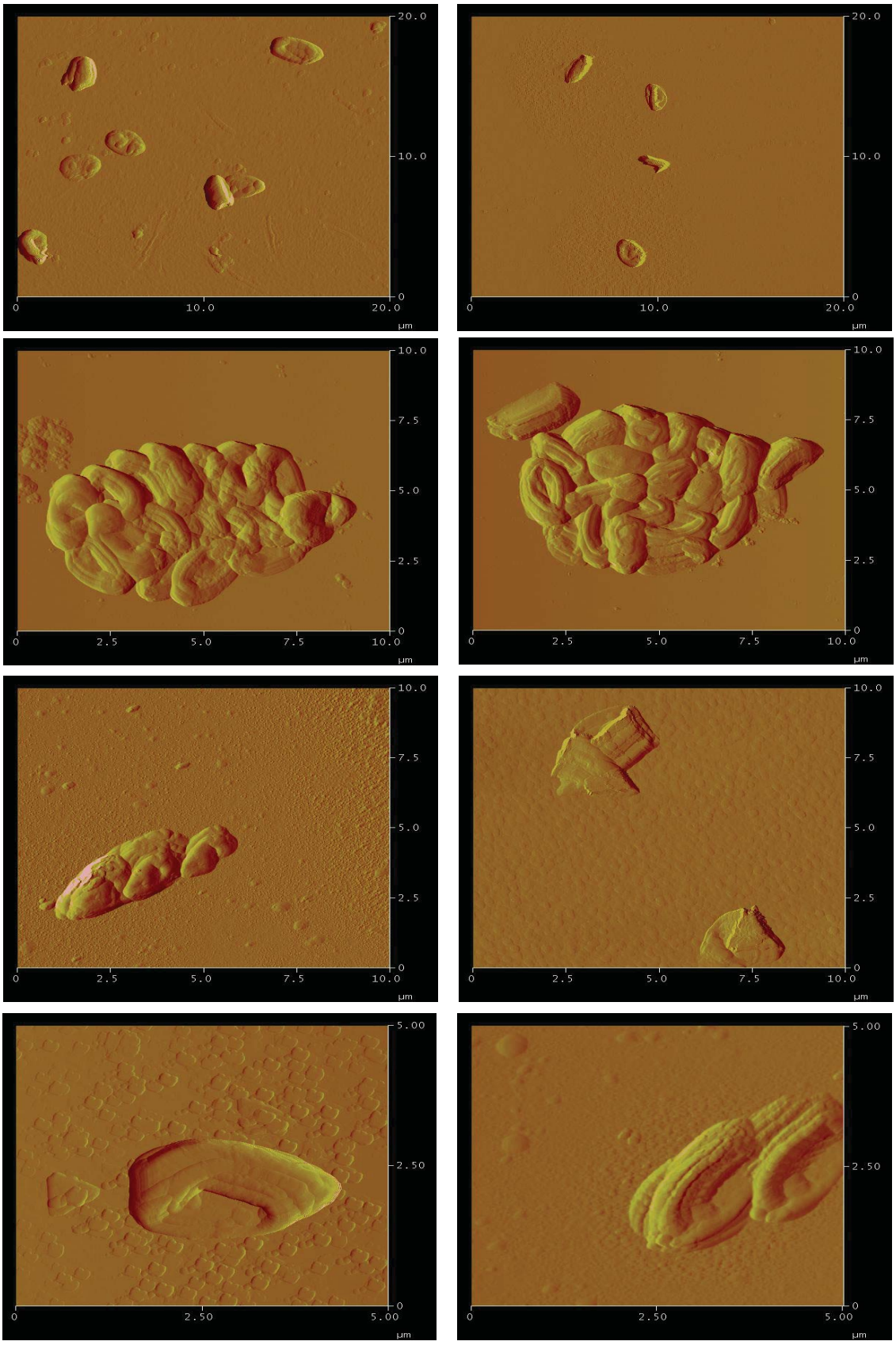

Figure 4.18. AFM images of wet–heated spores from BATA at 90 °C for 60 min

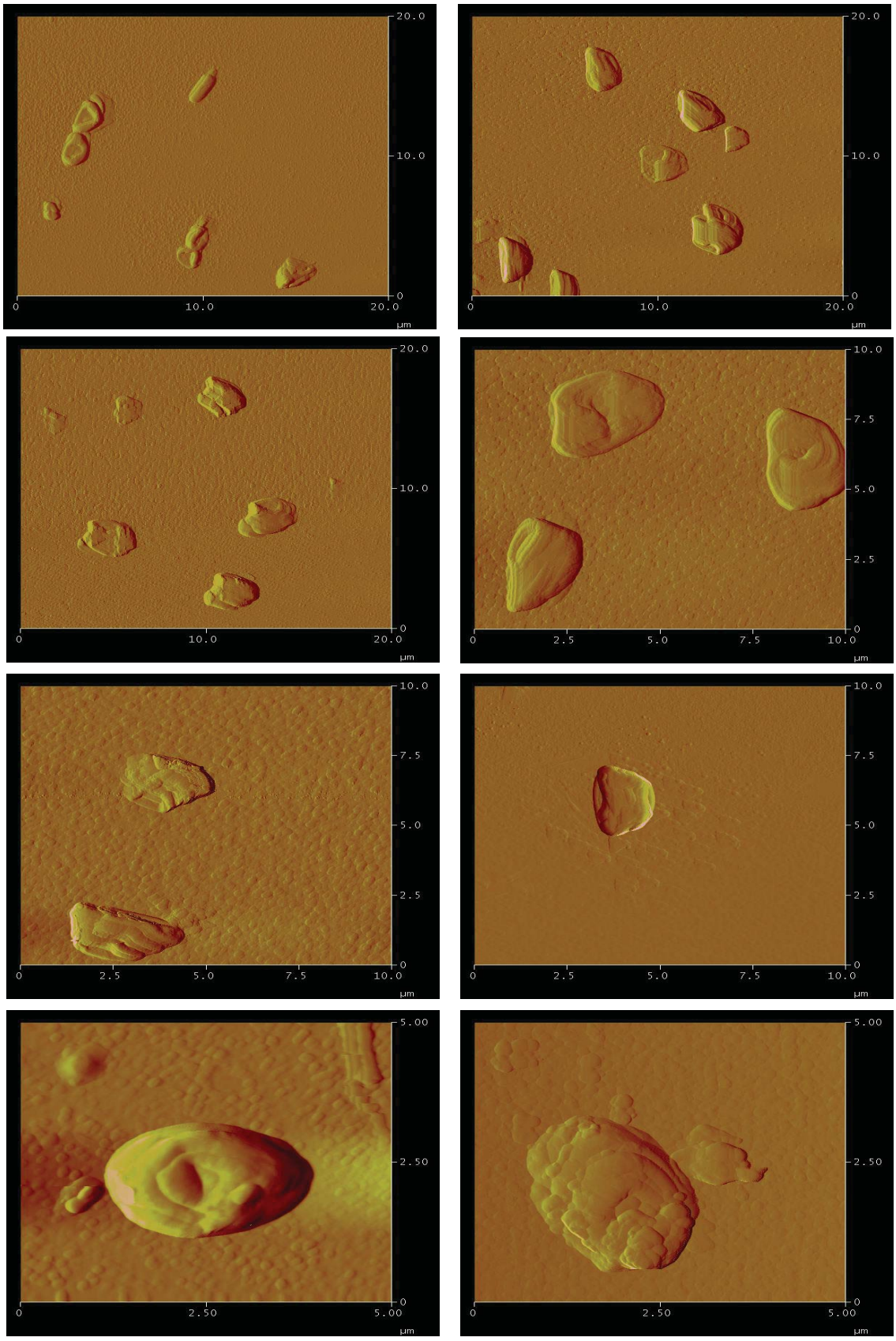

Figure 4.19. AFM images of wet–heated spores from BAA at 90 °C for 60 min

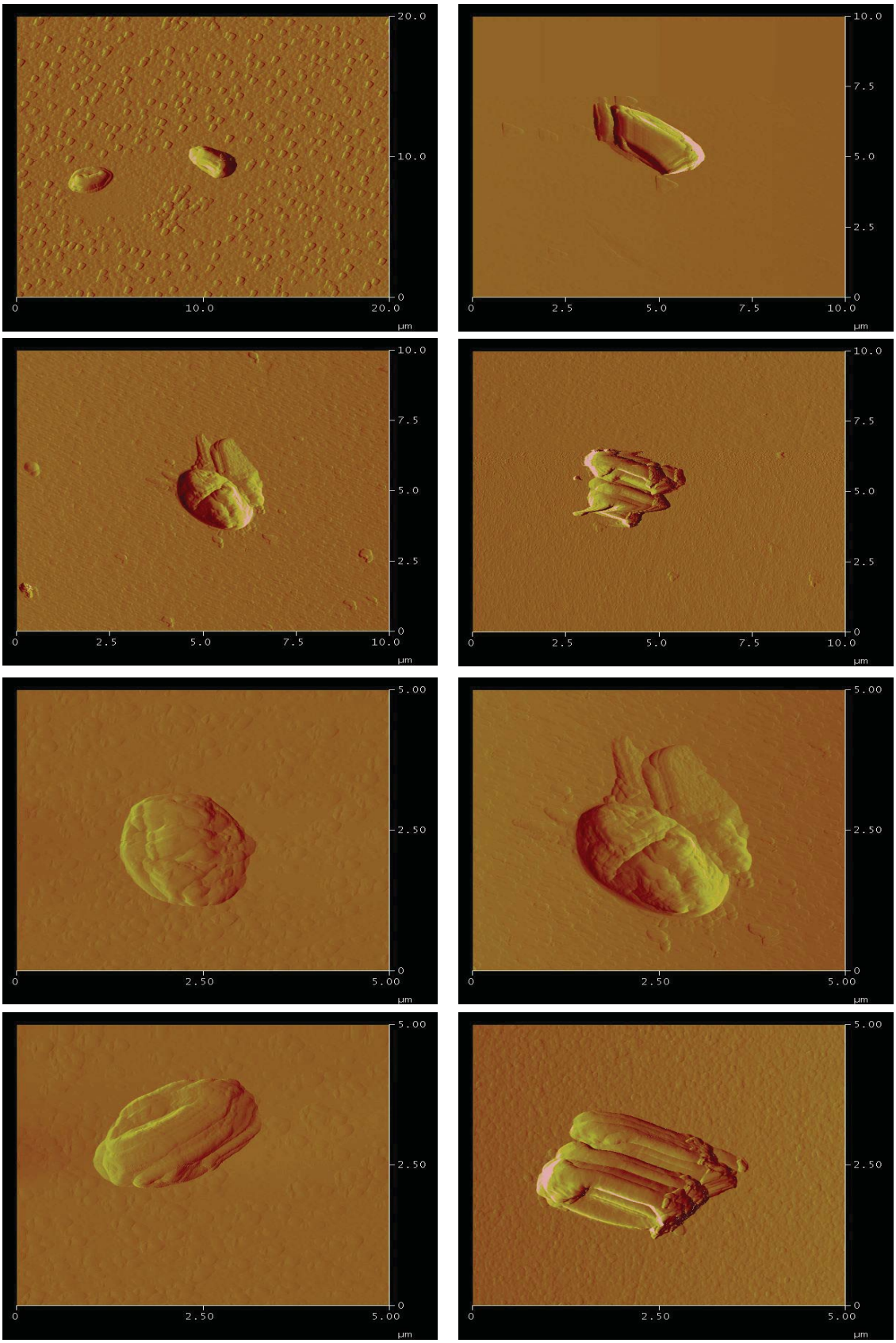

Figure 4.20. AFM images of wet–heated spores from MEA at 90 °C for 60 min

4.1.7. Leakage of Intracellular Components During Wet–Heat

Leakage of intracellular components (nucleic acids, proteins, amino acids and enzymes) indicates membrane damage (Chang et al., 2009). Therefore, OD_{260} and OD_{280} measurements of the materials leaked out from the spores are used to estimate the effects of the treatment on permeability of spore inner membrane (Zhang et al., 2012). Spores have significant amounts of protein in the spore coat and nucleoid structures (Driks, 1999). The presence of proteins in the spore suspension indicates the rupture of spore coat. Similarly, the presence of nucleic acid indicates the release of core components upon damage to spore inner membrane (Kim et al., 2009). The leakage of DNA (OD_{260}) and protein (OD_{280}) after wet–heat treatment is shown in Table 4.7. Heat shock treatment has been shown to increase absorbance values at 260 and 280 nm (Chang et al., 2009), therefore leakage of intracellular components was determined using non–heat activated spores.

Table 4.7. Leakage of cellular materials after wet–heat treatment at 90 °C

Absorbance (280 nm)				
Time (min)	PDA	BATA	BAA	MEA
0	$0.144\pm0.003^{Db\dagger}$	0.128 ± 0.006^{Db}	0.095 ± 0.004^{Db}	0.469 ± 0.058^{Ba}
15	0.285 ± 0.007^{Cb}	0.197 ± 0.003^{Cc}	0.145 ± 0.005^{Cd}	0.791 ± 0.006^{Aa}
30	0.295 ± 0.005^{Cb}	0.211 ± 0.004^{BCc}	0.161 ± 0.020^{BCd}	0.836 ± 0.022^{Aa}
45	0.306 ± 0.004^{BCb}	0.224 ± 0.005^{Bc}	0.179 ± 0.013^{Bc}	0.874 ± 0.035^{Aa}
60	0.320 ± 0.001^{Bb}	0.230 ± 0.008^{Bbc}	0.185 ± 0.004^{Bc}	0.923 ± 0.062^{Aa}
Autoclaved	0.374 ± 0.015^{Ab}	0.281 ± 0.018^{Ab}	0.221 ± 0.011^{Ab}	0.871 ± 0.158^{Aa}
Absorbance (260 nm)				
Time (min)	PDA	BATA	BAA	MEA
0	$0.079\pm0.013^{Cb\dagger}$	0.056 ± 0.015^{Bb}	0.043 ± 0.017^{Bb}	0.328 ± 0.053^{Ba}
15	0.158 ± 0.018^{Bb}	0.091 ± 0.015^{ABc}	0.075 ± 0.005^{Ac}	0.547 ± 0.044^{Aa}
30	0.177 ± 0.019^{ABb}	0.105 ± 0.019^{ABc}	0.086 ± 0.007^{Ac}	0.599 ± 0.025^{Aa}
45	0.184 ± 0.018^{ABb}	0.111 ± 0.024^{ABc}	0.091 ± 0.004^{Ac}	0.629 ± 0.037^{Aa}
60	0.211 ± 0.016^{Ab}	0.124 ± 0.016^{Ac}	0.099 ± 0.005^{Ac}	0.663 ± 0.039^{Aa}
Autoclaved	0.203 ± 0.019^{ABb}	0.116 ± 0.039^{ABb}	0.097 ± 0.008^{Ab}	0.630 ± 0.112^{Aa}

†Data were expressed as the mean±standard deviation from three experiments
‡Values with different capital letters in the same column are significantly different ($p<0.05$)
‡‡Values with different lower case in the same row are significantly different ($p<0.05$)

There were significant differences between the heating time and protein absorbance values ($p<0.05$). OD_{280} values of the materials from spores were significantly different from untreated controls at zero time ($p<0.05$). Protein absorbance values of treated spores from PDA, BATA and BAA media were significantly different

from the autoclaved samples (p<0.05). Interestingly, OD_{280} values of the materials after 60 min from spores produced on MEA was higher than that of autoclaved spores most probably due to seriously damaged inner membrane after wet–heat as previously well documented (Zhang et al., 2012). Also, OD_{260} values of the materials were significantly different from untreated controls (p<0.05). Based on the results, it can be concluded that the release of both proteins and nucleic acids into the spore suspension occurs due to the disruption of the spore coat and inner membrane during wet–heat treatment at 90 °C.

4.1.8. Determination of Elemental Composition on Spore Surface

Spores contain high amounts of Ca within the spore core and other elements (e.g.Mn) that are used to distinguish spores from other materials such as inorganic particles (Laue and Fulda, 2013). After drying spores, X–ray spectra of untreated individual spores were analysed using SEM–EDX (Fig.4.21). The spectra revealed the presence of Na, Mg, Al, P, S, K, Ca, Mn and Cu elements peaks (%).

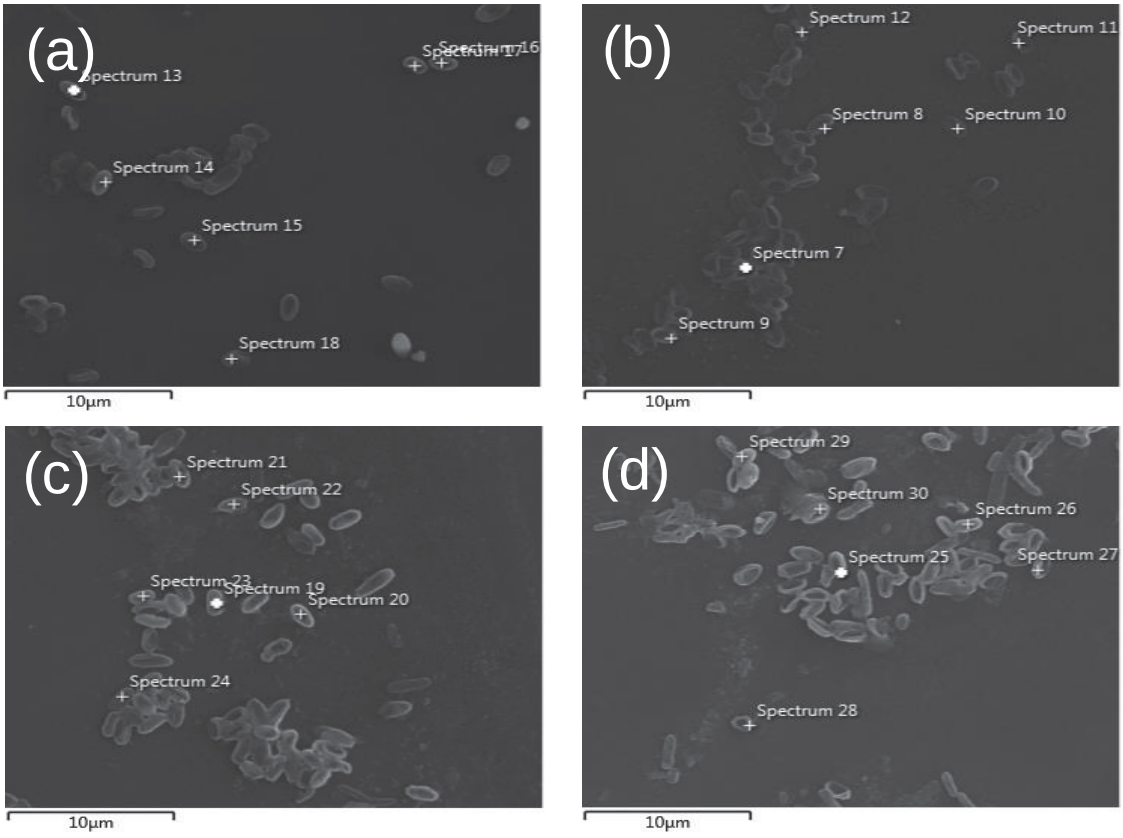

Figure 4.21. EDX spectra of spores from (a) PDA (b) BATA (c) BAA and (d) MEA

As seen in Table 4.8, the relative proportions of Na, Mg and Ca were higher than the other elements found on the surface of *A. acidoterrestris* spores. In addition, Mn and Cu levels were absent or at very low level.

Table 4.8. Elemental composition of spores by SEM–EDX (% relative proportion)

Spectrum	Na	Mg	Al	P	S	K	Ca	Mn	Cu
				PDA					
S13	42.58	16.56	5.82	1.59	2.33	0.92	30.20	0.00	0.00
S14	37.79	17.14	6.29	1.87	1.64	0.46	33.71	0.00	1.09
S15	40.14	17.28	6.42	2.27	1.79	0.73	31.36	0.00	0.00
S16	40.81	17.01	6.10	1.63	1.43	0.61	32.42	0.00	0.00
S17	43.88	17.34	4.95	1.65	1.55	0.99	29.31	0.23	0.00
S18	41.13	16.89	6.76	2.00	2.06	1.21	29.96	0.00	0.00
				BATA					
S7	40.17	16.45	5.02	2.70	1.86	1.30	32.06	0.22	0.23
S8	35.77	16.94	5.75	1.67	1.84	1.51	36.20	0.00	0.32
S9	40.58	16.79	5.25	2.21	1.93	0.93	32.31	0.00	0.00
S10	38.82	16.79	6.58	2.06	1.93	1.12	32.70	0.00	0.00
S11	37.58	17.45	6.85	1.55	0.88	0.89	33.98	0.37	0.34
S12	37.17	18.11	5.94	2.21	1.51	1.51	33.60	0.00	0.24
				BAA					
S19	46.53	14.20	4.70	2.23	1.67	1.04	29.41	0.20	0.00
S20	47.42	15.29	4.99	1.65	1.60	0.55	28.43	0.00	0.07
S21	43.13	16.26	5.41	1.91	1.91	0.96	29.50	0.33	0.57
S22	45.42	14.94	5.48	2.46	1.83	0.63	29.09	0.15	0.00
S23	40.58	15.87	5.77	2.99	1.72	0.72	31.99	0.08	0.28
S24	38.81	15.94	6.70	1.68	1.44	1.41	34.02	0.00	0.00
				MEA					
S25	42.40	17.43	5.94	1.58	2.83	0.81	28.61	0.41	0.00
S26	42.25	15.84	5.66	2.98	1.52	0.68	30.35	0.66	0.07
S27	47.70	15.31	5.92	1.74	1.70	1.18	25.88	0.00	1.17
S28	42.57	16.34	6.14	1.98	0.71	1.35	30.37	0.17	0.36
S29	44.28	15.07	5.74	1.90	2.06	1.12	29.53	0.31	0.00
S30	51.42	13.53	4.70	1.84	1.03	0.60	26.23	0.41	0.23

X–ray microanalysis in conjunction with SEM has been used to compare the Ca content among different species or to follow the Ca release during spore germination process (Bassi et al., 2009; Hintze and Nicholson, 2010). In a recent study, X–ray spectroscopy in conjunction with TEM was used to discriminate spores from other particles in environmental samples using the elemental composition of spores as a marker (Laue and Fulda, 2013). They revealed the presence of Ca, P and S elements regularly in several *Bacillus* sp. and *Clostridium* sp. spores. With all strains tested and preparations, the Ca peak was about the same or larger than P and S peaks. P and S peaks were also found as the smallest. Therefore, they concluded that the detection of P and S elements at similar or lower count rates than the Ca is necessary for a reliable spore discrimination from other particles having similar morphology.

Scanning–transmission electron microscopy (S–TEM) and X–ray spectroscopy using thin cryosections could reveal the main element composition of spores and their distribution among the different structures (Laue and Fulda, 2013). Thus, the use of ultrathin sections may be more useful then the other techniques to reveal the elemental distribution on spore structures.

4.2. Total Phenol Content

Phenolic compounds play an important role in the antimicrobial activity. Therefore, total phenol content (TPC) of the extracts and fruit juices was determined with the Folin–Ciocalteu colorimetric method and the results were expressed as Gallic acid Equivalents (GAE) using the calibration curve in Appendix B (Fig.B2).

Table 4.9. Total phenol content of the extracts and fruit juices

Sample	Total phenol content
	mg GAE/mL extract
Grape seed extract	21.24±0.36[†]
	mg GAE/g extract
Pomegranate extract	573±0.34
	mg GAE/L juice
Apple juice	264.81±11.30
Pomegranate juice	4004.82±94.14
80% pomegranate–apple blend juice	2432.31± 34.01
40% pomegranate–apple blend juice	1577.76± 52.29
20% pomegranate–apple blend juice	894.15± 25.38
10% pomegranate–apple blend juice	672.09± 14.66

[†]Data were expressed as the mean±standard deviation from three or more experiments

The TPC of GSE was found as 21.24±0.36 mg GAE/mL extract (Table 4.9). The TPC of the GSE obtained from juice and wine was reported to be 6.04±0.69 and 2.41±0.34 mg GAE/mL, respectively (Bijak et al., 2011; Delgado Adámez et al., 2012). The differences among the levels of phenolic compounds in the seeds from different varieties arise from the factors including climate, fruit ripeness degree, size and variety (Rockenbach et al., 2011). In fact, GSE is shown as a rich source of polyphenols such as phenolic acids, flavonoids (anthocyanidins, flavanols such as catechin and proanthocyanidins, flavanones, flavones, flavonols and isoflavones), stilbenes and lignans (Manach et al., 2004).

The TPC of pomegranate extract standardized to contain 70% total polyphenols (Patel et al., 2008) was found as 573 mg GAE/g extract in this study. The TPC in the pomegranate pulp and peel extracts varied from 11.62±0.63 to 21.03±1.51 and 98.28±4.81 to 226.56±18.98 mg GAE/g extract, respectively among nine different pomegranate cultivars (Shams Ardekani et al., 2011). Pomegranate peels have been found to contain the highest total polyphenol, flavonoid and tannin contents (Teixeira da Silva et al., 2013).

Polyphenols are the main components that determine the apple juice quality and extraction efficiency (Grimi et al., 2011). TPC of the apple juice was 264.81±11.30 mg GAE/L juice and this finding was in good agreement with previously published data (Caminiti et al., 2012). They found the phenol content of the untreated reconstituted apple juice as 299.7±12.03 mg/L juice. Overall, the variations in polyphenol content may be due to differences in varieties, climate, ripeness, and extraction method (Vasco et al., 2008). During processing of apples into juice, the loss of 50–90% of the polyphenols occurs. Therefore, the majority of commercial clear apple juices contain polyphenols in minor amounts (Kolniak-Ostek et al., 2013).

TPC of the pomegranate juice (4004.82 ±94.14 mg GAE/L) was found higher than apple juice. Pomegranate juice was shown to contain a 3–fold higher antioxidant activity than red wine or green tea and 2–, 6– and 8–fold higher levels than those detected in grape/cranberry, grapefruit, and orange juice, respectively (Ferrara et al., 2011). Most of the phenols in pomegranate juice are derived from the non–edible parts such as peels and inner lamellas. Among the commonly consumed fruit juices, pomegranate juice contains the highest amounts of ellagitannins contributing to its health beneficial effects. Punicalagin is a part of a family of the ellagitannins. It is unique to pomegranate juice and the most abundant soluble compound in peels. This compound is responsible for more than 50% of the juice's antioxidant activity. Pomegranate juice extracted from whole fruit also contains high amounts of gallagic, ellagic and gallic acids that have high antioxidant and anti–proliferative activities, and also health benefits indicated by *in vivo* and *in vitro* studies (Orgil et al., 2014). Polyphenols found in the pomegranate can alter bacterial populations in the mixed cultures and also bacteria can metabolize these polyphenols into smaller metabolites like urolithin (Bialonska et al., 2010). The phenolic content of pomegranate juices has been reported to be between 100.86 and 260.2 mg GAE/100 mL in commercial juices produced in Turkey (Tezcan et al., 2009). In another study, the TPC of pomegranate

juice was reported in the range of 784.4 to 1551.5 mg GAE/L depending on the cultivar (Gozlekci et al., 2011). In addition, the concentrations of phenolic substances vary depending on the different parts of the fruit. In fact, the differences between TPC are related to the method of fruit processing because some extraction methods can involve rubbing the internal part of the rind, and may contribute to the extraction of the phenolic contents (Vázquez-Araújo et al., 2011). Moreover, the TPC of the blend juices ranged from 672.09 to 2432.31 mg GAE/L juice (Table 4.9) and a strong correlation was obtained between the TPC and the concentration of pomegranate juice in the blend juice samples (R^2>0.956).

4.3. Antioxidant Activity

Antioxidant properties were determined as radical scavenging activity (% inhibition of DPPH) and ferric reducing/antioxidant power (FRAP). FRAP might give information about the capacity of an antioxidant to prevent the reactive radical species from reaching lipoproteins, polyunsaturated fatty acids, DNA, aminoacids, proteins and sugars in biological and food systems. DPPH is relatively stable organic radical commonly used to determine the antioxidant potential of compounds and different plant extracts. Antioxidants can deactivate or scavenge stable free DPPH radical in two ways. These are the reduction via electron transfer or by hydrogen atom transfer that may occur also in parallel, and steric accessibility is one of the major determinants of the reaction (Katalinić et al., 2010).

FRAP values were calculated according to the calibration curve given in Appendix B (Fig.B3). The pomegranate (1%, w/v) and grape seed extracts (1%, v/v) also had high power reduction of 65.70 and 110.90 mmole TE/L (Table 4.10). The highest antioxidant potential of GSE is primarily due to flavonoids that can perform scavenging activity on free radicals (superoxide, hydroxyl and DPPH), metal chelating properties, reduction of hydroperoxide formation and their effects on cell signaling pathways and gene expression. The presence of the functional –OH group in the structure and its position on the ring of the flavanoid determine the antioxidant capacity. Among the different parts of grape plant, grape seeds exhibit the highest antioxidant activity followed by the skin and the flesh (Perumalla and Hettiarachchy, 2011). The

reducing power in pomegranate extract was reported to be 4.7–fold higher than seed extract and 10.5–fold higher than juice extract (Teixeira da Silva et al., 2013).

Pomegranate juice displayed the highest antioxidant capacity according to FRAP assay among juice samples (40.05 mM TE). It is known that pomegranate juice has the highest total anthocyanin and tannin contents, and acidity which significantly affect antioxidant activity (Teixeira da Silva et al., 2013). Since punicalagin is water soluble, commercial pomegranate juice obtained by pressing this fruit contains significant amounts of this antioxidant (Patel et al., 2008). On the other hand, apple juice had lower antioxidant potential (0.93 mM TE). The reducing power of blend juices also ranged from 4.65 to 35.31 mM TE (Table 4.10). The obtained FRAP values were strongly correlated with the concentration of pomegranate juice in the apple juice (R^2>0.993).

Table 4.10. FRAP and DPPH–radical scavenging activities of extracts and juices

Sample	**FRAP mmole/L Trolox**	**DPPH % inhibition**
Grape seed extract (1%, v/v)	110.90±5.05	56.88±1.45
Pomegranate extract (1%, w/v)	65.70±1.99	94.09±0.09
Apple juice	0.93±0.03^{F}	19.01±1.16^{C}
Pomegranate juice	40.05±1.05^{A}	80.41±0.06^{B}
80% pomegranate–apple blend juice	35.31±1.93^{B}	83.81±0.94^{B}
40% pomegranate–apple blend juice	16.85±1.15^{C}	88.79±1.91^{A}
20% pomegranate–apple blend juice	8.21±0.63^{D}	89.74±1.55^{A}
10% pomegranate–apple blend juice	4.65±0.07^{E}	83.91±0.94^{B}

†Data were expressed as the mean±standard deviation from three experiments
‡Values with different capital letters in the same column are significantly different among juice samples (p<0.05)

The DPPH assay revealed that the apple juice had the lowest activity (19.01% inhibition of DPPH). However, the pomegranate juice showed 80.41% inhibition. On the other hand, the addition of pomegranate juice into the apple juice resulted in high scavenging activity ranging from 83.81% to 89.79% inhibition. As seen in Table 4.10, %inhibition of pomegranate juice, 80% and 10% blend juices were not statistically different. Whereas, 20% and 40% blend juices yielded the highest inhibition with 89.74% and 88.79%, respectively. In another study, the addition of black chokeberry concentrate to lemon juice enhanced its antioxidant activity against the most of the tested reactive species. The blend juice showed higher antioxidant activity than individual sources. It was suggested that the polyphenol content was not the only reason

for the antioxidant capacity, at the same time, the quality and the interactions between them are important similar to our findings (Gironés-Vilaplana et al., 2012).

The %inhibition of pomegranate extract (1%, w/v) was 94.09 and higher than that of the GSE (1%, v/v) and pomegranate juice. In the related literature, the DPPH scavenging activity values in pomegranate extract were 23.4–fold higher than the juice extracts, and the seed extracts had 2.3–fold higher than juice extract (Teixeira da Silva et al., 2013).

4.4. Antimicrobial Activity on Vegetative Cells

4.4.1. Antimicrobial Activity of Grape Seed Extract

The concentration of GSE in the apple juice ranged from 0 to 14.4% (v/v). The results showed that high concentrations of GSE (7.2% and 14.4%) reduced the cells immediately after inoculation; therefore it was not possible to estimate the exact log reduction. However, lower concentrations (0.23–3.6%, v/v) could be used for modeling (Table 4.11). GSE caused significant reductions in the cell counts but not completely eliminate the microbial population under the tested conditions. The highest reduction (4.63 log CFU/mL) was observed in apple juice with 3.6% concentration of GSE after 336 h. In addition, 0.23, 0.45, 0.9 and 1.8% (v/v) GSE reduced the log number of cells approximately 3.14, 3.55, 3.8, and 4.1, respectively. The lowest concentrations (0.06 and 0.12%) decreased the counts to a certain level and then the number of cells started to increase again. On the other hand, the control cells which were grown in apple juice without GSE (0%, v/v) attained approximately 7.09 log CFU/mL after 24 h incubation in apple juice (Table 4.11).

In food applications, GSE is used at fairly low concentrations (0.01–1%) (Perumalla and Hettiarachchy, 2011). The antimicrobial effect of GSE has been reported previously on different organisms (Delgado Adámez et al., 2012; Jayaprakasha et al., 2003; Kao et al., 2010; Rhodes et al., 2006; Silván et al., 2013; Sivarooban et al., 2008). However, no data have been published on the antimicrobial activities of GSE against spoilage caused by *A. acidoterrestris* in fruit juices. Takahashi et al. (2004) tested the antimicrobial activity of leaf extracts and flavanoids from *Eucalyptus* spp. and

obtained the minimum inhibitory concentrations ranging from 7.8 g/L (*E. globosa*) to 31 g/L (*E. saligna*).

Table 4.11. Counts of cells (log CFU/mL) in apple juice with GSE at 37 °C

Treatment (h)	0.23%	0.45%	0.9%	1.8%	3.6%	Control
0	5.45(0.12)^{Acd}	5.47(0.06)^{Ac}	5.69(0.05)^{Ab}	6.01(0.03)^{Aa}	6.01(0.01)^{Aa}	5.27(0.05)^{Dd}
24	4.27(0.13)^{Bb}	4.16(0.04)^{Bb}	4.07(0.12)^{Bb}	4.00(0.07)^{Bb}	4.01(0.08)^{Bb}	7.09(0.33)^{ABa}
48	4.19(0.06)^{Bb}	3.90(0.04)^{BCbc}	3.75(0.16)^{Bc}	3.64(0.12)^{Bc}	3.79(0.12)^{Bc}	6.99(0.14)^{ABa}
72	3.88(0.06)^{Bb}	3.48(0.19)^{Cbc}	3.31(0.01)^{Cc}	3.19(0.08)^{Cc}	3.28(0.14)^{Bc}	6.87(0.25)^{ABa}
168	3.22(0.23)^{Cb}	2.43(0.19)^{Dc}	2.29(0.11)^{Dc}	2.28(0.16)^{Dc}	2.32(0.13)^{Cc}	7.23(0.14)^{Aa}
240	2.98(0.11)^{Cb}	2.33(0.20)^{DEbc}	2.01(0.20)^{DEc}	2.11(0.10)^{Dc}	1.65(0.58)^{CDc}	6.14(0.13)^{Ca}
336	2.31(0.24)^{Db}	1.92(0.14)^{Ebc}	1.89(0.14)^{Ebc}	1.91(0.27)^{Dbc}	1.38(0.43)^{Dc}	6.66(0.06)^{BCa}

† : The experiments were repeated three times, and data are expressed as mean ± standard deviation
†† : Values with different capital letters in the same column are significantly different (p<0.05)
‡: Values with different lower case in the same row are significantly different (p<0.05)

In the present study, the growth of *A. acidoterrestris* vegetative cells was inhibited in the apple juice with GSE when compared to the control. There might be three reasons for higher cell reductions of *A. acidoterrestris* in the apple juice with GSE. Firstly, Gram–positive bacteria are more sensitive to natural extracts than Gram–negative due to the absence of lipopolysaccharide layer that might reduce the sensitivity (Delgado Adámez et al., 2012). Second, the susceptibility of bacteria to plant extracts increases as the pH of the food decreases (Gutierrez et al., 2009). Also, it might be the susceptibility of *A. acidoterrestris* to phenolic compounds found in GSE.

4.4.1.1. Modeling Inactivation Data

The use of plant extracts with known antimicrobial properties is focused in food preservation. Most of the literature presents inactivation data from model foods or laboratory media (Negi, 2012), but natural extracts are less active in food systems due to the strong interaction between extracts and food components (Kim et al., 2004). In addition, the extrapolation of results obtained from *in vitro* experiments with laboratory media to food products is complicated as foods are complex, multicomponent systems consisting of different interconnecting microenvironments (Negi, 2012). Therefore, the

antimicrobial activity of GSE was carried out in the apple juice rather than model fruit juice or broth system to see the interactions between GSE and fruit juice components. The survival data of *A. acidoterrestris* cells in the presence of GSE is presented in Fig.4.22. As the data did not follow a first–order kinetics, two mathematical models, the log–linear tail and the Weibull models were used to model the inactivation as a function of time (h).

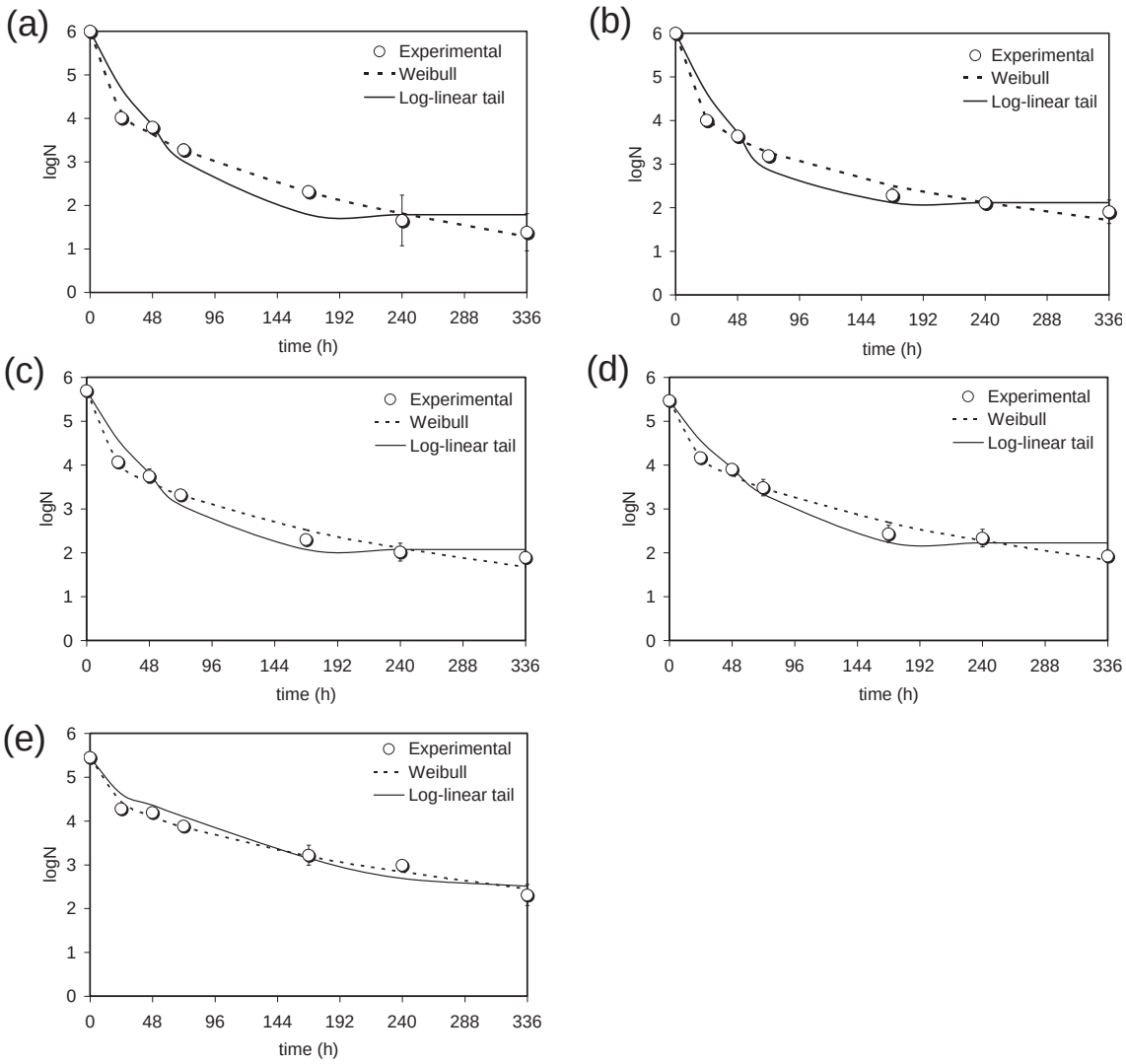

Figure 4.22. Survival curves of vegetative cells in the apple juice with GSE (a) 3.6% (b) 1.8% (c) 0.9% (d) 0.45% (e) 0.23%

The parameters of both models as well as the statistical indices are shown in Table 4.12. Visual evaluation of the fitted curves (Fig.4.22) and statistical indices (lower RMSE and higher R^2 values) in Table 4.12 showed that the Weibull model fitted the experimental data better than the log–linear tail model. The RMSE values of the

Weibull model ranged from 0.138 to 0.175. In addition, the Weibull model showed higher R^2 values (>0.988). For all treatments, the shape parameter (β) was smaller than 0.5 indicating upward concavity. This tailing behaviour has been observed under thermal and non–thermal treatments (Hereu et al., 2012; Mañas and Pagán, 2005; Patterson, 2005). As mentioned earlier, the scale parameter (δ) represents the first decimal reduction. In general, the time for 4–log reduction (t_{4D}) of the Weibull model is a better parameter than δ for evaluation or comparison of the susceptibility of pathogens to environmental stress (Yang et al., 2013). For this reason, t_{4D} values for the Weibull model were calculated. It was observed that as the concentration increased, t_{4D} values decreased during storage. As an example, at the concentration of 3.6% GSE, t_{4D} was found as 204.1 h. At the lowest concentration (0.23%), it increased to 675.9 h. In previous studies, the Weibull model has also been used to estimate bacterial inactivation by the use of other antimicrobial compounds (Bialka et al., 2008; Chun et al., 2009; De Oliveira et al., 2013; Gonzalez et al., 2009; San Martín et al., 2007).

Table 4.12. Parameters of the Weibull and the log–linear tail models

GSE (%)	**Weibull**				**Log–linear tail**		
	δ (h)	β	t_{4D}[a](h)	Statistical indices	$k_{max}(h^{-1})$	$\log N_{res}$ (CFU/mL)	Statistical indices
0.23	24.91	0.42	675.9	RMSE[b]=0.138 R^2[c]=0.988 R^2–adj[d]=0.982	0.03	2.49	RMSE=0.405 R^2=0.897 R^2–adj=0.845
0.45	12.12	0.39	423.9	RMSE=0.156 R^2=0.990 R^2–adj=0.984	0.06	2.22	RMSE=0.314 R^2=0.958 R^2–adj=0.937
0.9	5.63	0.34	332.1	RMSE=0.175 R^2=0.989 R^2–adj=0.984	0.07	2.07	RMSE=0.361 R^2=0.953 R^2–adj=0.930
1.8	2.34	0.29	278.8	RMSE=0.154 R^2=0.992 R^2–adj=0.989	0.09	2.12	RMSE=0.442 R^2=0.937 R^2–adj=0.905
3.6	4.34	0.36	204.1	RMSE[b]=0.142 R^2[c]=0.995 R^2[d]–adj=0.992	0.08	1.79	RMSE=0.550 R^2=0.922 R^2–adj=0.882

[a]: Predicted time for 4–log reduction calculated by the Weibull model
[b]: Root mean square error
[c]: Coefficient of determinaton
[d]: Adjusted regression coefficient

The R^2 and RMSE values obtained by fitting the log–linear tail model are also given in Table 4.12. The R^2 and RMSE values were in the ranges of 0.897–0.958 and 0.314–0.550, respectively. The residual population density ($\log N_{res}$) was 2.49, 2.22,

2.07, 2.12 and 1.79 CFU/mL for the concentrations of 0.23%, 0.45%, 0.9%, 1.8% and 3.6% (v/v), respectively.

In the present study, the stability of active components in GSE may be lost resulting in lower inactivation rates through the end of incubation period. In a similar study (Kao et al., 2010), the inhibitory effect of GSE has been suggested to be affected by the nature of the food and prolong incubation. These researchers also suggested that the reverse of *Staphylococcus aureus* growth inhibition might occur due to the instability of active compounds present in GSE at higher temperatures.

4.4.1.2. Scanning Electron Microscopy

Vegetative *A. acidoterrestris* cells are rod–shaped, intact and show smooth surfaces (Fig.4.23).

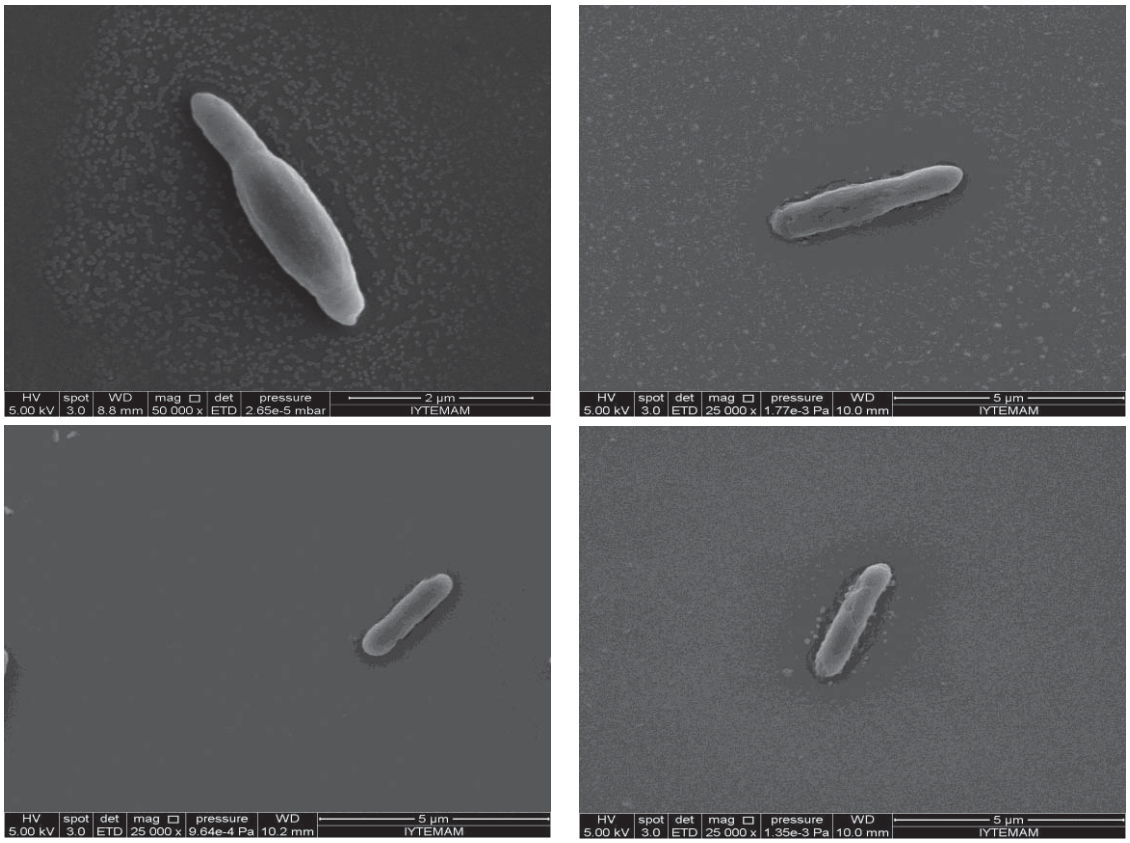

Figure 4.23. SEM images of 24 h grown vegetative cells

The cell size is reported to be 0.6–0.8 μm in width and 2.9–4.3 μm in length (Bahçeci et al., 2003). Also, the vegetative cells of DSM 3922 strain were measured as 0.65–1.08 μm in width and 2.47–3.98 μm in length. Overall, the average cell size was calculated as 0.79±0.08 μm in width and 3.21±0.84 μm in length (Fig.4.23).

The ultrastructural changes on *A. acidoterrestris* cells after GSE treatments were determined using SEM analysis. In fact, the antimicrobial effect of natural plant extracts on *Alicyclobacillus* spp. or *A. acidoterrestris* has not been previously investigated. The cells inoculated into apple juice containing GSE indicate altered bacterial cell morphology compared to the control cells which are intact and show smooth surface (Fig.4.23). In addition, the growth control in apple juice without GSE indicates the presence of both cells and spores after 168 and 336 h (Fig.4.24).

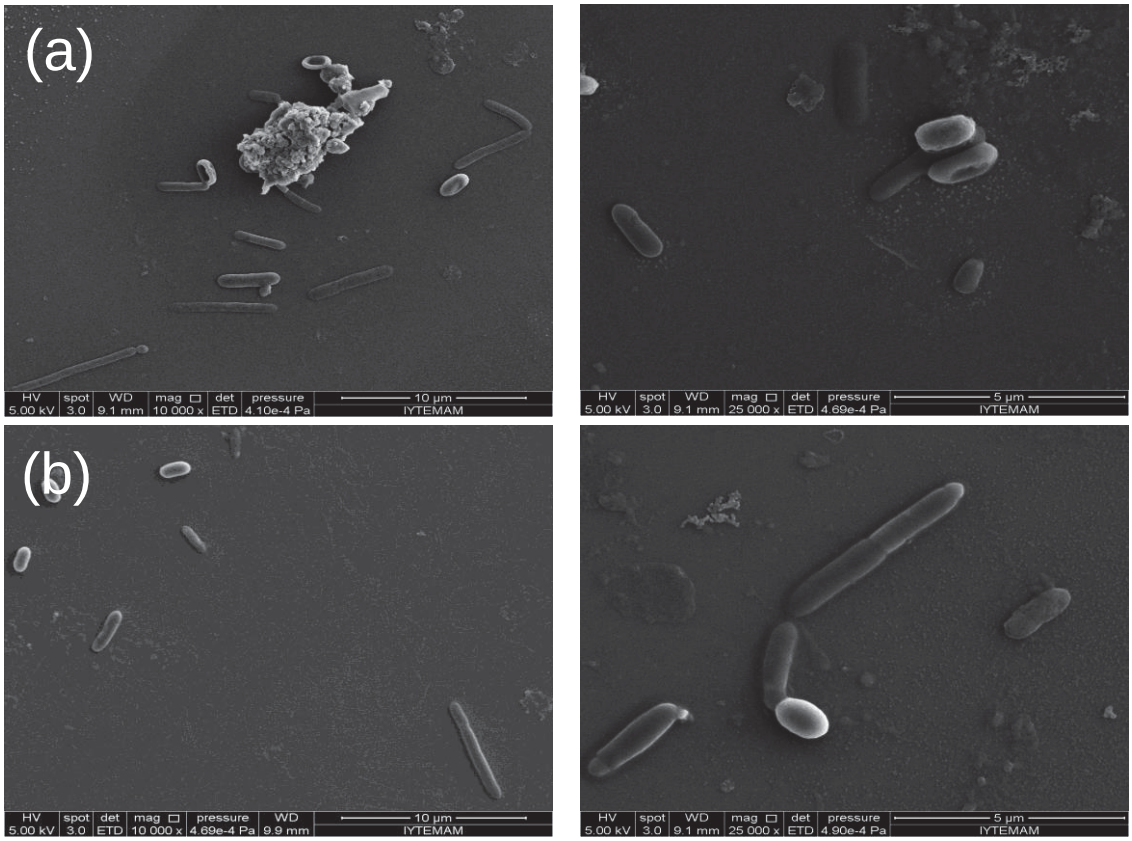

Figure 4.24. SEM images of vegetative growth control in apple juice after (a) 168 h (b) 336 h

SEM images of treated cells showed different surface morphologies depending on the GSE concentration and treatment time. Fig.4.25 represents the morphology of treated cells after 168 h. The use of 7.2% and 14.4% concentrations of GSE may destroy the cell membrane integrity by the release of cellular constituents. Since the

main target of phenolics is the cell membrane, the antimicrobial action of GSE is more obvious with the presence of holes in the cell membrane. Lower concentrations affected the cell deformity especially roughness (Fig. 4.25c–d and 4.27a). Indeed, polyphenols alter the cell membrane structure producing leakage of cell constituents such as proteins, nucleic acids and inorganic ions in the first stages (García-Ruiz et al., 2011). The morphological changes were found more severe after 336 h treatment (Fig.4.26a and 4.26b). The treated cells show cell content coagulation and appear empty of contents (Fig.4.26a), have small punctuated holes and wrinkled cell surface (Fig.4.26b–d and 4.27b).

In the related literature (Guendez et al., 2005), catechin (49.8%) and epicatechin (26.0%) were found as the major constituent of the grape seeds followed by epicatechin gallate (9.3%), procyanidin B1 (5.8%) and B2 (5.1%), epigallocatechin gallate (1.9%) and gallic acid (1.3%). The tested GSE contains epicatechin (9827.07 ppm), gallic acid (700.93), quercetin–3–galactoside (536.52 ppm), rutin (277.25 ppm) and epicatechin gallate (249.22 ppm) as the major components (Yavuzdurmaz, 2013). Indeed, phenolic compounds attack bacterial cell wall and membranes. They can interact with the membrane proteins by means of hydrogen bonding through their hydroxyl groups which can cause changes in membrane permeability and result in cell destruction. They can also penetrate into bacterial cells and coagulate cell content (Tian et al., 2009). In addition, they interfere with membrane function such as electron transport, nutrient uptake, protein and nucleic acid synthesis and enzyme activity (Paul et al., 2011). In the previous studies, different individual phenolic components have also been tested to reveal their antimicrobial action. Gallic acid can effectively permeabilize the outer membrane of *Salmonella* spp. inducing its disintegration based on chelation of divalent cations (Nohynek et al., 2006). The antibacterial mechanism of catechins have been mainly attributed to cytoplasmic membrane damage, although other mechanisms could be involved (Cushnie and Lamb, 2011). In addition, the mechanism of the antimicrobial activity of epigallocatechin gallate (EGC) has been attributed to its effect on cell wall components (Zhao et al., 2002). The antibacterial mechanism of EGC is a result of reduced permeability due to the adsorption of this compound to the cell surface. It acts on cells after germination through inhibiting division and proliferation (Shigemune et al., 2012). Based on the SEM images (Fig.4.25–4.27), it can be concluded that GSE might also disrupt the cellular metabolism preventing *A. acidoterrestris* cell division and forespore formation in the apple juice.

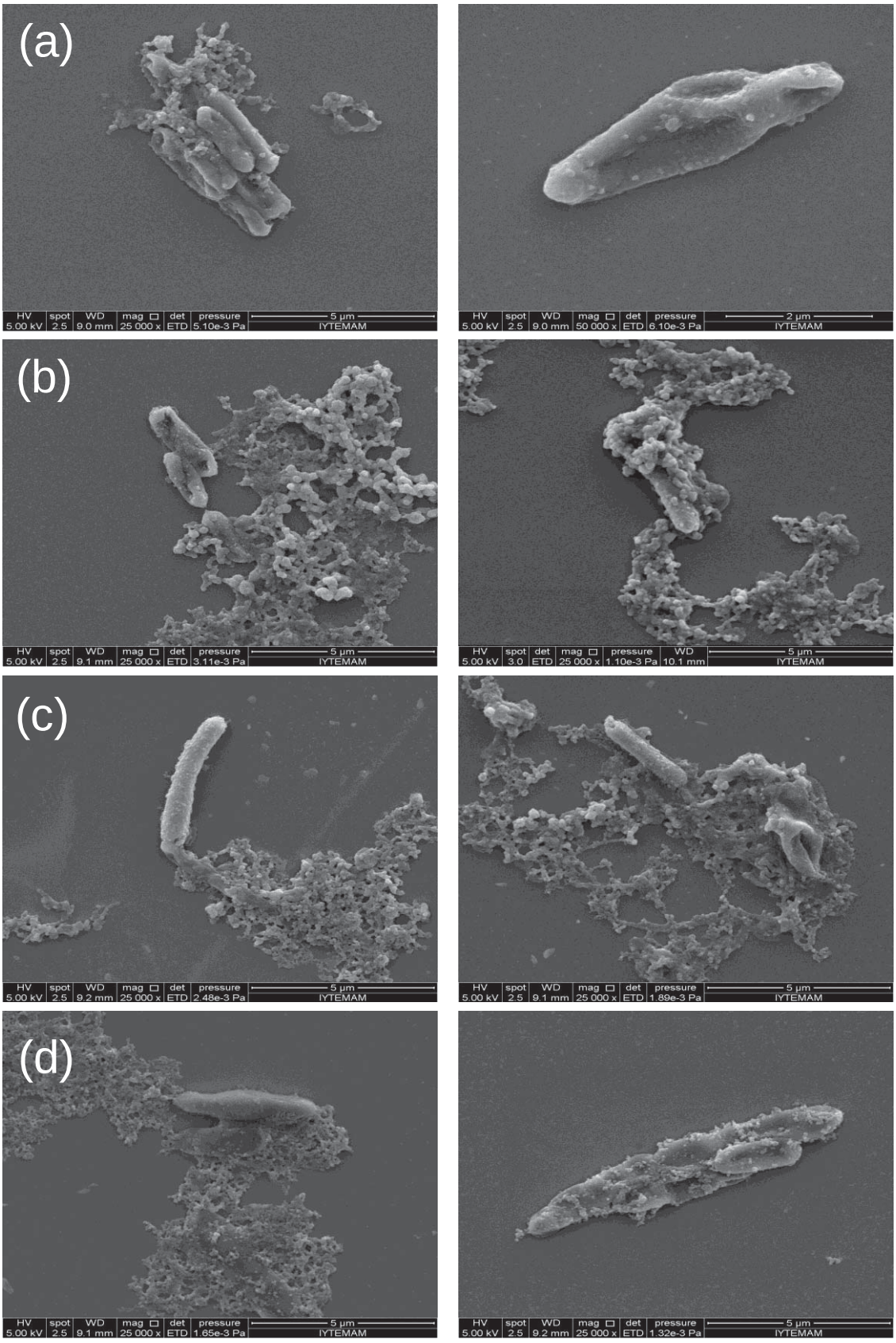

Figure 4.25. SEM images of vegetative cells in the apple juice with GSE after 168 h (a,b) 14.4% (c,d) 7.2% (e,f) 3.6% (g,h) 1.8%

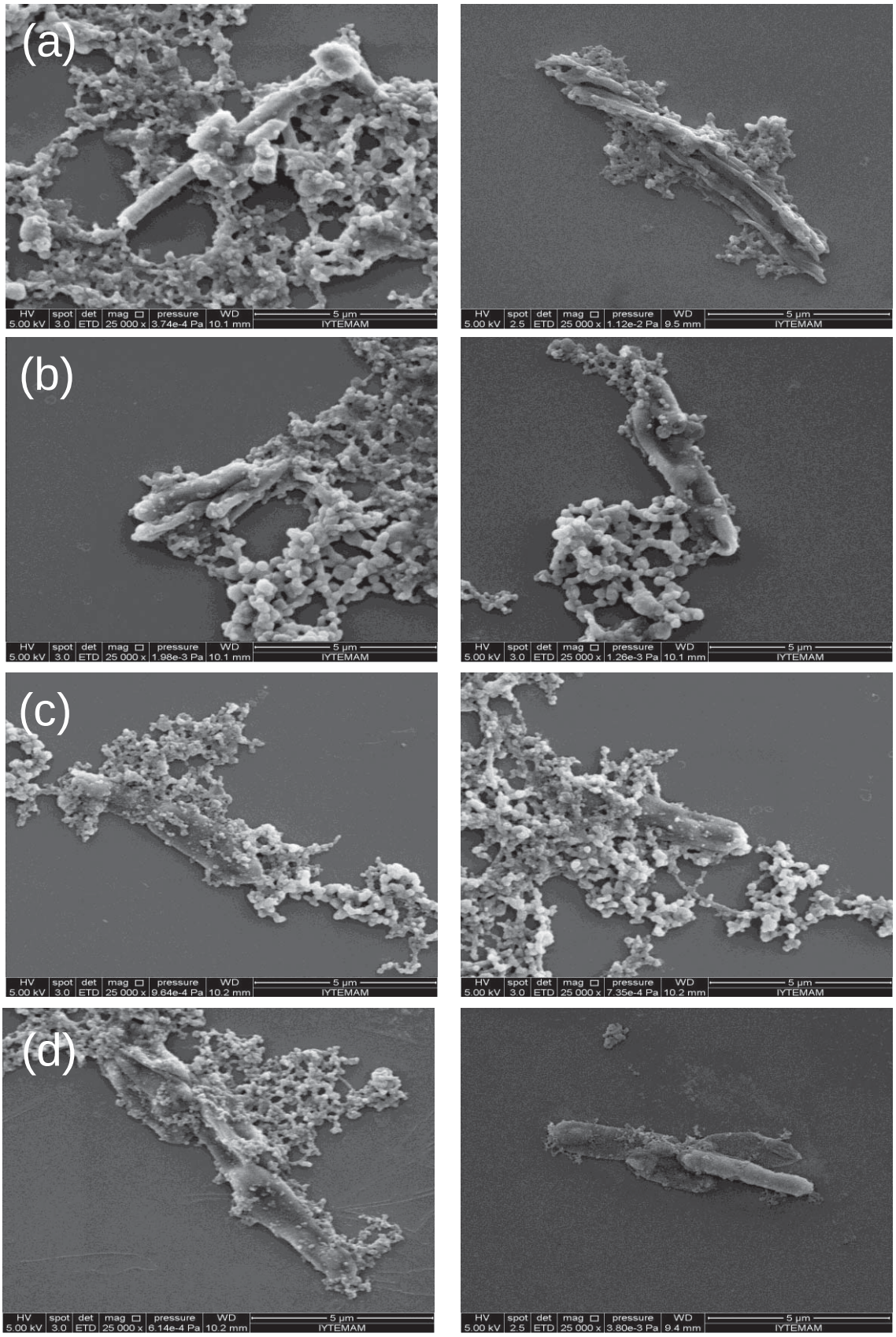

Figure 4.26. SEM images of vegetative cells in the apple juice with GSE after 336 h (a) 14.4% (b) 7.2% (c) 3.6% (d) 1.8%

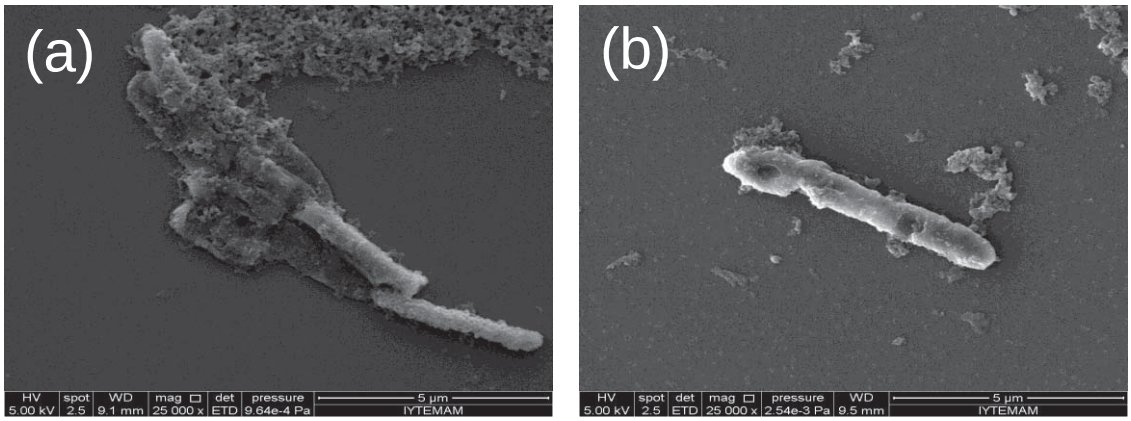

Figure 4.27. SEM images of vegetative cells in the apple juice with 0.9% GSE after (a) 168 h (b) 336 h

4.4.1.3. Atomic Force Microscopy

AFM is a suitable technique for investigation of cell morphology and structures (Stoimenov et al., 2002). It has been used to image bacterial nanostructures, genetic variation and antibacterial effects (Fernandes et al., 2009).

As indicated in Fig. 4.28, the concentrations of 0.23 and 0.45% (v/v) GSE caused morphological damage by changing the surface roughness when compared to control cell. As seen in Fig.4.28a, cell division and lysis are observed in the growth control while treated cells lost their integrity by the adsorption of GSE on the cell surface. Three–dimensional height images also indicate the distortion of the cells (Fig.4.29b), blebs on the surface (Fig.4.29c and d), shrinkage (Fig.4.29e) when compared to the control cells (Fig.4.29a). In fact, AFM imaging was achieved by the lowest concentrations having antimicrobial activity due to contamination of the tip by higher concentrations.

Similar to SEM, AFM imaging also indicate that the main target of the GSE may be the cell membrane of *A. acidoterrestris* inhibiting the cell division and subsequently spore formation.

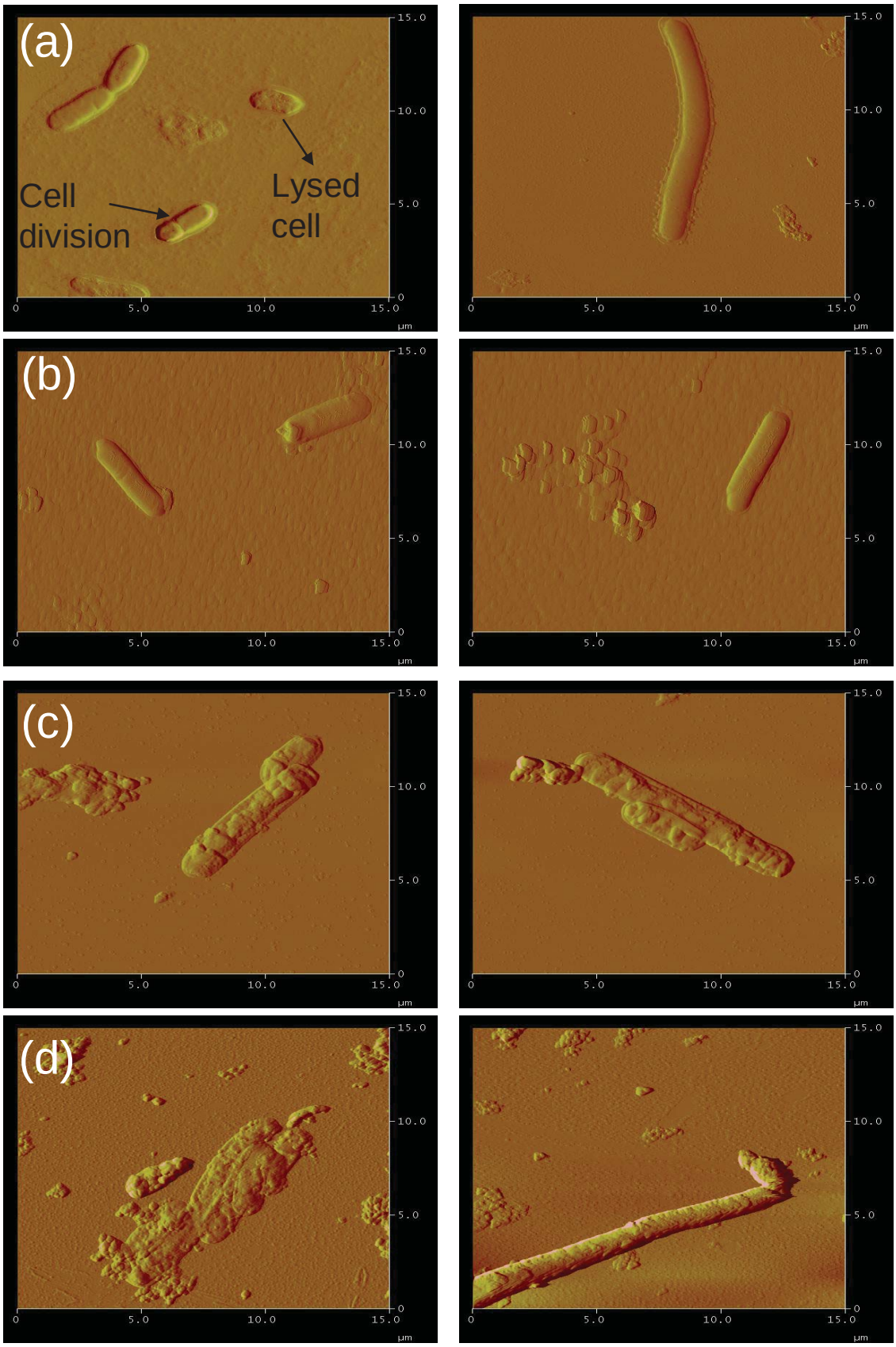

Figure 4.28. AFM images of vegetative cells in the apple juice with GSE after 336 h (a,b) control (c) 0.45% (d) 0.23%

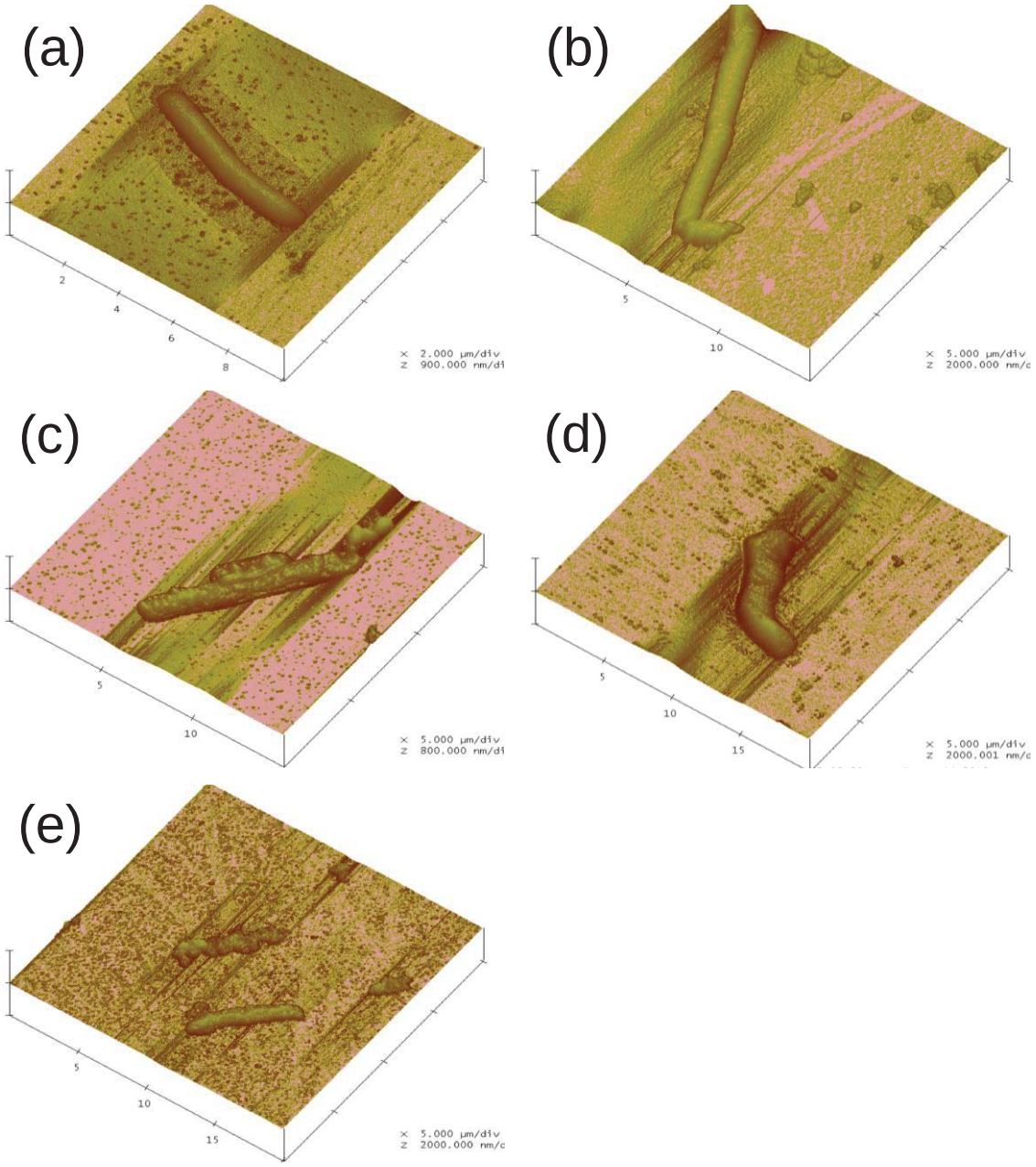

Figure 4.29. Three–dimensional AFM images of vegetative cells in the apple juice with GSE after 336 h (a) control (b) 0.23% (c) 0.45% (d) 0.9% (e) 1.8%

4.4.2. Antimicrobial Activity of Pomegranate Extract

Although many studies have been carried out *in vitro* to evaluate the antimicrobial activity of plant extracts, very few studies are conducted for food products, probably because plant extracts did not produce as considerable inhibition as many of the pure compounds in foods. As the crude extracts generally contain flavonoids in glycosidic form, where the sugar present in the extracts decreases

effectiveness against some bacteria (Kapoor et al., 2007; Parvathy et al., 2009; Rhee et al., 1994).

In the present study, the growth of *A. acidoterrestris* vegetative cells was inhibited in the apple juice with lower concentrations of pomegranate extract (PE) (Table 4.13). At the end of 240 h storage, the presence of 2% and 4% (w/v) concentrations of PE in the apple juice caused 3.46 and 3.56 log reductions, respectively. Also, the reductions were 2.56, 2.84, 3.26 and 3.32 log CFU/mL in the apple juice with 0.125, 0.25, 0.5 and 1% (w/v) PE, respectively. In the control samples, the population attained approximately 7.36 log CFU/mL after 24 h incubation in the apple juice.

Table 4.13. Counts of cells (log CFU/mL) in apple juice with PE at 37 °C

Treatment (h)	**0.125%**	**0.25%**	**0.5%**	**1%**	**2%**	**4%**	**Control**
0	5.45(0.07)^{Abc}	5.56(0.07)^{Abc}	5.65(0.01)^{Aab}	5.64(0.04)^{Aab}	5.73(0.04)^{Aa}	5.75(0.01)^{Aa}	5.57(0.04)^{Ac}
24	4.43(0.01)^{Bb}	4.41(0.09)^{Bb}	4.34(0.19)^{Bb}	4.34(0.04)^{Ab}	4.19(0.07)^{Bb}	4.10(0.25)^{Bb}	7.36(0.09)^{ABa}
48	4.29(0.10)^{Bb}	4.17(0.11)^{Cb}	4.05(0.05)^{BCb}	3.94(0.13)^{Bbc}	3.66(0.11)^{Cc}	3.57(0.09)^{Cc}	7.21(0.22)^{ABa}
96	3.96(0.06)^{Cb}	3.87(0.07)^{Dbc}	3.67(0.18)^{Cbcd}	3.56(0.04)^{Ccde}	3.47(0.01)^{Cde}	3.28(0.21)^{Ce}	7.59(0.12)^{ABa}
144	3.57(0.11)^{Db}	3.44(0.01)^{Ebc}	3.19(0.04)^{Dcd}	3.13(0.20)^{Dcde}	2.99(0.06)^{Dde}	2.83(0.12)^{De}	7.54(0.11)^{ABa}
168	3.43(0.06)^{Db}	3.28(0.04)^{Ebc}	3.04(0.03)^{Dcd}	2.89(0.03)^{Ede}	2.72(0.06)^{Ee}	2.66(0.15)^{De}	7.50(0.19)^{Ba}
192	3.37(0.01)^{Db}	3.26(0.07)^{Eb}	3.12(0.22)^{Dbc}	2.85(0.11)^{Ecd}	2.71(0.11)^{Ede}	2.48(0.09)^{DEe}	7.64(0.07)^{Ba}
240	2.89(0.05)^{Eb}	2.72(0.08)^{Fb}	2.39(0.06)^{Ebc}	2.32(0.07)^{Ecd}	2.27(0.09)^{Fde}	2.19(0.02)^{Ee}	7.21(0.17)^{Ca}

†: The experiments were repeated three times, and data are expressed as mean ± standard deviation
††:Values with different capital letters in the same column are significantly different (p<0.05)
‡: Values with different lower case in the same row are significantly different (p<0.05)

PE indicates both antioxidant and antibacterial properties. In the literature, the efficiency of various extracts from different parts of pomegranate plant against Gram–positive and Gram–negative bacteria have been studied (Ahmad and Aqil, 2007; Burapadaja and Bunchoo, 1995; De et al., 1999; Machado et al., 2002; Navarro et al., 1996; Negi et al., 2003; Prashanth et al., 2001; Rani and Khullar, 2004).

Pomegranate juice production yield is 40% of the whole fruit. Remaining of the waste products can be used to produce pomegranate peel extract since peel extracts contain high amounts of phenolics than the pulp and seed extracts (Hayrapetyan et al., 2012). Components of PE such as gallic acid, ellagic acid and punicalagin besides their free radical scavenging activities possess antimicrobial activities against intestinal flora

especially enteric pathogens such as *E. coli*, *Salmonella* spp., *Shigella* spp. and *Vibrio cholerae* (Ismail et al., 2012).

Aqueous and ethanolic fruit shell extracts were found to have antibacterial activity against *E. coli* strains (Voravuthikunchai et al., 2004) and used to inhibit the production of staphylococcal enterotoxin A (Braga et al., 2005). Other solvent extracts from the rind of *P. granatum* also showed antibacterial activity against enterohaemorrhagic *E. coli* (Prashanth et al., 2001) and food spoilage bacteria (Negi et al., 2003). Peel extracts were also used to enhance the shelf life of chicken meat products by controlling oxidative rancidity and bacterial growth due to their antioxidant potential (Kanatt et al., 2010).

In a recent study, Hayrapetyan et al. (2012) found that inhibitory effect of PE against the organisms in the following order of increasing sensitivity: *L. monocytogenes*, *B. cereus*, *B. subtilis*, *E. coli* and *S. aureus* by disc diffusion method. Then, they chose *L. monocytogenes* as the target organism because of its low sensitivity. No viable *L. monocytogenes* cells were detected after incubation in laboratory broth containing 7.5% v/v (24.7 mg PE/mL) liquid pomegranate extract. On the other hand, pure components of gallic and ellagic acids in PE were not effective against *L. monocytogenes* in broth system (Hayrapetyan et al., 2012). In the same study, the extract inhibited the growth of this organism at 4 °C during 46 days by 4.1 log CFU/mL in meat paté although the control reached to 9.2 log CFU/mL at 18^{th} day of incubation. They also suggested that there was a good correlation between the total phenol content of PEs and inhibitory effect. The antimicrobial activity of PE in broth was attributed to the components of extract showing antimicrobial activity. The inhibitory effect was not solely due to pH reduction and dilutions of nutrients in broth. These researchers also indicated that the extraction procedures, isolation source, and solubility of the extract could affect the antimicrobial activity of the PE.

Similarly, our results indicated the possibility of using PE as natural preservative against *A. acidoterrestris* in apple juice most probably due to its high total phenol content (573 mg GAE/g extract). In a recent study, the temperature dependency of the inhibitory effect of PE on *L. monocytogenes* has been revealed (Hayrapetyan et al., 2012). Moreover, the antibacterial activity of phenolics is pH dependent (Friedman et al., 2003). Therefore, the antimicrobial activity of PE might change depending on the storage temperature and the type of the juice.

4.4.2.1. Modeling Inactivation Data

The inactivation curves of *A. acidoterrestris* cells in the apple juice with PE are presented in Fig.4.30. The Weibull and the log–linear tail models were used to model the inactivation as a function of time (h). In previous studies, the Weibull model has also been used to describe the antibacterial inactivation kinetics of lemon juice, oregano and lemongrass essential oils (De Oliveira et al., 2013; Yang et al., 2013).

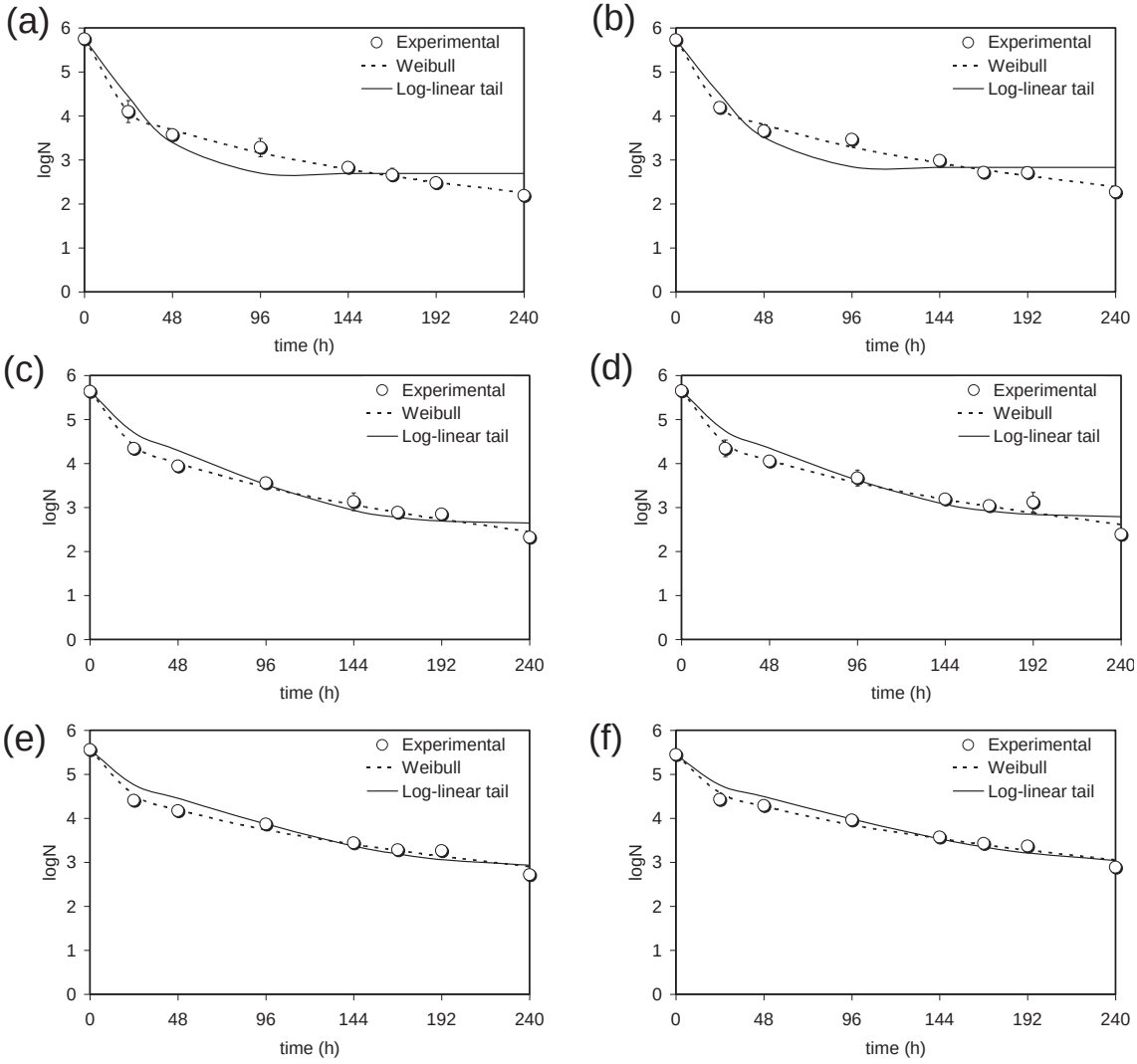

Figure 4.30. Survival curves of vegetative cells in the apple juice with PE (a) 4% (b) 2% (c) 1% (d) 0.5% (e) 0.25% (f) 0.125%

The parameters of the models as well as the statistical indices are shown in Table 4.14. Visual evaluation of the fitted curves (Fig.4.30) and statistical indices (lower RMSE and higher R^2 values) in Table 4.14 showed that the Weibull model fitted

well the experimental data. The RMSE values ranged from 0.0826 to 0.1651. Also, the Weibull model showed higher R^2 values (>0.983). For all treatments, the shape parameter (β) was smaller than 1 indicating upward concavity. Estimated t_{4D} values were also found as 352 h and 422 h for 4% and 2% PE, respectively.

Table 4.14. Parameters of the Weibull and the log–linear tail models

PE (%)	**Weibull**				**Log–linear tail**		
	δ (h)	β	t_{4D}[a](h)	Statistical indices	$k_{max}(h^{-1})$	$\log N_{res}$ (CFU/mL)	Statistical indices
0.125	35.18	0.45	766.0	RMSE[b]=0.120 R^2[c]=0.984 R^2–adj[d]=0.978	0.03	2.95	RMSE=0.278 R^2=0.913 R^2–adj=0.879
0.25	23.70	0.42	643.0	RMSE=0.128 R^2=0.985 R^2–adj=0.979	0.03	2.89	RMSE=0.330 R^2=0.900 R^2–adj=0.860
0.5	16.74	0.41	492.3	RMSE=0.165 R^2=0.981 R^2–adj=0.973	0.04	2.78	RMSE=0.397 R^2=0.889 R^2–adj=0.844
1.0	15.88	0.42	430.9	RMSE=0.106 R^2=0.993 R^2–adj=0.990	0.04	2.63	RMSE=0.379 R^2=0.908 R^2–adj=0.871
2.0	7.16	0.34	422.4	RMSE=0.127 R^2=0.991 R^2–adj=0.987	0.10	2.83	RMSE=0.425 R^2=0.893 R^2–adj=0.851
4.0	5.28	0.33	352.4	RMSE=0.083 R^2=0.996 R^2–adj=0.995	0.11	2.69	RMSE=0.408 R^2=0.910 R^2–adj=0.874

[a]: Predicted time for 4–log reduction calculated by the Weibull model
[b]: Root mean square error
[c]: Coefficient of determination
[d]: Adjusted regression coefficient

4.4.2.2. Scanning Electron and Atomic Force Microscopy

In the control samples, the production of forespore due to cell division is clearly observed (Fig.4.31). In response to nutrient depletion, vegetative cells undergo asymmetric cell division to produce mother cell and forespore. The smaller cell is the forespore that differentiates into the endospores while mother cell lyses to release mature spore (Meisner et al., 2008). SEM images demonstrated the effect of PE at the point of cell division after 240 h (Fig.4.32). The mechanism of antimicrobial activity of pomegranate peel phenolics involves precipitation of membrane proteins resulting in microbial lysis. Phenolics react with microbial cell membrane proteins and/or protein sulfhydryl groups resulting in bacterial cell death due to membrane precipitation and

inhibition of enzymes such as glycosyltransferases (Ismail et al., 2012). Based on the SEM images (Fig.4.32a–d), most of the treated cells indicate cell elongation to their multiple cell size that is the evidence of the effect of PE on the cell division. Moreover, during growth in the apple juice with 0.25% PE, shrinkage and leakage of cellular materials are observed (Fig.4.32e). Therefore, it can be concluded that PE might affect the cell membrane, disrupt the cellular function most probably cell division and prevent the forespore formation. The spore formation is divided into seven stages. In the first stage, the nuclear material is disposed axially into filaments. In the second stage, completion of DNA segregation occurs and a septum is formed. In the third stage, the septum begins to curve, and the immature spore is surrounded by a double membrane of the mother cell in an engulfment process, and the smaller forespore becomes entirely within the mother cell. In the next stage, the mother cell intermediates the formation of the forespore into the spore (Sella et al., 2014). Therefore, the production of forespore is a key factor for the spore formation.

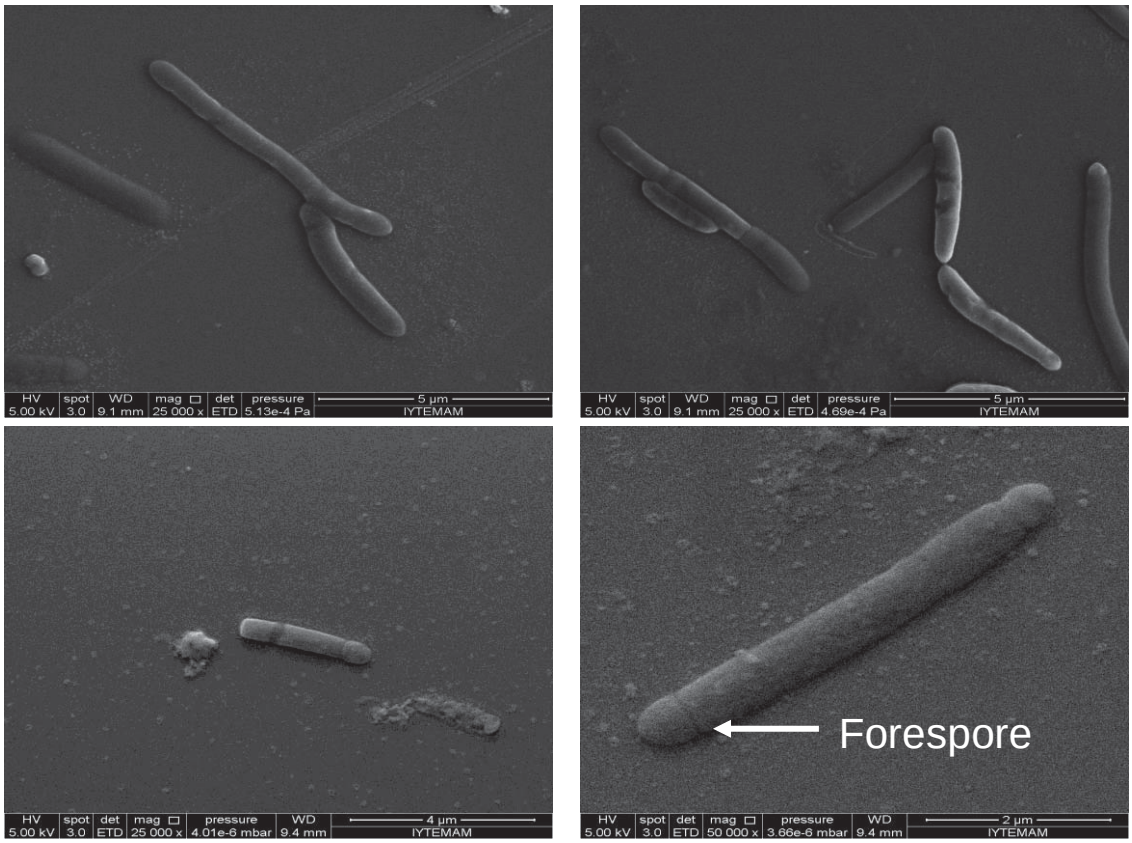

Figure 4.31. SEM images of forespore formation during cell division in apple juice after 240 h

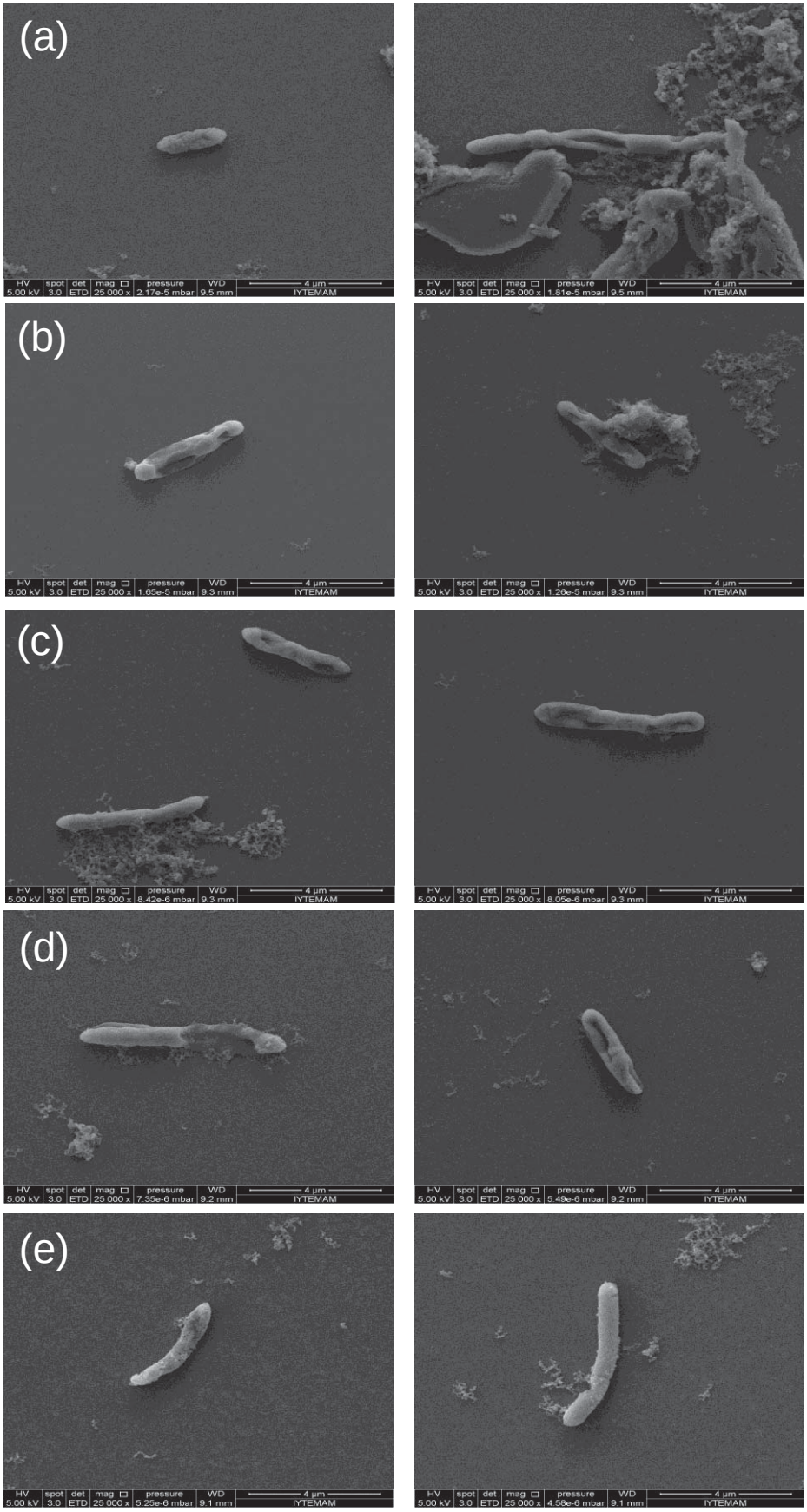

Figure 4.32. SEM images of vegetative cells in the apple juice with PE after 240 h (a) 4% (b) 2% (c) 1% (d) 0.5% (e) 0.25%

AFM images are also inconsistent with the SEM images of PE–treated vegetative cells. The use of PE also appeared to affect the structural integrity of cells. The treated *A. acidoterrestris* cells show the evidence of mainly shrinkage (Fig.4.33b–c and 4.34b–c) when compared to the control cells with smooth surface (Fig.4.33a and 4.34a).

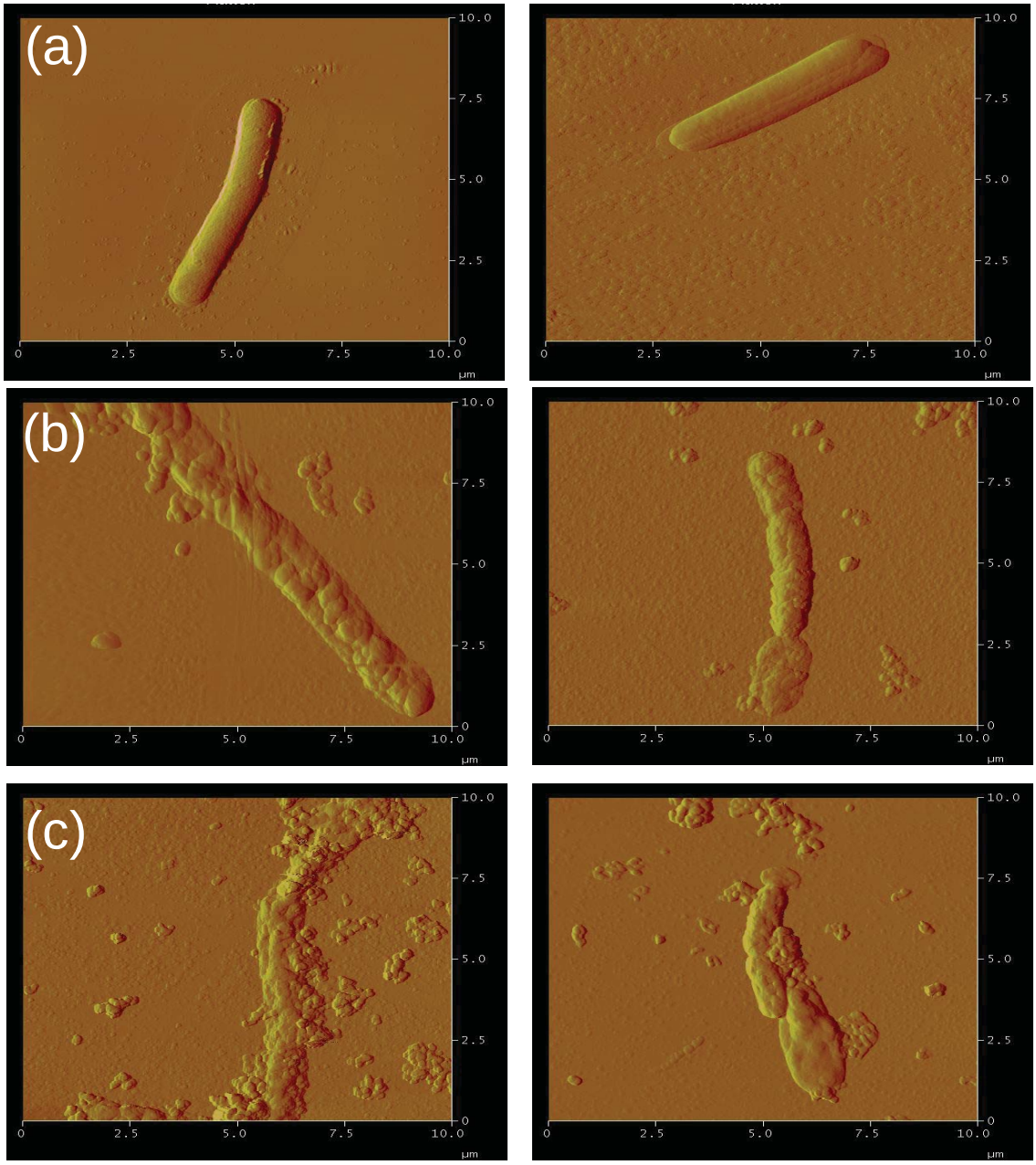

Figure 4.33. AFM images of vegetative cells in the apple juice with PE after 240 h (a) control (b) 0.25% (c) 0.5% PE

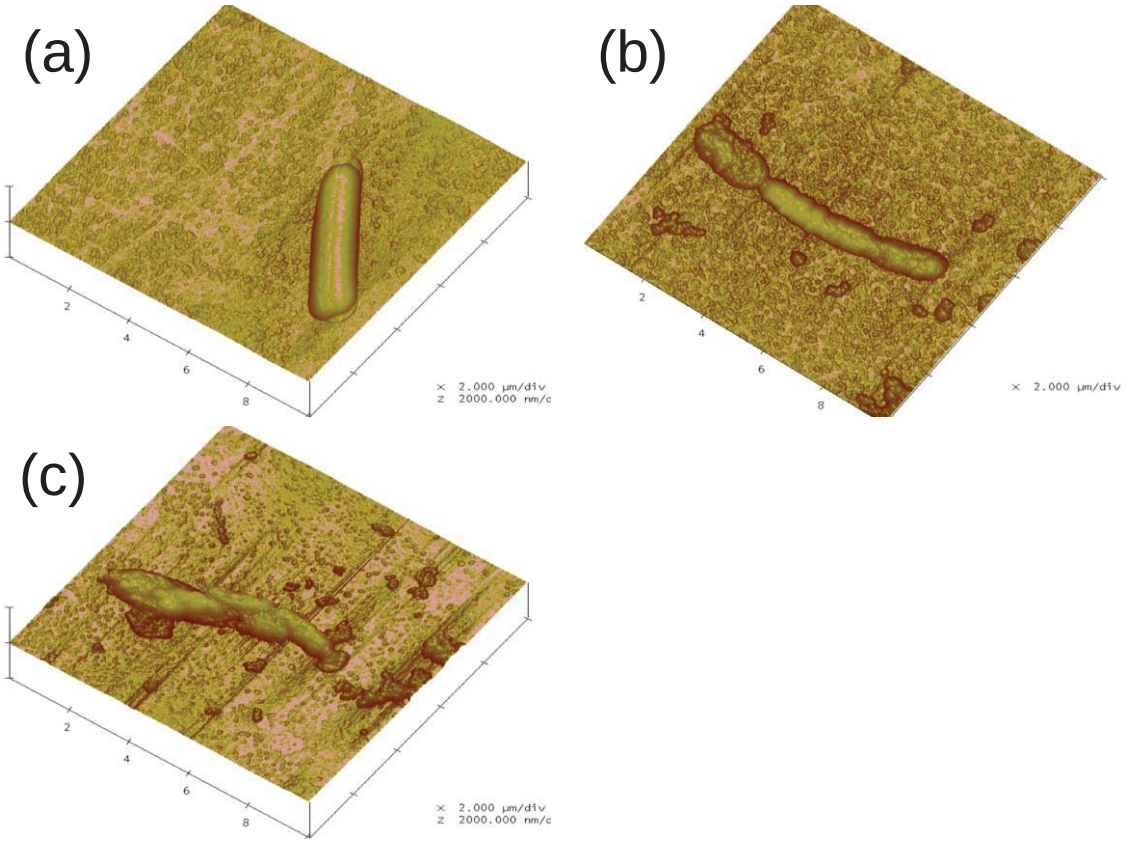

Figure 4.34. Three–dimensional AFM images in the apple juice with PE after 240 h (a) control (b) 0.25% (c) 0.5%

4.4.3. Antimicrobial Activity of Pomegranate and Blend Juices

Since pomegranate juices contain high polyphenolic compounds, they exhibit astringent taste and consequently the sensory acceptance of the pomegranate juice is reduced. Because of this reason, pomegranate juices are generally mixed with other fruit juices to enhance sensory properties for consumption. Therefore, the growth characteristics of *A. acidoterrestris* were determined in apple, pomegranate and pomegranate–apple blend juices (10, 20, 40 and 80%, v/v) during storage at 37 °C for 336 h. Table 4.15 presents the effect of pomegranate juice on the growth inhibition of vegetative cells. The average initial cell population was about 5.43, 5.49, 5.53, 5.55, 5.44 and 5.62 log CFU/mL at the concentrations of 10%, 20%, 40%, 80% blend, apple and pomegranate juices, respectively. Furthermore, *A. acidoterrestris* cells reached 7.27 log CFU/mL within 24 h incubation in the apple juice. In general, the number of cells decreased significantly with increasing pomegranate juice concentration and storage time in the apple juice (p<0.001). The use of 40% and 80% blend juices and

pomegranate juice caused 3.17, 3.53, 3.72 log reductions, respectively. On the other hand, in the presence of 10% and 20% blend juices, the growth was inhibited for a certain time, and then the cell numbers started to increase after 72 h and 144 h storage, respectively. The population attained approximately 7.17 log CFU/mL in apple juice after 336 h while the cell numbers reached 7.12 and 7.00 log CFU/mL in 10% and 20% pomegranate–apple blend juices, respectively (Table 4.15). In the related literature, 40% pomegranate–apple juice blend was found better than apple juice based on the sensory evaluations using a 9 hedonic scale (Ibrahim et al., 2011). They also suggested that pomegranate juice could be used as a natural antimicrobial in different food products including juices to inhibit the growth of *E. coli* O157:H7.

Table 4.15. Counts of cells (log CFU/mL) in the juice samples at 37 °C

Storage (h)	**PJ**	**80%**	**40%**	**20%**	**10%**	**AJ**
0	5.62(0.09)^{Aa}	5.55(0.07)^{Aa}	5.53(0.00)^{Aa}	5.49(0.05)^{Da}	5.43(0.18)^{Ca}	5.44(0.06)^{Ba}
24	4.42(0.19)^{Bb}	4.19(0.03)^{Bb}	4.51(0.12)^{Bb}	4.29(0.05)^{Eb}	4.53(0.09)^{Db}	7.27(0.67)^{Aa}
48	4.02(0.04)^{Cd}	3.89(0.04)^{Ce}	4.01(0.01)^{Cd}	4.15(0.03)^{Ec}	4.78(0.01)^{Db}	7.30(0.00)^{Aa}
72	3.87(0.21)^{Cb}	3.72(0.01)^{Db}	3.92(0.04)^{Cb}	4.09(0.18)^{Eb}	7.78(0.07)^{Aa}	7.59(0.12)^{Aa}
144	3.07(0.10)^{Dc}	2.99(0.08)^{Ec}	3.12(0.00)^{Dc}	6.59(0.08)^{Cb}	6.84(0.16)^{Bb}	7.17(0.08)^{Aa}
168	2.93(0.00)^{DEb}	2.91(0.00)^{Eb}	2.95(0.04)^{Eb}	7.56(0.08)^{Aa}	7.67(0.13)^{Aa}	7.54(0.13)^{Aa}
240	2.67(0.13)^{Eb}	2.71(0.02)^{Fb}	2.69(0.00)^{Fb}	7.14(0.09)^{Ba}	6.87(0.04)^{Ba}	6.95(0.24)^{Aa}
336	1.90(0.08)^{Fc}	2.02(0.09)^{Gbc}	2.36(0.01)^{Gb}	7.00(0.16)^{Ba}	7.12(0.06)^{Ba}	7.17(0.19)^{Aa}

† : The experiments were repeated three times, and data are expressed as mean ± standard deviation
†† :Values with different capital letters in the same column are significantly different (p<0.05)
‡: Values with different lower case in the same row are significantly different (p<0.05)

It was reported that apple–grape–cherry blend, apple–raspberry–grape blend and apple–red grape blend, a cranberry cocktail and prune juice were unable to support the growth. On the other hand, apple juice, apple–orange–pineapple blend, grapefruit juice, orange juice, pineapple juice, pineapple–orange juice, tomato juice, a tropical fruit blend were able to support the growth of *A. acidoterrestris* (Splittstoesser et al., 1994). In another study, *A. acidoterrestris* was unable to grow in apple–cranberry, pineapple and 10% fruit juice, or salsa. Similarly, growth occurred in grapefruit, apple, orange, pear, white grape and tomato juices. The reason for growth and spoilage only in specific juices is unclear, but it could be because of the different concentrations of guaiacol precursors or growth inhibitors in juices (Walls and Chuyate, 2000). Similarly, the

growth inhibition of *A. acidoterrestris* in red grape juice has been reported. This inhibitory effect is not only attributed to the synergistic effects of polyphenols showing antimicrobial activity. These compounds have also stronger antimicrobial effects at lower pHs and lower effect in the presence of fibers that adhere to polyphenols and does not show any effect at the higher pH values (Takahashi et al., 2007). In another study, water–extracted powder of Acerola fruit (*Malpighia emarginata* DC.) has been reported to inhibit the growth of *A. acidoterrestris* DSM 3922 in 35% (w/v) apple juice when used at the concentration of 0.1% (w/v). It has been suggested that the growth inhibition occurs because of the combined effect of major (vitamin C, glucose, fructose and malic acid) and minor components such as polyphenols in this fruit (Takahashi et al., 2007).

The antimicrobial activity of pomegranate juice was reported to be due to tannins such as ellagitannins and other high molecular weight tannins (Al-Zoreky, 2009; Machado et al., 2003). In a recent study (Türkyılmaz et al., 2013), antimicrobial activity of pomegranate juice was tested against Gram–positive (*B. megaterium*, *B. subtilis*, *S. aureus*, and *L. plantarum*) and Gram–negative bacteria (*Pseudomonas* spp, *E. coli*, *E. coli* O157:H7, *S.* Enteritidis, *E. cloaceae*, and *C. freundii*) and molds (*A. niger*, *Aspergillus* spp., *Penicillum* spp.) using agar well diffusion method. The antimicrobial activity of juice indicating the highest yield was tested and found that pomegranate juice showed higher activity on Gram–positive than Gram–negative bacteria. While pomegranate juice showed antimicrobial activity on *B. megaterium*, *B. subtilis*, *S. aureus* and *Pseudomonas* spp., the activity was not detected on the other tested organisms.

Different fruit juices such as blackberry, blackcurrant, cranberry, pomegranate, lemon, and red grape juices have been tested for their antimicrobial activity against many organisms. Growth inhibition occurs due to low pH and the presence of antimicrobial compounds such as phenolics in cranberries (Côté et al., 2011). In a recent study, the growth of *L. monocytogenes*, *Salmonella* Typhimurium and *E. coli* O157:H7 were significantly inhibited in both milk and broth supplemented with 10% blackberry juice by 1–3 log CFU/mL. On the other hand, blackberry juice significantly stimulated the growth of *Lactobacillus* in a time dependent manner (Yang et al., 2014). While the growth of probiotics was not inhibited, the growth inhibition of *L. monocytogenes*, *Salmonella* Typhimurium and *E. coli* O157:H7 was observed in blueberry juice (Biswas et al., 2012). Apple cider (1.0–1.4 log reduction) was less inhibitory than grapefruit juice (1.8 to 2.2 log reduction) against *Salmonella* Typhimurium after 24 h incubation.

Differences in the composition of organic acids in the grapefruit juice (citric acid) and apple cider (malic acid) were shown to be effective for the inactivation (Yin et al., 2012). In another study, blackcurrant juice inhibited the proliferation and adhesion to gut cells but enhanced the proliferation of *Lactobacillus rhamnosus* (Parkar et al., 2014).

The grape juice obtained from Ribier black table grapes was active against all tested *Listeria* spp. but not effective against *B. cereus*, *Salmonella* Menston, *E. coli*, *S. aureus* and *Y. enterocolitica*. Grape juice decreased the cell numbers from 10^6–10^7 CFU/mL to undetectable level within 10 min (Rhodes et al., 2006). Similarly, the survival of *S.* Typhimurium in red grape juice and apple cider was examined (Yin et al., 2012). The results showed that both grape juice and apple cider inactivated the organism resulting in 0.8–2.2 log reductions compared to control. The population in grape juice was 1.0–1.4 log lower than in apple cider after 24 h incubation. They suggested that although acidity is important for antimicrobial activity and both juices analyzed were acidic, the presence of certain compounds in the grape fruit juice is responsible for its antimicrobial and/or antiviral activities that are not found in the apple cider.

4.4.3.1. Modeling Inactivation Data

The survival curves of *A. acidoterrestris* cells in 40%, 80% blend and pomegranate juices were fitted by the Weibull model as a function of time (h) (Fig.4.35). The parameters of the Weibull model with corresponding R^2, adj–R^2 and RMSE values are presented in Table 4.16. Cell inactivation in these juice samples can also be well described by the Weibull model according to the visual evaluation of the predicted survival curves (Fig.4.35) and good measures of fit (Table 4.16). Lower RMSE (0.1125≤), higher R^2 (>0.992) and adj–R^2 (>0.989) values indicated that the Weibull model satisfactorily described the inactivation kinetics of *A. acidoterrestris* cells in 40%, 80% blend and pomegranate juices (Table 4.16). The shape parameter (β) was found 0.41, 0.37 and 0.45 for 40% and 80% blend juices and pomegranate juice, respectively. As can be seen in Fig.4.35, the survival curves were upward concave in which β values were smaller than 1. According to the Eq. 3.8, t_{4D} values were calculated as 533, 524, 427 h for 40% and 80% blend juices and pomegranate juices, respectively.

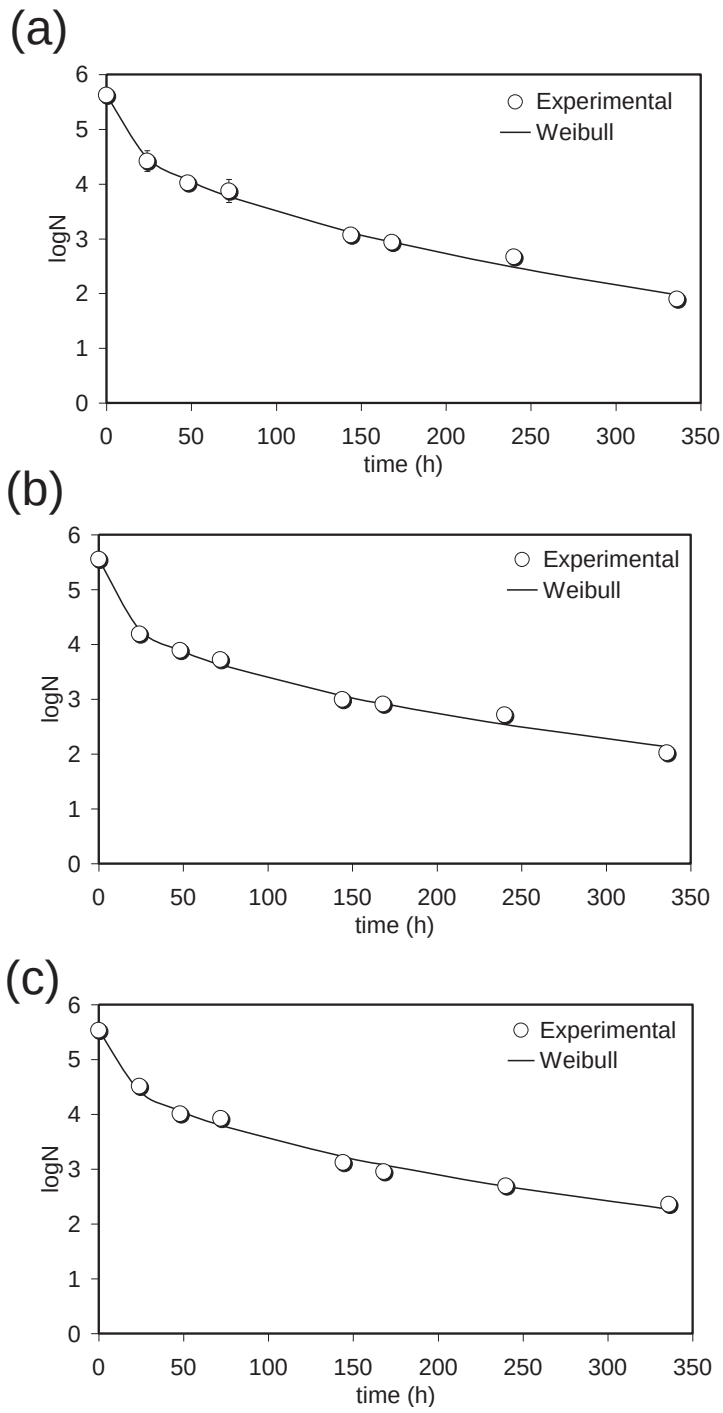

Figure 4.35. Survival curves of vegetative cells fitted by the Weibull model in (a) pomegranate juice (b) 80% blend juice (c) 40% blend juice

Table 4.16. Parameters and statistical indices of the Weibull model

Juice	Weibull			Statistical indices		
	δ (h^{-1})	β	t_4[a](h)	RMSE[b]	R^2[c]	R^2–adj[d]
PJ	19.65	0.45	427.8	0.111	0.994	0.991
80% blend juice	12.81	0.37	524.9	0.113	0.992	0.989
40% blend juice	18.15	0.41	533.1	0.110	0.992	0.989

[a]: Predicted time for 4–log reduction calculated by the Weibull model
[b]: Root mean square error
[c]: Coefficient of determination
[d]: Adjusted regression coefficient

4.4.3.2. Scanning Electron and Atomic Force Microscopy

SEM images indicate that during growth in 40%, 80% pomegranate–apple blend juices and pomegranate juices, structural deformations have occurred such as leakage of cellular constituents; perforations and blebs on the cells surface after 168 h (Fig.4.36). The growth only in apple juice was used as a control to determine the addition of pomegranate juice into the apple juice (Fig.4.36a and 4.37a). Leakage of intracellular components is the indication of damage on the bacterial cytoplasmic membrane (Kong et al., 2010).

As the storage time increased to 336 h, the loss of structural integrity is more obvious (Fig.4.37). Most of the cells seem ruptured leaving only the membrane (Fig.4.37b–d). Since pomegranate juice has high TPC than apple juice, the antimicrobial mechanism of the pomegranate juice should be attributed to the action of phenolics.

In a similar study, the antimicrobial activity of blueberry proanthocyanidins (5 mg/mL) and commercial blueberry juice against two strains of *Cronobacter sakazakii* was tested (Joshi et al., 2014). Both proanthocyanidins and berry juice reduced the cell numbers to undetectable levels and resulted in clumping and formation of blebs on the cell surface. Blueberry phenolics are also known to affect membrane fluidity, change fatty acid profile, and disrupt cellular metabolism (Joshi et al., 2014). Growth inhibition by blueberry juice could be due to the partial hydrophobicity of phenolics, anthocyanins and proanthocyanidins which allows them to bind to the outer membrane of the bacteria causing changes in fluidity (Biswas et al., 2012).

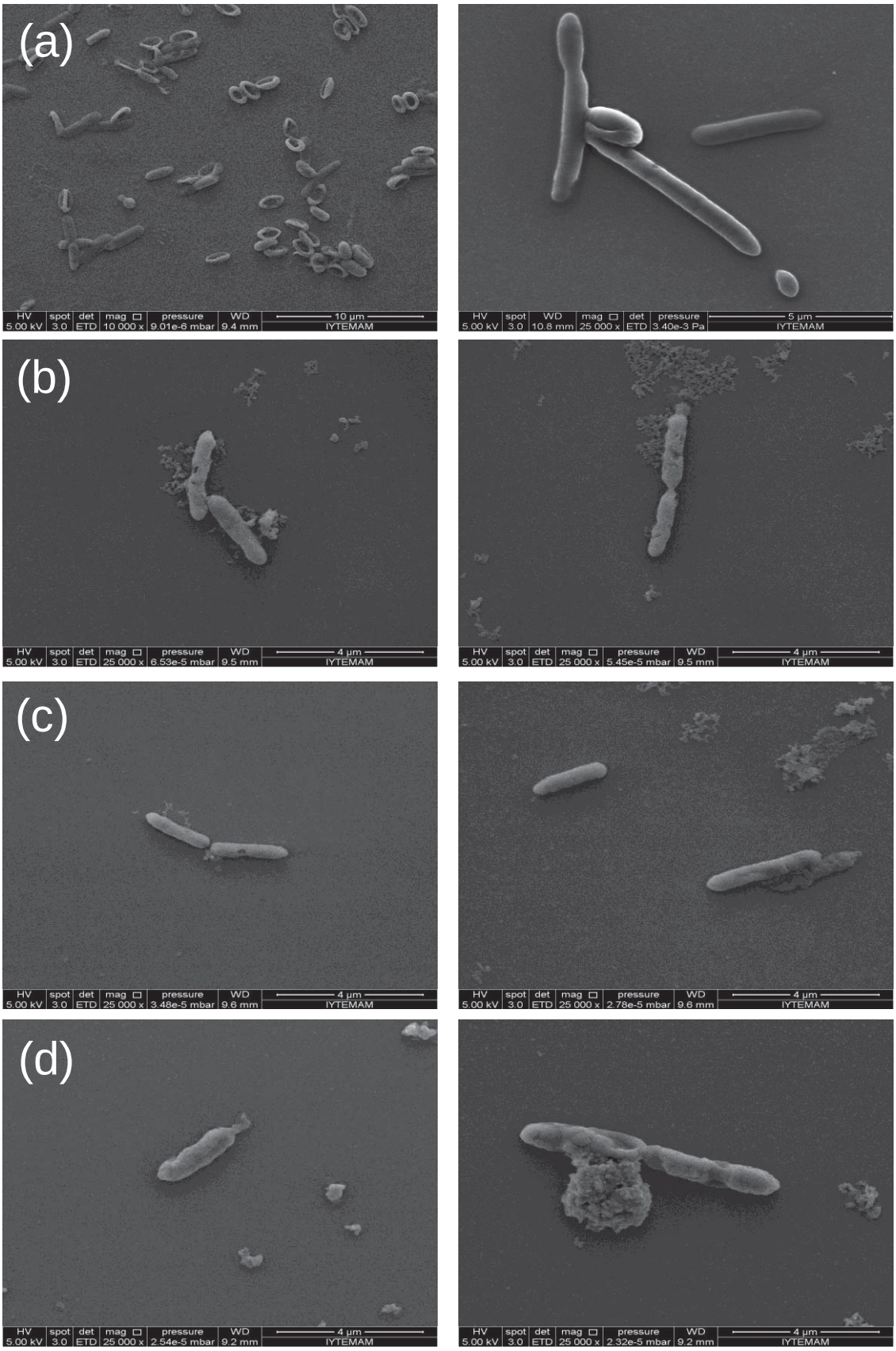

Figure 4.36. SEM images of vegetative cells after 168 h in (a) apple juice (b) pomegranate juice (c) 80% blend juice (d) 40% blend juice

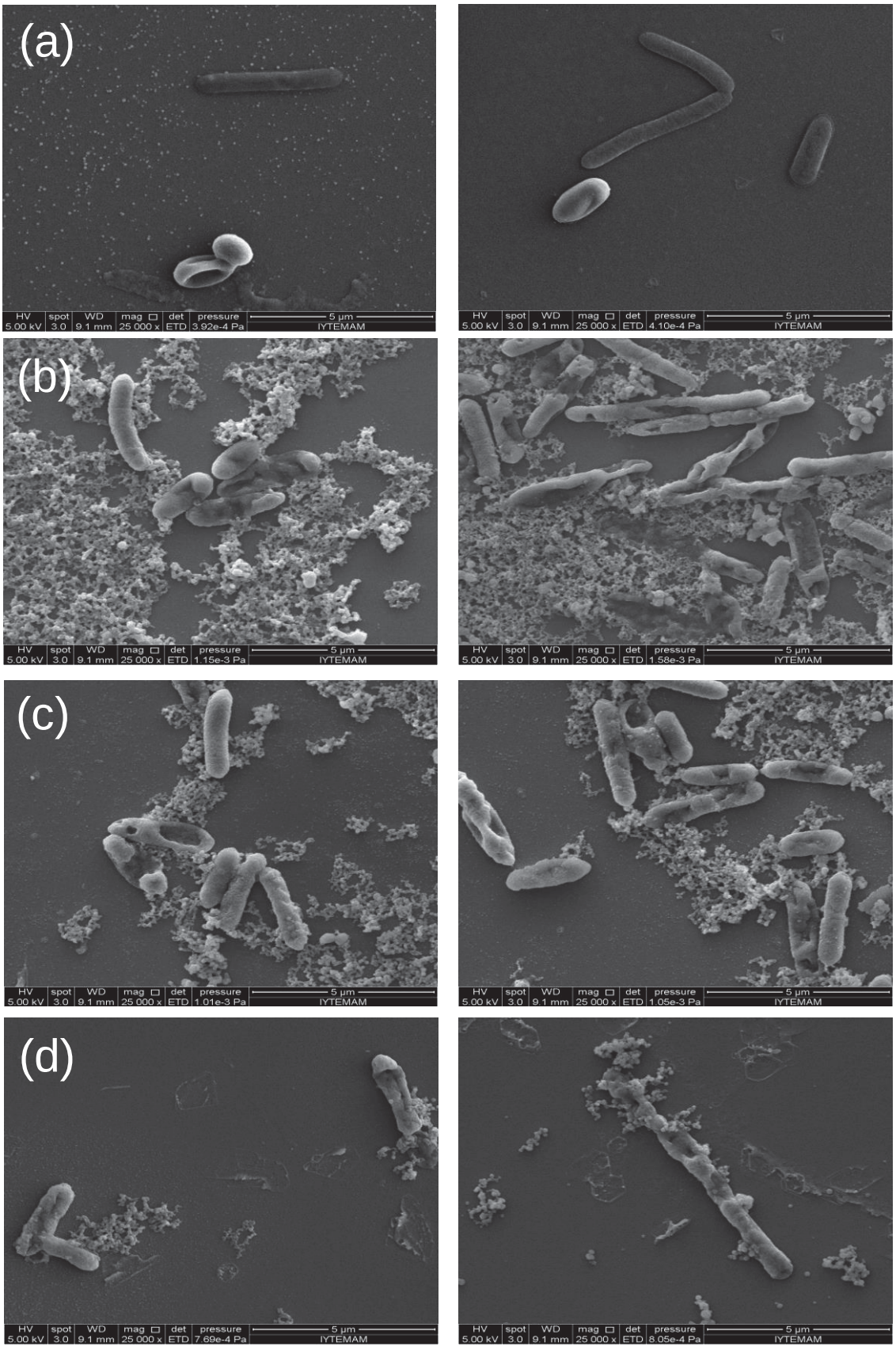

Figure 4.37. SEM images of vegetative cells after 336 h in (a) apple juice (b) pomegranate juice (c) 80% blend juice (d) 40% blend juice

Detrimental changes on external cell surface during growth in 40% and 80% blend juices and pomegranate juice were also determined using AFM analysis. The untreated cells are intact with smooth surface and clearly round border (Fig.4.38a and 4.39a) but treated cells lost structural integrity becoming grainy (Fig.4.38b–d and 4.39 b–d).

Tannins in pomegranate juice could exhibit antimicrobial activity by forming complexes with proteins found in cell walls thus causing a decrease in both cell permeability and substrate transportation into cells. The second mechanism might be the inhibition of important bacterial enzymes. They can form stable complexes with metal ions such as Fe^{2+} and Cu^{2+}, resulting in a decrease in their availability to bacteria, thus affecting the activity of metalloenzymes (Puupponen-Pimiä et al., 2005).

The SEM and AFM images were consistent with these findings indicating the effect of tannins found in pomegranate juice on the cell wall structure.

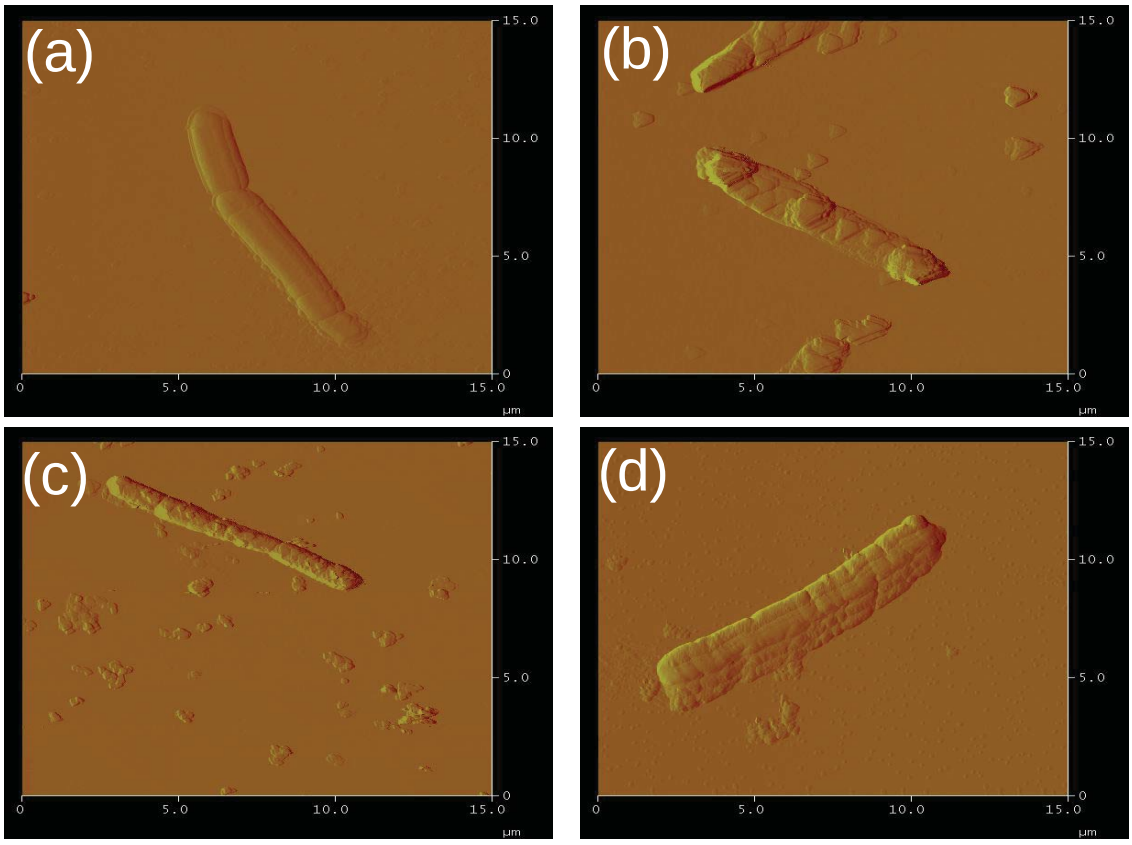

Figure 4.38. AFM images of vegetative cells after 336 h in (a) apple juice (b) 40% blend juice (c) 80% blend juice (d) pomegranate juice

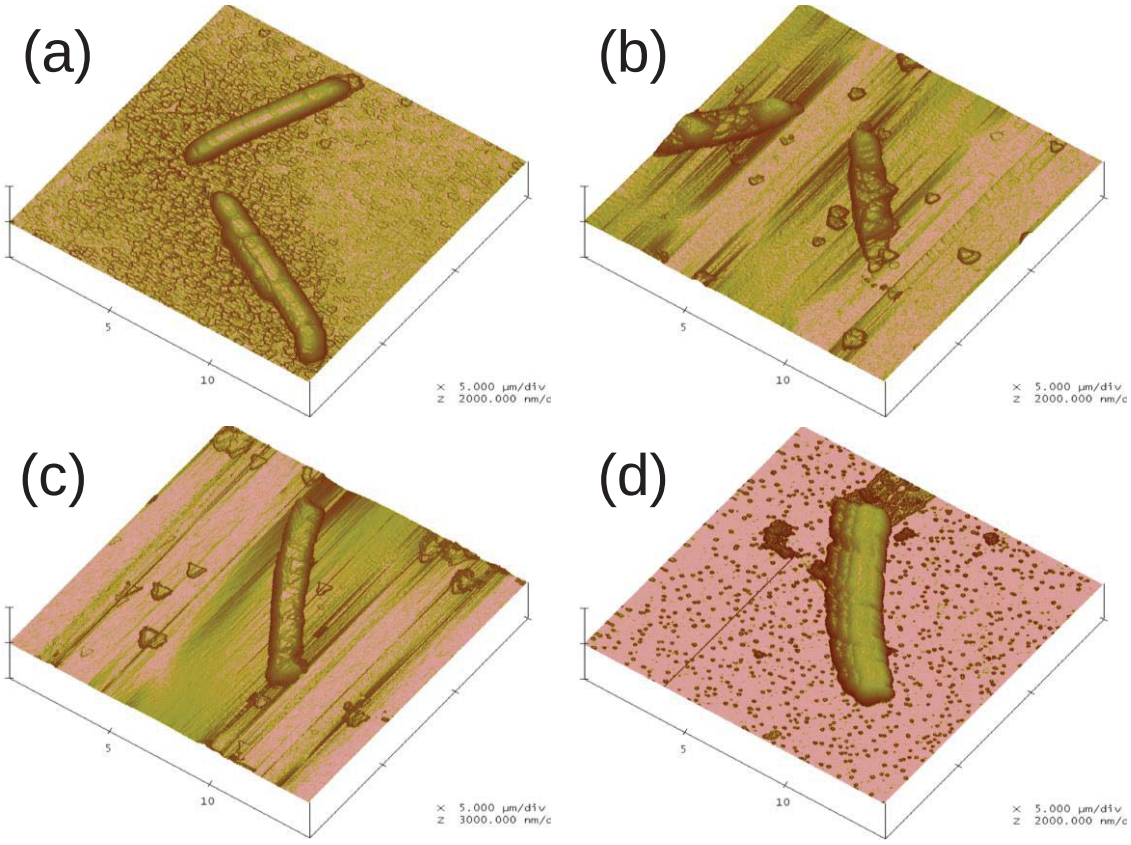

Figure 4.39. Three–dimensional AFM images of vegetative cells after 336 h in (a) apple juice (b) 40% blend juice (c) 80% blend juice (d) pomegranate juice

4.5. Antimicrobial Activity on Spores

4.5.1. Guaiacol Production of Spores in Apple Juice

For the determination of guaicol production, spores produced on PDA and BATA were inoculated separately into apple juice supplemented with 100 ppm vanillin or 100 ppm vanillic acid. The inoculum levels were 10^1, 10^3 and 10^5 CFU/mL for each spore suspension. During storage at 37 °C, guaiacol concentrations were measured by PECA at certain time intervals (Table 4.17) using guaiacol curve given in Appendix B (Fig.B4). This method was used because it is easy to apply and requires less time than HPLC. On the other hand, the concentrations below 10 mg/L can not be determined exactly (Bahçeci and Acar, 2007a). At the lowest inoculum level (10^1 CFU/mL), the guaiacol concentration was always below the detection limit in the apple juice containing 100 mg/mL vanillin. When the vanillic acid was used as guaiacol precursor

instead of vanillin, the concentration of guaiacol exceeded 10 mg/L even though the inoculum level was 10^1 CFU/mL.

If the inoculum level was increased to 10^3 CFU/mL, the guaiacol production was faster in the juice samples containing vanillic acid. As seen in Table 4.17, the concentrations were found as 58.51 and 56.09 mg/L for spores produced on PDA and BATA, respectively after 48 h. In the case of vanillin, the spores produced on PDA and BATA produced 30.19 and 29.27 mg/L guaiacol after 264 h. At the highest inoculum level (10^5 CFU/mL), the spores from PDA and BATA yielded 30.13 and 29.90 mg/L guaiacol in the apple juice supplemented with vanillin after 48 and 72 h incubation, respectively. In addition, at the highest inoculum level, 59.76 mg/L and 60.66 mg/mL guaiacol were produced in apple juice spiked with vanillic acid from spores PDA and BATA, respectively after 24 h.

Table 4.17. Guaiacol production (mg/L) during storage at 37 °C

Initial spore count (CFU/mL)	**Time (h)**	**PDA**	**BATA**	**PDA**	**BATA**
		100 ppm vanillin		**100 ppm vanillic acid**	
10^1	0	nd	nd	nd	nd
	24	7.41±0.03	5.91±0.52	11.86±0.43	12.73±0.34
	48	5.27±0.14	6.96±0.00	13.10±0.91	11.82±1.64
	72	7.81±0.03	6.98±0.69	13.80±0.02	12.68±0.05
	144	7.80±0.04	6.96±0.02	14.90±1.05	15.78±0.09
	168	8.13±0.49	8.85±0.88	14.71±1.62	17.76±1.31
	264	7.13±0.49	6.37±0.78	12.60±1.40	13.95±0.26
10^3	0	nd	nd	nd	nd
	24	7.26±0.79	8.29±0.19	14.01±1.17	11.54±0.95
	48	6.19±1.26	7.00±0.68	58.51±3.67	56.09±1.05
	72	6.86±0.61	7.88±0.71	58.41±1.07	52.47±0.19
	144	6.40±0.30	7.90±0.20	59.30±0.53	55.04±0.16
	168	6.18±0.52	10.14±0.91	60.54±0.19	55.10±0.03
	264	30.19±0.53	29.27±3.35	55.52±1.97	43.16±1.03
10^5	0	nd	nd	nd	nd
	24	9.58±0.26	7.68±0.09	59.76±1.07	60.66±4.38
	48	30.13±0.21	29.78±0.47	59.02±1.40	57.59±0.02
	72	30.70±0.52	29.90±1.12	55.49±1.43	52.34±0.03
	144	31.24±1.78	30.38±2.59	57.87±2.17	50.00±0.22
	168	29.94±0.66	29.26±0.17	60.13±0.16	54.14±1.36
	264	28.30±1.41	30.70±4.66	53.34±1.69	49.48±1.48

† The experiments were repeated four times, and data are expressed as mean ± standard deviation

In fact, the relationship between the guaiacol production and growth of Alicyclobacilli has not been well documented. It has been supposed that limited amount of guaiacol is produced in logarithmic growth phase. In the stationary phase or exposure

to stress conditions, the production may be triggered (Orr et al., 2000). The obtained results here showed that initial spore level, type of precursor, sporulation media used and incubation time were important for guaiacol concentration. In another study, the guaiacol formation has been found to be related to vanillin concentration, inoculated spore levels and temperature in apple juice (Bahçeci et al., 2005). Similar to the results obtained in this study, the metabolic conversion of vanillic acid to guaiacol was found to be faster than the conversion of vanillin to guaiacol (Witthuhn et al., 2012).

4.5.2. Antimicrobial Activity of Grape Seed Extract

The surviving spores of *A. acidoterrestris* after juice pasteurization turn into vegetative cells subsequently producing off–flavor compounds during juice storage. And, taint compounds in fruit juices can be produced in the presence of about 10^4–10^5 CFU/mL of *A. acidoterrestris* cells (Bahçeci et al., 2005; Gocmen et al., 2005; Pettipher et al., 1997). Therefore, the inhibition and control of *A. acidoterrestris* spore germination/outgrowth in fruit juices is necessary.

The effect of GSE was evaluated on spores from different media in the apple juice with 0–1.8% (v/v) GSE at 37 °C for 336 h (Fig.4.40). Untreated control (without GSE) increased by 2.2–2.6 log CFU/mL in the apple juice after 48 h. On the other hand, the use of 0.9% and 1.8% GSE decreased continuously the number of spores produced on PDA, BATA, BAA and MEA during storage suggesting the addition of GSE might inhibit the spore germination/outgrowth. After 336 h, the total population at the highest tested concentration (1.8%) decreased 1.8, 1.9, 1.4, and 2.7 log among spores from PDA, BATA, BAA and MEA, respectively. And, the use of 0.9% GSE caused 1.8, 1.6, 1.5 and 2.6 log reductions, among spores from PDA, BATA, BAA and MEA, respectively. At the lower concentrations, the spore counts from BATA, BAA and MEA also decreased. In all cases, total population in apple juice containing GSE was less than the control after 48 h incubation. On the other hand, the growth was not inhibited among spores produced on PDA in the presence of 0.06, 0.12, 0.23 and 0.45% concentrations of GSE in apple juice (Fig.4.40).

Spore germination starts with the interaction of germinants with receptors such as Ger A, Ger B and Ger C. After the germination process, the bursting of the spore coat and the subsequent emergence of the vegetative cell called outgrowth occurs (Pandey et

al., 2014; Setlow, 2003). Germination may take place in a short time (within minutes) depending on the strains and sporulation conditions and can initiate in the presence of certain amino acids, sugars or other compounds in the complex culture media in bacilli (Abbas et al., 2014).

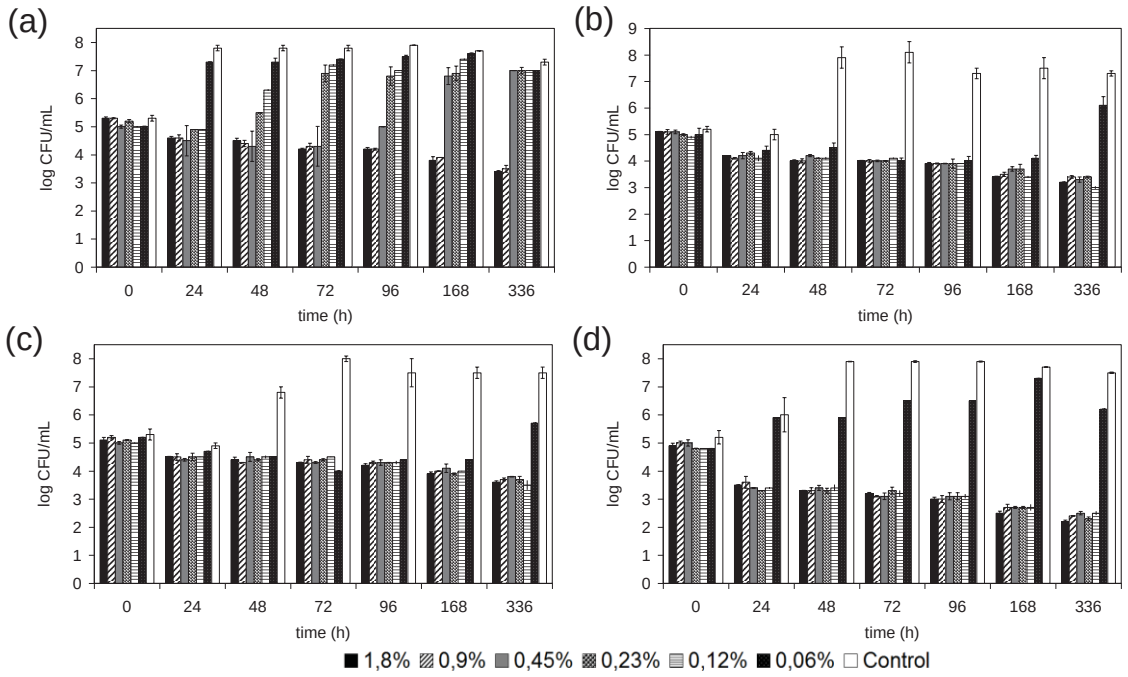

Figure 4.40. Log numbers of spores grown on (a) PDA (b) BATA (c) BAA (d) MEA in the apple juice with GSE

GSE used in this study was found to contain epicatechin (9827.07 ppm), gallic acid (700.93), quercetin–3–galactoside (536.52 ppm), rutin (277.25 ppm) and epicatechin gallate (249.22 ppm) (Yavuzdurmaz, 2013).

Polyphenols especially catechins have been shown to inhibit the growth of vegetative cells but not germination of *Bacillus* spp. spores (Friedman et al., 2006; Hara-Kudo et al., 2005; Pandey et al., 2014; Shigemune et al., 2012). Similarly, the catechins indicated antimicrobial activity on *Clostridium botulinum* and *C. butyricum* spores but not on *B. cereus* spores (Hara-Kudo et al., 2005). They also showed that lower concentrations of catechins were found to inhibit the bacterial spores with a longer incubation period. After exposure to catechins, damage to the spore membrane of *C. butyricum* was observed by fluorescence microscopy.

In a recent study (Pandey et al., 2014), the effects of tea compounds such as gallic acid, gallocatechin gallate, Teavigo (>90% epigallocatechin gallate) and the

theaflavin 3,3'–digallate on the spore germination, outgrowth and subsequent vegetative cell growth of *B. subtilis* were evaluated. Incubation of spores in the presence of different concentrations of tea compounds did not result in any apparent differences in germination behavior when compared to the control. However, gallocatechin gallate and theaflavin 3,3'–digallate clearly caused more extended outgrowth stage with higher concentrations. Also, gallic acid strongly reduced the ability to grow out and theaflavin 3,3'–digallate influenced the growth of vegetative cells.

4.5.2.1. Scanning Electron Microscopy

The growth control of spores in apple juice without GSE contains both vegetative cells and spores after 336 h (Fig.4.41).

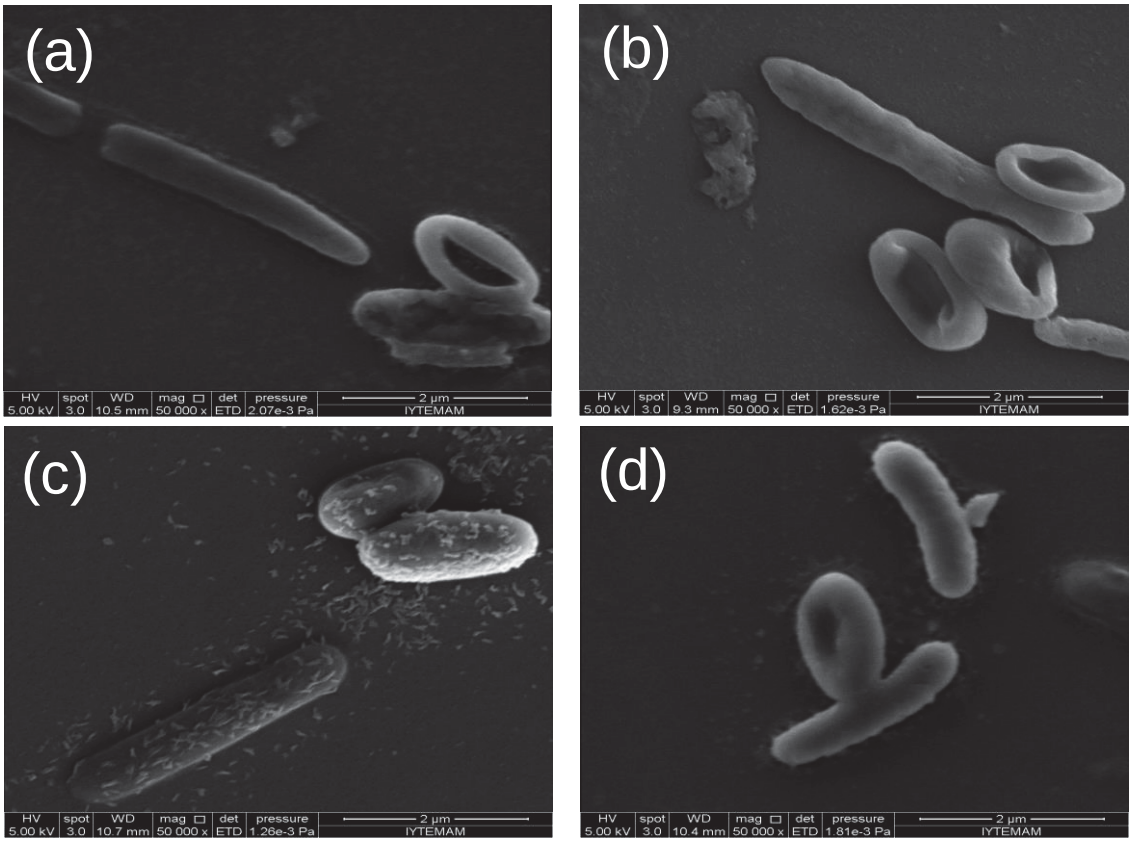

Figure 4.41. SEM images of untreated control spores in the apple juice after 336 h (a) PDA (b) BATA (c) BAA (d) MEA

After 168 h incubation, 0.9% and 1.8% GSE did not affect the integrity of spores (Fig.4.42). As the treatment time increased to 336 h, the spore shape still seems to be

unaffected (Fig.4.43). In some cases, shrinkage (Fig.4.42h) or collapsed spores (Fig.4.43a and d) are observed. Indeed, damage to the spore membrane of *C. butyricum* was observed after exposure to catechins by fluorescence microscopy (Hara-Kudo et al., 2005). On the other hand, the development of spores into vegetative cells is not visualized among treated spores. Neutral phenolic compounds in red grape prevented the spoilage by *Alicyclobacillus* spp. Similar to our findings, the addition of catechin gallate into the apple juice was found to inhibit the endospore germination of *Alicyclobacillus* (Splittstoesser et al., 1994).

Since the majority of the antimicrobial compounds in plant extracts are composed of water soluble phenolics, adsorption of the antimicrobial compounds onto the spores was supposed to be important for the induction of inactivation (Cui et al., 2011).

The antibacterial action of catechins on cells and spores differs due to differences in their catechin adsorption. The outer layer of *Bacillus* spp. contains peptidoglycan cell wall. ECG may pass through the cell wall and bind hydrophobically to the lipid bilayer. In contrast, ECG is not readily adsorbed by either the exosporium or the spore coat. Therefore, catechins do not suppress spore germination or inactivate spores because they are not adsorbed by spores and thus can not act on them (Shigemune et al., 2012).

Among the phenolic components of GSE, gallic acid might also involve in lowering the intracellular pH and disturb the build up of a proton gradient in the germinating spore (Ter Beek and Brul, 2010). In another study (Pandey et al., 2014), gallic acid was also supposed to interfere with the interaction of germinant receptor necessary for the initiation of germination.

Therefore, it has been suggested that the certain phenolic components in GSE might prevent the germination/outgrowth of *A. acidoterrestris* spores in apple juice.

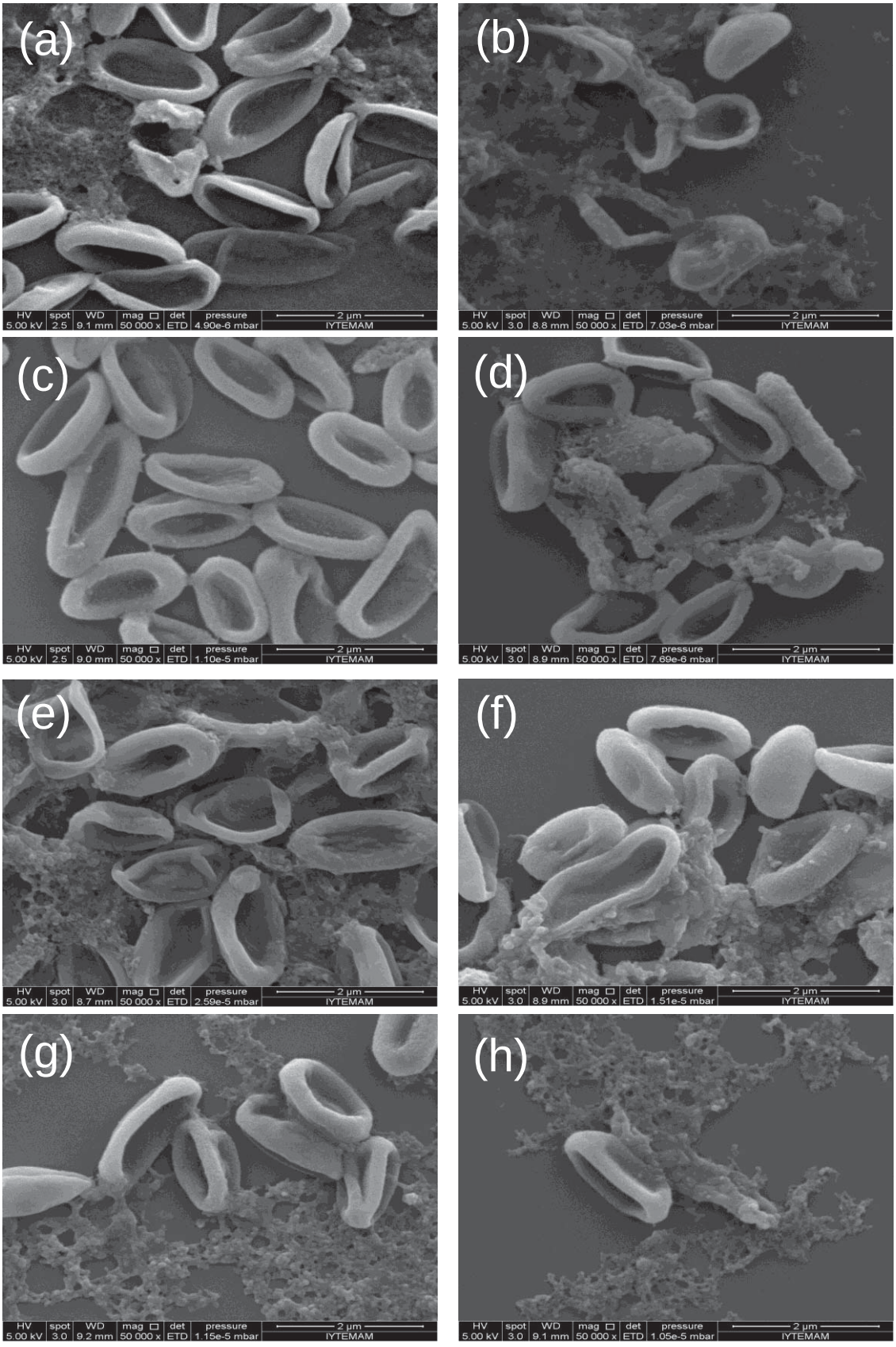

Figure 4.42. SEM images of spores in the apple juice with 0.9% GSE (a) PDA (b) BATA (c) BAA (d) MEA and 1.8% GSE (e) PDA (f) BATA (g) BAA (h) after 168 h

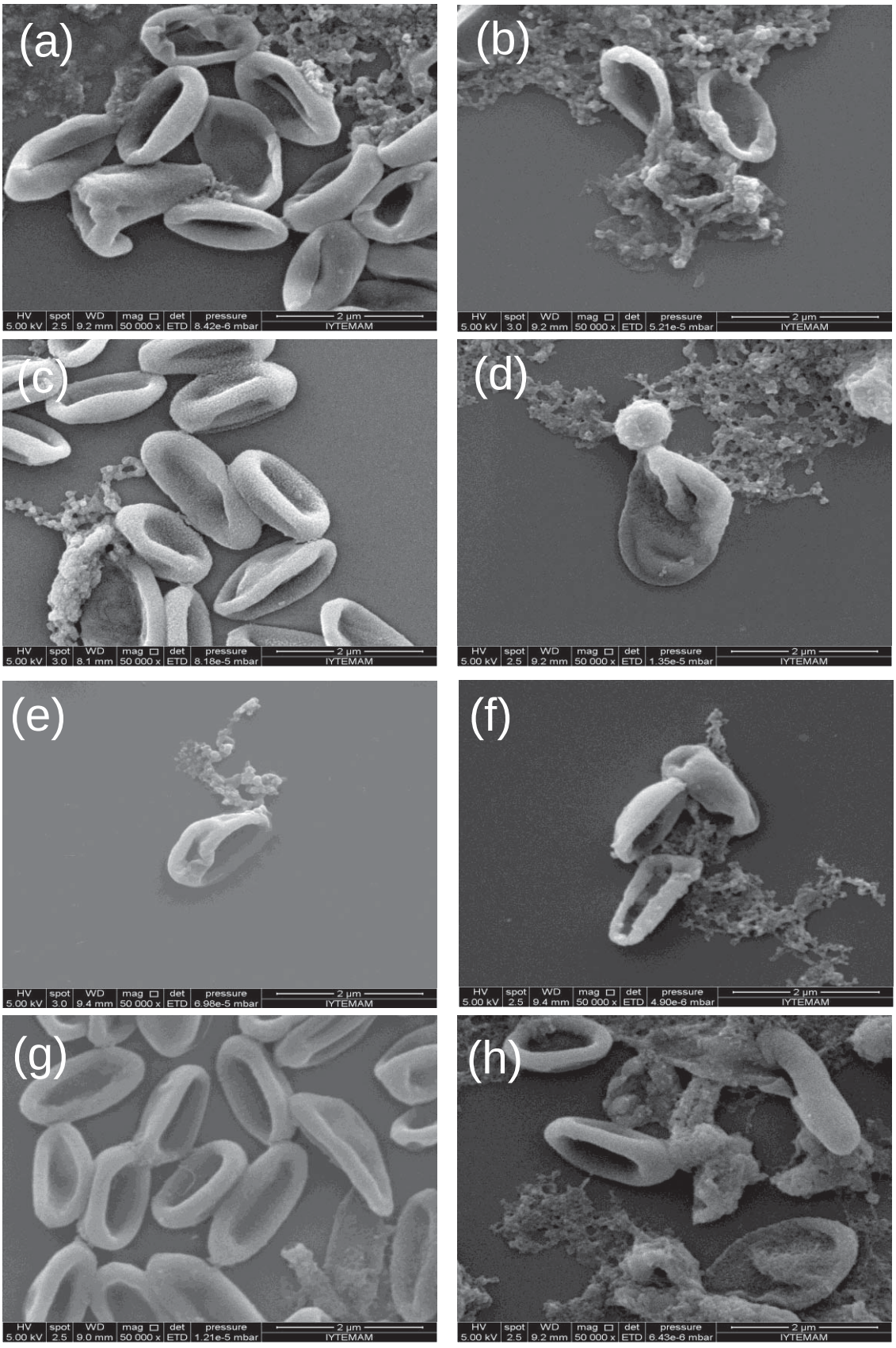

Figure 4.43. SEM images of spores in the apple juice with 0.9% GSE (a) PDA (b) BATA (c) BAA (d) MEA and 1.8% GSE (e) PDA (f) BATA (g) BAA (h) after 336 h

4.5.3. Antimicrobial Activity of Pomegranate Fruit Extract

Pomegranates have the highest level of punicalagin among the most commonly consumed fruits. The antioxidant, antifungal and antimicrobial properties of punicalagin have been shown (Ismail et al., 2012). The spores were incubated in the apple juice with 0–4% PE at 37 °C (Fig.4.44). In apple juice, approximately, 1.9, 2.1, 2.2, and 2.1 log CFU/mL increase in spore counts was obtained with respect to untreated controls from PDA, BATA, BAA and MEA, respectively after 336 h. Compared to untreated control, at all tested concentrations, PE inhibited the germination/outgrowth of spores produced on PDA, BATA, BAA and MEA and decreased continuously during treatment. At the end of the treatment, the number of spores at the highest tested concentration (4%) decreased 1.6, 1.65, 1.4, and 1.6 log among spores from PDA, BATA, BAA and MEA, respectively. At the lowest concentration tested (0.125%), the spore counts from PDA, BATA, BAA and MEA showed 1.4, 0.9, 0.7 and 1.3 log reductions. In all cases, total population in apple juice containing PE was less than the control after 48 h (Fig.4.44). Although many reports on the antimicrobial activity of pomegranate extracts exist for vegetative cells, there is no study related to its activity on spores.

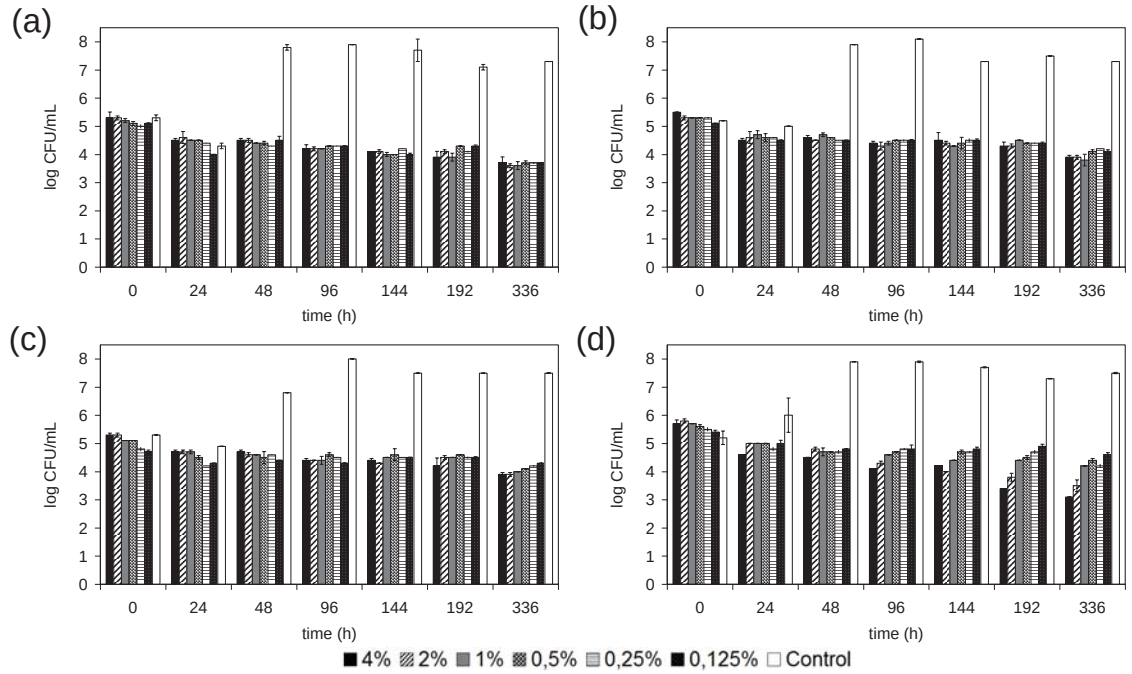

Figure 4.44. Log numbers of spores grown on (a) PDA (b) BATA (c) BAA (d) MEA in the apple juice with PE

4.5.3.1. Scanning Electron Microscopy

The untreated controls indicate the presence of spores and vegetative cells that maintain the structural integrity (Fig.4.45a and 4.46a). On the other hand, the treated samples indicate only the presence of spores (Fig.4.45b–c and 4.46b–c). Spore shape and integrity do not seem to be destroyed in the apple juice with PE. The use of PE as a natural compound against *A. acidoterrestris* spores could prevent additionally the formation of vegetative cells and as consequent off–flavor compounds even if it is added at lower concentrations into the apple juice. Similarly, phenolics originated from spices such as gingeron, zingerone and capsaicin have been found to inhibit the germination of bacterial spores (Leon and Eddy, 2007).

Fernandes et al. (2009) found that chitosan was not effective on the morphology of *B. cereus* spores with only around 1 log–reduction. Chitosan was found to prevent the nutrient uptake of cells by AFM imaging but its penetration into the spores was difficult. They indicated that spores did not seem to be destroyed or collapsed as observed among vegetative cells after chitosan treatments. Chitosan only caused the loss of exosporium which is essential for spore germination. In this study, it was also interesting that the spore morphology was not changed after PE treatments but reductions were higher than 1 log CFU/mL at the concentrations used for SEM imaging (1.7 and 1.4 log CFU/mL for spores produced on PDA and BATA, respectively in the presence of 2% extract).

Pomegranate peel contains ellagitannin, punicalagin, ellagic and gallic acids. Punicalagin is the active compound for the antimicrobial activity of pomegranate. Pomegranate peels were found to precipitate membrane proteins and inhibit enzymes such as glycosytransferases causing to cell lysis (Ismail et al., 2012; Parashar et al., 2009).

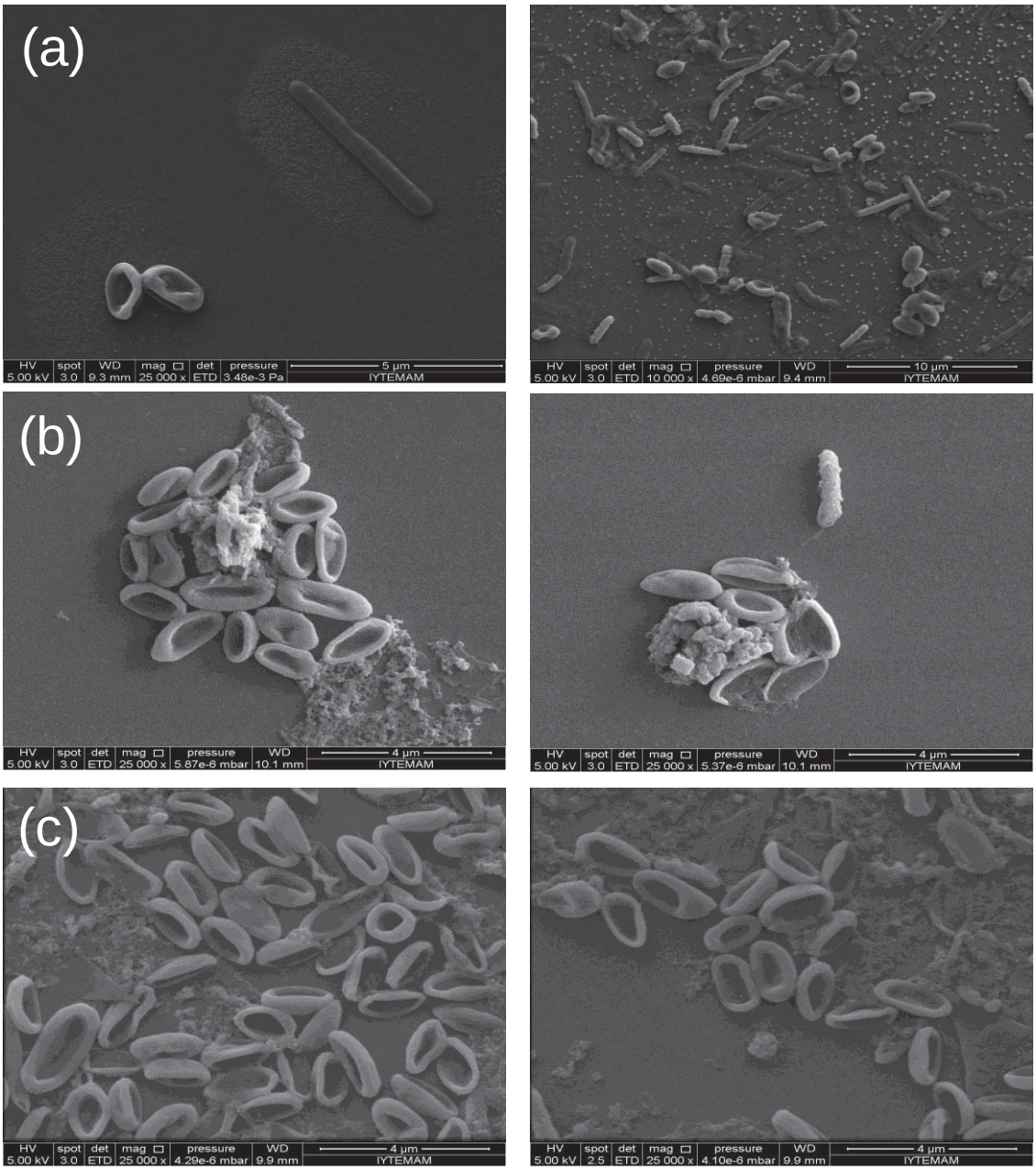

Figure 4.45. SEM images of spores grown on PDA in the apple juice with PE after 336 h (a) control (b) 2% (c) 1%

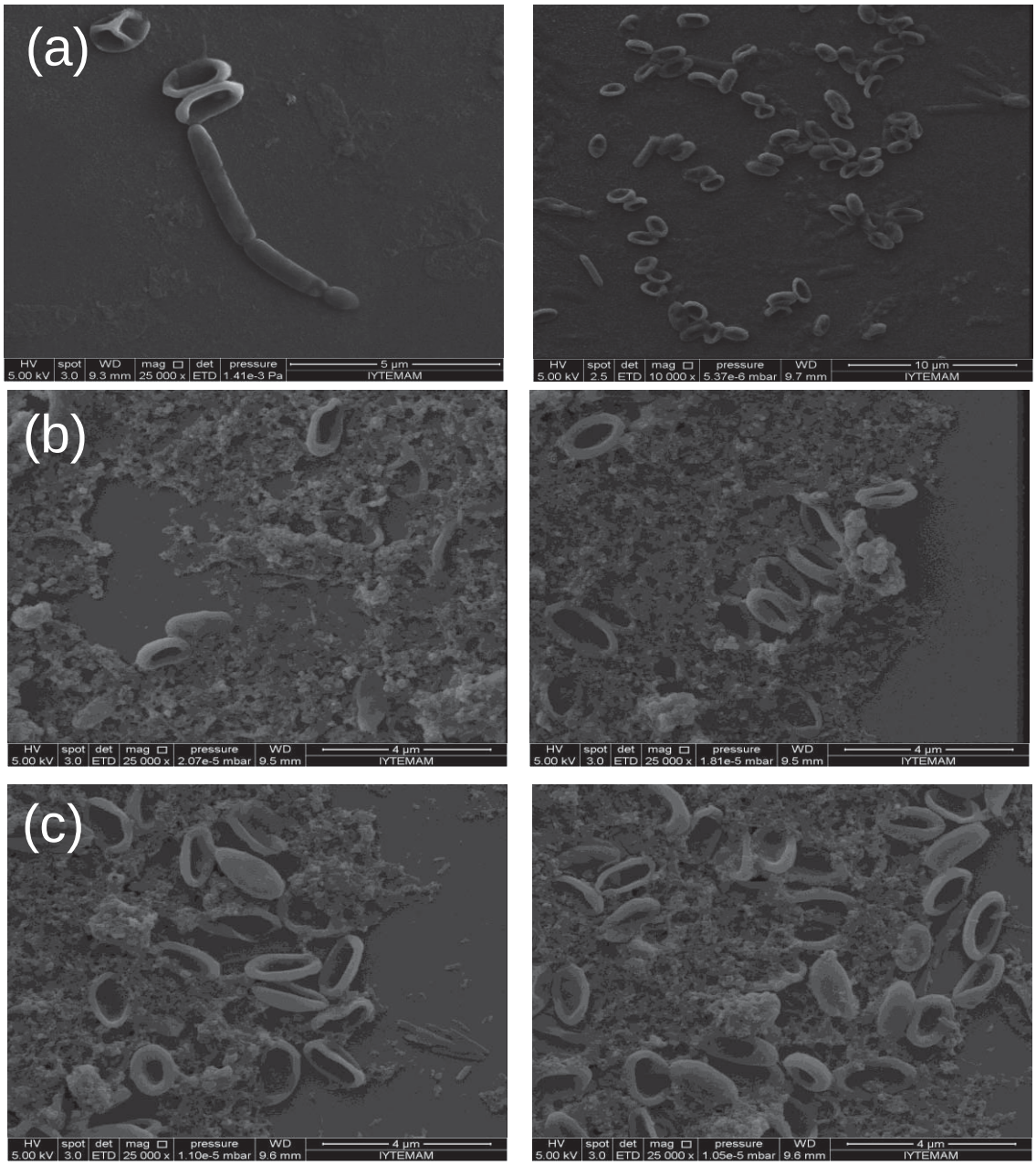

Figure 4.46. SEM images of spores grown on BATA in the apple juice with PE after 336 h (a) control (b) 2% (c) 1%

4.5.4. Antimicrobial Activity of Pomegranate and Blend Juices

Due to high acidity, astringency, bitterness and other factors present in fruits and vegetables, the utilization of these fruits for the production of processed products is limited although they have high nutritional values. Therefore, blending of two or more fruit or vegetable juices for the preservation of ready–to–drink beverages may be used as alternative for the utilization of these fruits and vegetables (Bhardwaj and Pandey,

2011). Blending of different fruit, vegetable and spiced juices improve storability and microbial growth in juice and thus can be stored effectively for long periods without chemical changes (Bhardwaj and Pandey, 2011).

All tested juice samples containing pomegranate juice decreased the counts among spores produced on mineral containing media (BATA and BAA). On the other hand, total population from nonmineral containing media (PDA and MEA) differed depending on the concentration used. While total population from PDA was increased in the presence of 10%, 20% and 40% blend juice, only the use of 10% juice resulted in an increase after 168 h among spores produced on MEA (Fig.4.47). A similar result was obtained for spores produced on PDA treated by GSE. Only the use of 1% and 2% GSE in the apple juice inhibited the spores from PDA. The counts in pomegranate juice indicated 1.49, 1.65, 1.67, 1.28 log reductions among spores produced on PDA, BATA, BAA and MEA, respectively (Fig.4.47). In addition, 1.51, 1.38, 1.4 and 1.16 log reductions were obtained in 80% blend juice among spores from PDA, BATA, BAA and MEA, respectively. Based on the results, pomegranate and blend juices have been suggested to inhibit spore germination/outgrowth.

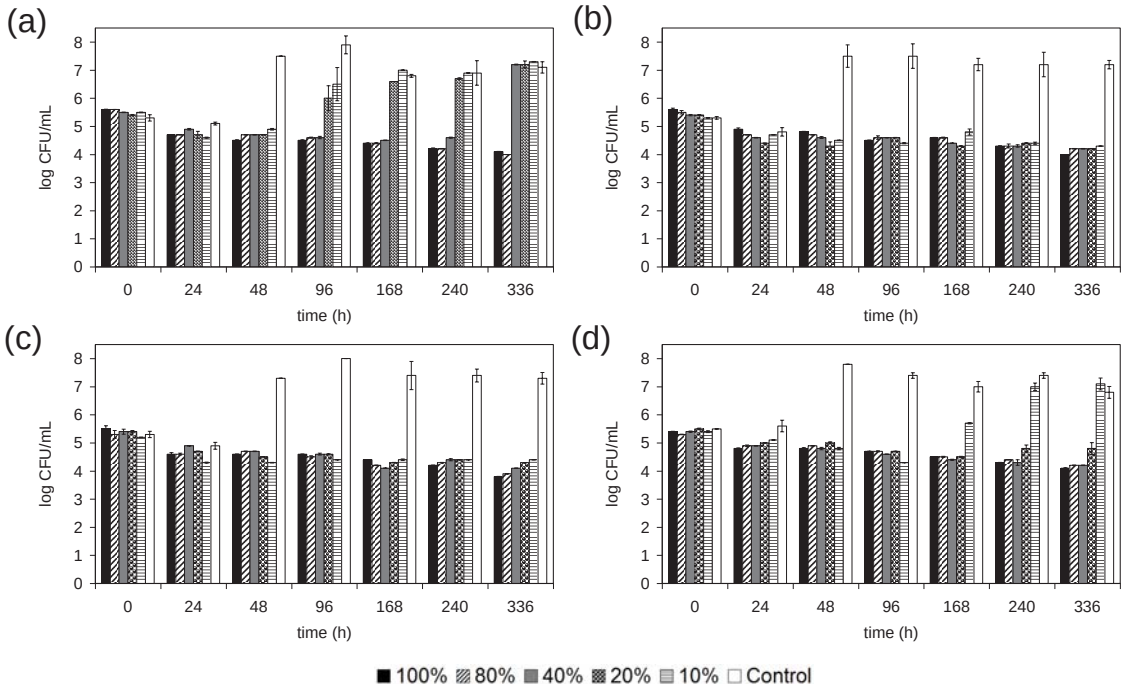

Figure 4.47. Log numbers of spores produced on (a) PDA (b) BATA (c) BAA (d) MEA in pomegranate and pomegranate–apple blend juices

Growth of Alicyclobacilli was not detected in mixed juices such as apple/orange/pineapple, apple/raspberry/grape and cranberry cocktail. The reason for this might be the low pH of these juices (2.5–3.0). Also, the growth of *A. acidoterrestris* was not detected apple/cranberry, 10% fruit juices, pineapple juice and salsa (Walls and Chuyate, 2000). In another study, the growth was inhibited with the use of 1000 ppm catechin gallate and phenolic compounds naturally found in red grape juice were found to affect the ability of *A. acidoterrestris* spores to germinate and grow (Splittstoesser et al., 1994). Polyphenols extracted from grapes such as cumaric and ferulic acids indicated strong inhibitory effect (Oita and Koyama, 2002). The reason for growth and spoilage only occurring in certain juices is unclear, but these juices contain might contain different concentrations of guaiacol precurcors or growth inhibitors (Walls and Chuyate, 2000). Possibly, these inhibitory compounds inhibit spore germination or cell growth (Tokuda, 2007).

4.5.4.1. Scanning Electron Microscopy

The representative spores from PDA, BATA and BAA were examined by SEM to visualize the spore morphology that occurred during growth in apple juice and pomegranate juice. As expected from viable count data, the injury of spore was very low that could not be detected visually by SEM (Fig.4.48–4.50). As seen, both vegetative cells and spores are present during growth in apple juice but no vegetative cells are formed during growth in pomegranate juice. Therefore, it can be concluded that the mode of action of phenolics present in pomegranate juice does not inactivate or injure *A. acidoterrestris* spores. It seems that phenolic substances might prevent the germination, the development of the vegetative cells and eventually growth. In this way, the potential risk of apple juice spoilage due to production of off–odor compounds in the fruit juices can be inhibited. On the other hand, the site of action of the phenolics found in pomegranate juice and natural extracts to prevent spore germination was not fully understood according to the obtained results. If no effects are observed on cell structure and membrane functionality, it is supposed that the site of action is intracellular. The target can be proteins and enzymes in general, or essential cellular processes involved in biosynthesis or energy generation. An intracellular site of action

can be determined incorporating radioactively labeled substrates particularly used in biosynthesis pathways (Hyldgaard et al., 2012).

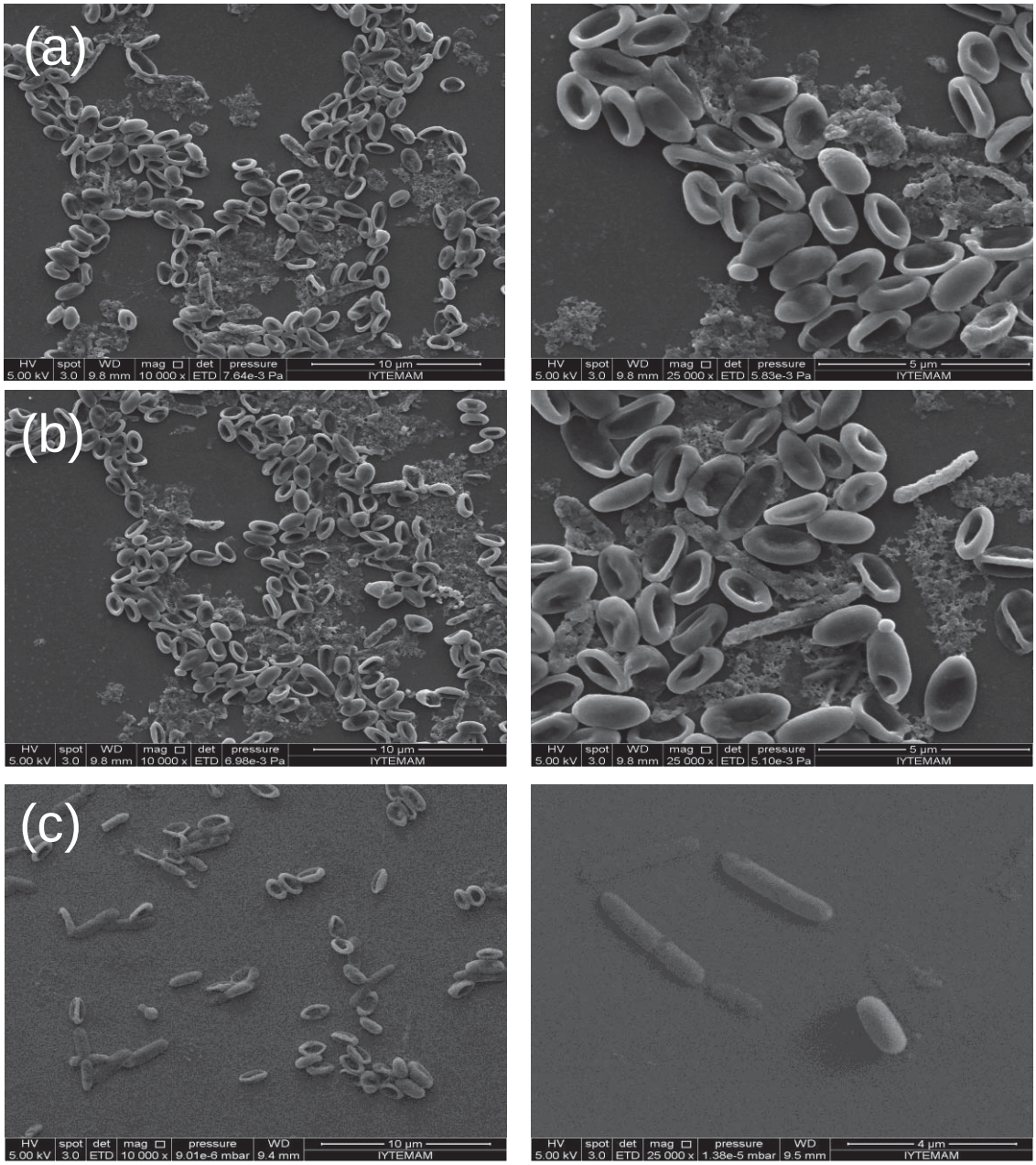

Figure 4.48. SEM images of spores grown on PDA after 336 h in (a,b) pomegranate and (c) apple juices

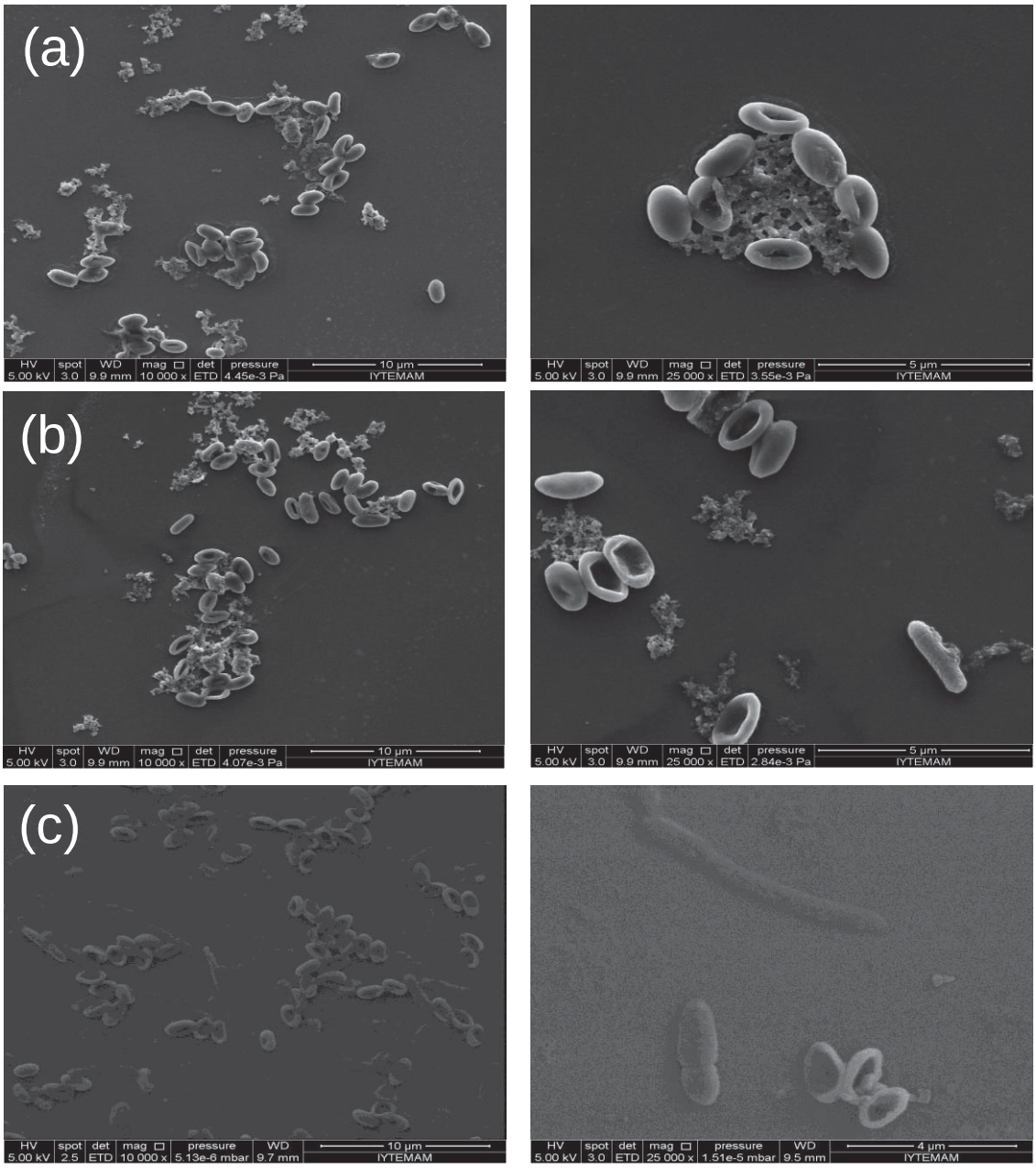

Figure 4.49. SEM images of spores grown on BATA after 336 h in (a,b) pomegranate and (c) apple juices

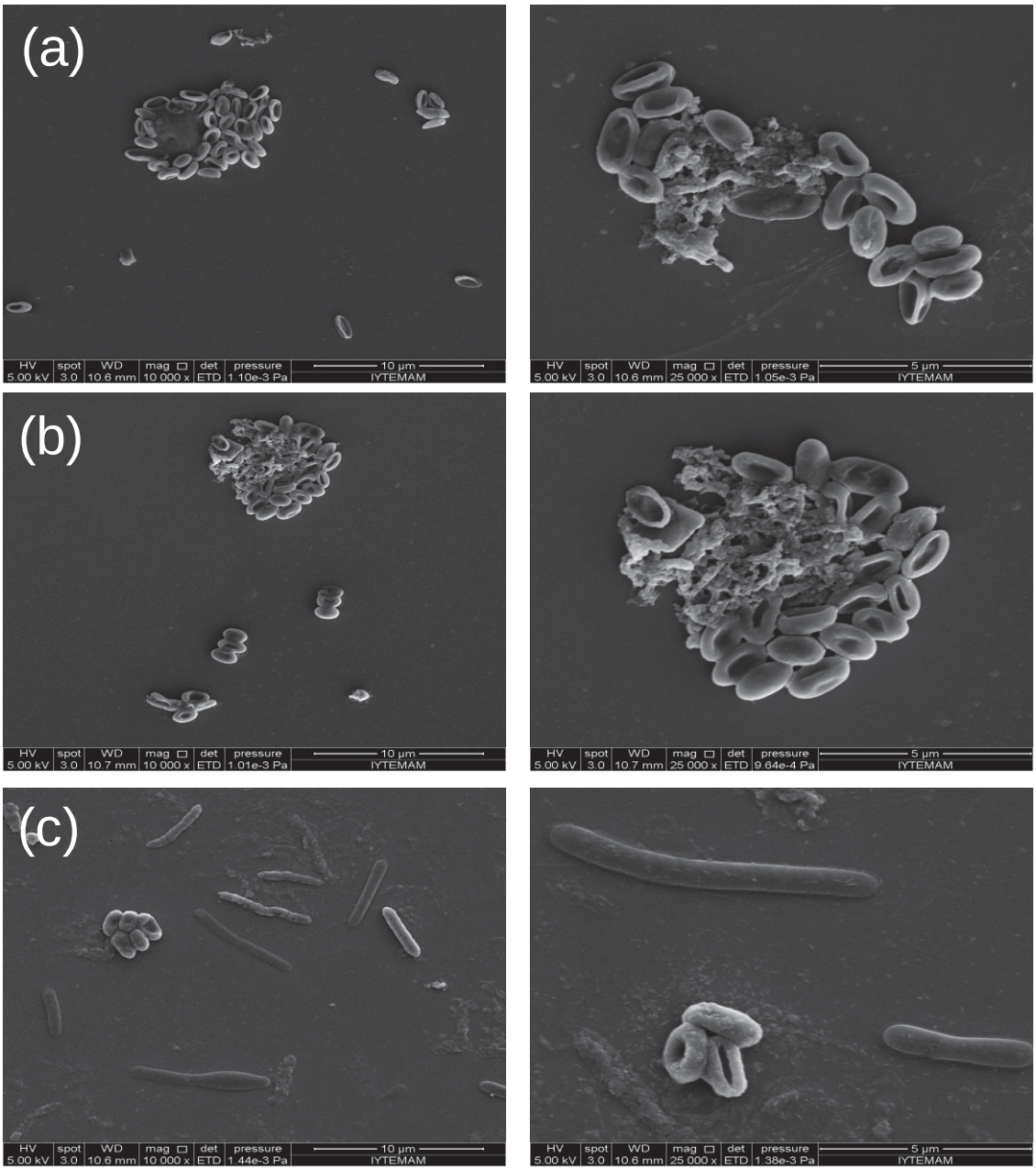

Figure 4.50. SEM images of spores grown on BAA after 336 h in (a,b) pomegranate and (c) apple juices

4.5.5. Surface Hydrophobicity

Thermal treatment is not recommended to eliminate vegetative cells from spore suspensions that will be used to determine the spore hydrophobicity because of the increase in hydrophobicity after sublethal heat treatment (Wiencek et al., 1990). The relative hydrophobicity of cells and spores was determined using MATH assay (Table 4.18). It is known that if the organism is more hydrophobic, it will be more sensitive to

hydrophobic antimicrobials (Chaibi et al., 1997). *A. acidoterrestris* DSM 3922 cells were found highly hydrophobic (71.73±0.98%). Indeed, vegetative cells of *Bacillus* spp. are not thought to possess significant amounts of surface protein. In contrast, spores have proteinaceous coats that confer the sites for the adherence to hydrocarbon (Doyle et al., 1984). Therefore, spores are thought to be more hydrophobic than vegetative cells. However our results indicated that the surface hydrophobicity of vegetative cells was higher than the spores most probably due to the presence of ω–fatty acids on the cell surface which might explain the possible interaction with the tested antimicrobials. As an example, phenolic contents found in grape seed are partially hydrophobic and are considered to interact with the bacterial cell wall and lipopolysaccharide interfaces by decreasing membrane stability (Shrestha et al., 2012). Sporulation media and conditions were reported to have little effect on spore hydrophobicity. On the other hand, exposure to thermal treatment was found to increase the spore hydrophobicity (Wiencek et al., 1990). Similar to this finding, there was no clear correlation between the composition of the sporulation media and *A. acidoterrestris* spore hydrophobicity. Based on the results of antimicrobial activity, the inhibition of spore germination produced on PDA was achieved at higher concentrations of GSE in apple juice (0.9% and 1.8%), pomegranate and 80% pomegranate–apple blend juices (v/v) than other spore suspensions. It is known that the hydrophobic compounds are also known to inhibit the spore germination by hydrophobic interaction between the compound and hydrophobic region of the spore (Yasuda-Yasaki et al., 1978).

Table 4.18. Adherence to n–hexadecane of cells and spores (%)

	Hydrophobicity (%)	Classification
Vegetative cells	71.73±0.98	Strong hydrophobic
Spores grown on		
PDA	49.50±4.07	Moderately hydrophobic
BATA	25.55±3.86	Moderately hydrophilic
BAA	34.56±2.49	Moderately hydrophobic
MEA	3.77±1.73	Strong Hydrophilic

Additionally, a change in the amount of cells that can adhere to a solvent is an expression of a change in surface structure. If it increases, lipids are thought to be released into the medium making the cells more susceptible to other stress conditions (Tsuchido and Shibasaki, 1980). Therefore, the combination of antimicrobial with other

thermal or nonthermal methods may be more effective to inactivate cells and spores due to the change in their surface structure.

4.6. Chemical Treatment

NaOH solutions are the most commonly used chemical cleaning detergents on industrial scale. It is effective in dissolving both proteinaceous and fatty soils, also has emulsifying properties. H_2O_2 is active against Gram–positive and Gram–negative bacteria. Since anaerobes are unable to produce catalase, they are particularly susceptible. At high concentrations, H_2O_2 is sporicidal and this activity increases as the temperature is increased (Russell, 2010). This compound is environmentally friendly because its decomposition products are oxygen and water (Fraise, 2002). H_2O_2 in the vapor phase is mostly used for surface sterilization purposes (Russell, 2010). While sanitization processes, along with pasteurization and/or evaporation, have apparently been adequate to control most microbial spoilage and pathogenic organisms, these protocols are not sufficient for the control of spore–forming organisms such as *Alicyclobacillus* spp. (Friedrich et al., 2009). The obtained results align with the related literature indicating the ineffectiveness of chemical agents against spores. Chemical treatments did not cause significant log reductions (CFU/mL)among spores produced on different media. In all cases, the log reduction was lower than 1; 90 min treatment with NaOH resulted in 0.83, 0.67, 0.55 and 0.57 log reductions among the spores produced on PDA, BATA, BAA and MEA, respectively; the use of 0.2% fumaric acid caused approximately 0.3 log reduction among spores and 4% H_2O_2 caused approximately 0.54, 0.96, 0.41 and 0.63 log reductions among spores produced on PDA, BATA, BAA and MEA, respectively (Table 4.19).

Table 4.19. Log reductions obtained after chemical treatments

Treatment	**Sporulation media**			
	PDA	BATA	BAA	MEA
1 N NaOH–30 min	$0.61\pm0.11^{A\dagger}$	0.53 ± 0.01^{AB}	0.34 ± 0.02^{C}	0.36 ± 0.04^{BC}
1 N NaOH–60 min	0.80 ± 0.11^{A}	0.59 ± 0.00^{B}	0.43 ± 0.00^{C}	0.45 ± 0.02^{C}
1 N NaOH–90 min	0.83 ± 0.15^{A}	0.67 ± 0.01^{AB}	0.55 ± 0.01^{B}	0.57 ± 0.01^{B}
0.2% Fumaric acid–60 min	0.33 ± 0.01^{A}	0.29 ± 0.02^{A}	0.31 ± 0.03^{A}	0.21 ± 0.03^{B}
2% H_2O_2–10 min	0.17 ± 0.01^{A}	0.62 ± 0.04^{B}	0.21 ± 0.01^{A}	0.18 ± 0.04^{A}
4% H_2O_2–10 min	0.54 ± 0.07^{BC}	0.96 ± 0.02^{A}	0.41 ± 0.05^{C}	0.63 ± 0.06^{B}

†Different capital letters within each rows differ significantly ($p<0.05$)

The proteinaceous spore coats, low permeability of the spore core to hydrophilic chemicals, low spore core water content and protection of spore DNA by α/β–type play a significant role in spore resistance to chemicals (Nicholson et al., 2000). It has been shown that at very high concentrations of H_2O_2, especially at elevated temperatures, it can cause major dissolution of spores with loss of structure of coats, cortex and core (Shin et al., 1994). However, at lower concentrations of H_2O_2, it can kill spores without inducing microscopically evident cytological changes. To visualize the effect of H_2O_2 on spore structure, 8% H_2O_2 was applied without neutralization by bovine liver catalase. As seen in Fig.4.51, H_2O_2 led to the extrusion of the inner material of spores produced on BAA.

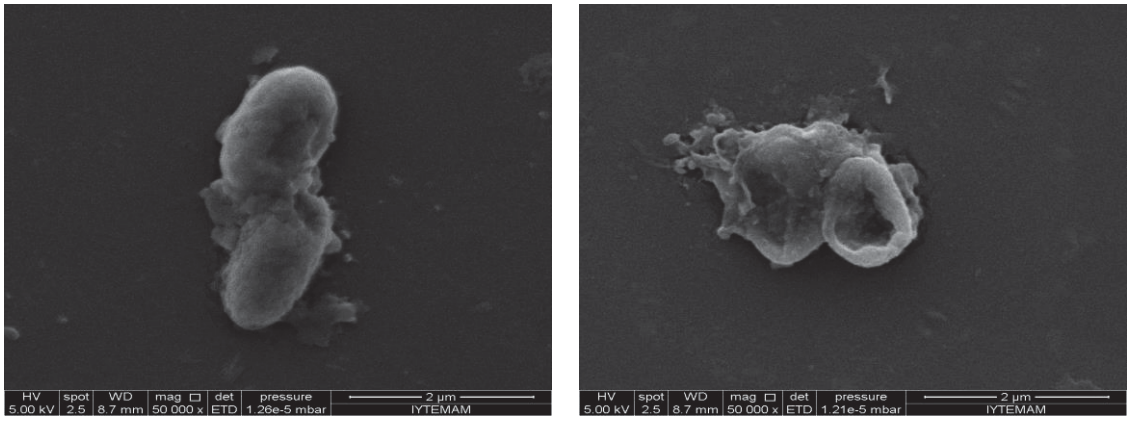

Figure 4.51. SEM images of 8% H_2O_2 treated spores produced on BAA

CHAPTER 5

CONCLUSION

Fruit juice industry has been suffering from spoilage problems due to the growth of *A. acidoterrestris* in their products since the early 1980s. Therefore, research is still ongoing to develop new methods to prevent the spoilage caused by *A. acidoterrestris* in fruit juices and understand the mechanism of the resistance against applied treatments.

Although the wet–heat resistance characteristics of *A. acidoterrestris* spores in various fruit juices were determined by many researchers in the literature; somehow, the effect of sporulation media on the wet–heat resistance properties of *A. acidoterrestris* has not been studied. Therefore, the comparison of resistance properties is difficult to control the process conditions in fruit juice industry. In the present study, it has been suggested that sporulation media affects the wet–heat resistance properties of *A. acidoterrestris* in the reconstituted apple juice depending on the heating temperature. Based on the results, wet–heat resistance of spores produced on mineral containing media (BATA and BAA) were found higher than that of spores formed on nonmineral containing media (PDA and MEA). The higher heat resistance among spores was correlated with the spore core's high levels of DPA and Ca^{2+}. DPA release took place at a much slower rate than spore killing by wet–heat. Although the exact mechanism of spore resistance to wet–heat is not clear, the presence of proteins and nucleic acids in the wet–heat treated spore suspensions indicated the rupture of spore coat and damage to spore inner membrane, respectively. Based on the SEM and AFM images, the sporulation media was also found to be effective on the spore external structure before and after wet–heat treatments. Since the main reservoir of *A. acidoterrestris* is soil that is rich in minerals, the results from mineral containing media will be more useful to control process conditions during pasteurization treatments in the fruit juice industry.

Nowadays, consumer demand for the use of natural preservatives instead of chemicals to control the growth of spoilage and pathogenic microorganisms in foods. Although the current pasteurisation treatments inactivate vegetative cells, these treatments are not sufficient to eliminate spores. The surviving spores can germinate and produce vegetative cells during storage leading to spoilage. However, most of the

studies investigated the antimicrobial activity of natural compounds against non–spore forming bacteria. The findings obtained in this study emphasizes the potential use of by–products of the juice industry such as grape seed and pomegranate extracts to inhibit the growth of *A. acidoterrestris* vegetative cells and spore germination in apple juice. Differences in the antimicrobial action of extracts could be explained by variations in their total phenol contents or the presence of certain growth inhibitors. From the results obtained, these natural extracts may be used as additives in the apple juice to prevent the spoilage.

Due to high polyphenolic compounds, pomegranate juices exhibit astringent taste. Thus, pomegranate juices can be blended with other juices to enhance sensory acceptance for consumption. There are few studies related to growth inhibition in mixed fruit juices but the mechanism of action on the growth inhibition has not been revealed yet. The blending of pomegranate juices with apple juices (40% and 80%, v/v) resulted in the growth inhibition of vegetative cells and spore germination as observed in the apple juice with natural extracts. While the cell numbers increased in the apple juices even after 24 h incubation, the log reductions were obtained in the pomegranate and blended juices (40% and 80%, v/v) indicating the antimicrobial action of phenolics present in pomegranate juice.

To our knowledge, there is no study about the modeling of inactivation kinetics of *A. acidoterrestris* cells in the presence of antimicrobial compunds. Also, most of the literature represent the modeling inactivation data in the laboratory media instead of using food systems. The inactivation rate in foods is expected to be lower due to the interaction with food components. Therefore, models were developed to describe the inactivation kinetics of *A. acidoterrestris* cells in apple juice with these extracts, and during growth in pomegranate and blend juices (40% and 80%) to fill the gap in the related literature. Among the tested models, the Weibull model was found best fitted model to the cell inactivation data.

Regarding data obtained in the production of guaiacol, the level of guaiacol production in the apple juice depends on the initial spore level, the type of guaiacol precursor and storage time. Besides wet–heat resistance, sporulation media was also found to be effective on the growth characteristics in the apple juice spiked with natural extracts and in pomegranate and blend juices.

Damages to the cell structure (leakage of cellular constituents; presence of perforations and blebs) due to the phenolic compounds in natural extracts and

pomegranate juice were observed using SEM and AFM techniques. Based on the results, it can be concluded that the mode of action of phenolic compounds in the tested extracts and pomegranate juice against spores is to prevent the germination, the development of the vegetative cells and eventually growth when compared to the control spores in the apple juice. Finally, natural extracts and pomegranate juice have been suggested to contain certain growth inhibitors to affect the ability of *A. acidoterrestris* vegetative cell growth and spore germination.

Nowadays, industry and consumers are interested in the health beneficial effects of products. Products with high phenolic content have been popular because of its functional and nutritional properties in human diet. The results of this study clearly showed that apple juice supplemented with grape seed or pomegranate extracts or blended with pomegranate juices could not only prevent the spoilage but may also enhance the nutraceutical value of juice resulting in the production of functional fruit juice/beverages for human health with higher antioxidant activity determined by FRAP and DPPH assays.

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APPENDIX A

GLNAFIT DATA OF WET–HEAT INACTIVATION KINETICS

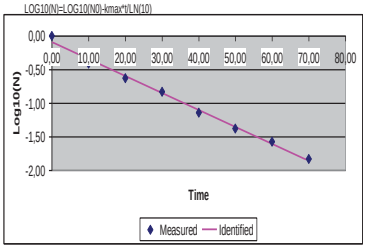
(a)

Time	Measured LOG10(N)	Identified LOG10(N)	Squared difference
0,00	0,00	-0,09	0,01
10,00	-0,42	-0,34	0,00
20,00	-0,63	-0,60	0,00
30,00	-0,83	-0,85	0,00
40,00	-1,14	-1,10	0,00
50,00	-1,38	-1,35	0,00
60,00	-1,57	-1,61	0,00
70,00	-1,83	-1,86	0,00
Least Sum of Squared Error			0,02

Parameters	Parameter	Standard Error
kmax	0,06	0,00
LOG10(N0)	-0,09	0,04

Mean Sum of Squared Error	0,0031
Root Mean Sum of Squared Error	0,0558
R-Square	0,9930
R-Square adjusted	0,9919

Inactivation model identified
N= N0 * exp(-kmax * t)
For identification purposes reformulated as
LOG10(N)=LOG10(N0)-kmax*t/LN(10)



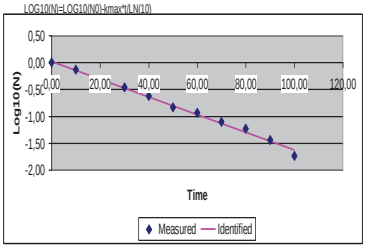
(b)

Time	Measured LOG10(N)	Identified LOG10(N)	Squared difference
0,00	0,00	0,02	0,00
10,00	-0,13	-0,15	0,00
20,00	-0,36	-0,31	0,00
30,00	-0,47	-0,48	0,00
40,00	-0,62	-0,64	0,00
50,00	-0,83	-0,81	0,00
60,00	-0,93	-0,97	0,00
70,00	-1,11	-1,14	0,00
80,00	-1,24	-1,30	0,00
90,00	-1,44	-1,47	0,00
100,00	-1,74	-1,63	0,01
Least Sum of Squared Error			0,02

Parameters	Parameter	Standard Error
kmax	0,04	0,00
LOG10(N0)	0,02	0,03

Mean Sum of Squared Error	0,0025
Root Mean Sum of Squared Error	0,0499
R-Square	0,9926
R-Square adjusted	0,9918

Inactivation model identified
N= N0 * exp(-kmax * t)
For identification purposes reformulated as
LOG10(N)=LOG10(N0)-kmax*t/LN(10)



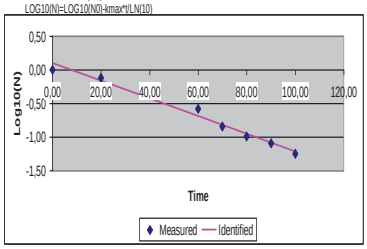
(c)

Time	Measured LOG10(N)	Identified LOG10(N)	Squared difference
0,00	0,00	0,10	0,01
20,00	-0,12	-0,16	0,00
40,00	-0,35	-0,42	0,01
60,00	-0,58	-0,69	0,01
70,00	-0,84	-0,82	0,00
80,00	-1,00	-0,95	0,00
90,00	-1,10	-1,08	0,00
100,00	-1,25	-1,22	0,00
Least Sum of Squared Error			0,03

Parameters	Parameter	Standard Error
kmax	0,03	0,00
LOG10(N0)	0,10	0,05

Mean Sum of Squared Error	0,0056
Root Mean Sum of Squared Error	0,0750
R-Square	0,9778
R-Square adjusted	0,9741

Inactivation model identified
N= N0 * exp(-kmax * t)
For identification purposes reformulated as
LOG10(N)=LOG10(N0)-kmax*t/LN(10)



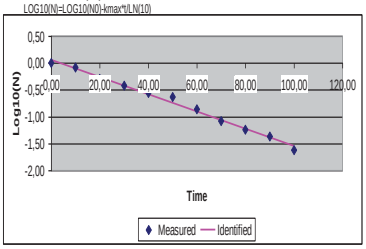
(d)

Time	Measured LOG10(N)	Identified LOG10(N)	Squared difference
0,00	0,00	0,06	0,00
10,00	-0,09	-0,10	0,00
20,00	-0,29	-0,26	0,00
30,00	-0,42	-0,42	0,00
40,00	-0,55	-0,58	0,00
50,00	-0,63	-0,74	0,01
60,00	-0,86	-0,90	0,00
70,00	-1,08	-1,06	0,00
80,00	-1,24	-1,22	0,00
90,00	-1,37	-1,38	0,00
100,00	-1,62	-1,54	0,01
Least Sum of Squared Error			0,03

Parameters	Parameter	Standard Error
kmax	0,04	0,00
LOG10(N0)	0,06	0,03

Mean Sum of Squared Error	0,0028
Root Mean Sum of Squared Error	0,0531
R-Square	0,9911
R-Square adjusted	0,9901

Inactivation model identified
N= N0 * exp(-kmax * t)
For identification purposes reformulated as
LOG10(N)=LOG10(N0)-kmax*t/LN(10)



(e)

Time	Measured LOG10(N)	Identified LOG10(N)	Squared difference
0,00	0,00	0,01	0,00
10,00	-0,14	-0,12	0,00
20,00	-0,25	-0,25	0,00
30,00	-0,38	-0,37	0,00
40,00	-0,47	-0,50	0,00
50,00	-0,65	-0,62	0,00
60,00	-0,72	-0,75	0,00
70,00	-0,83	-0,88	0,00
80,00	-1,03	-1,00	0,00
90,00	-1,11	-1,13	0,00
100,00	-1,29	-1,25	0,00
Least Sum of Squared Error			0,01

Parameters	Parameter	Standard Error
kmax	0,03	0,00
LOG10(N0)	0,01	0,02

Mean Sum of Squared Error	0,0008
Root Mean Sum of Squared Error	0,0284
R-Square	0,9959
R-Square adjusted	0,9954

Inactivation model identified
N= N0 * exp(-kmax * t)
For identification purposes reformulated as
LOG10(N)=LOG10(N0)-kmax*t/LN(10)

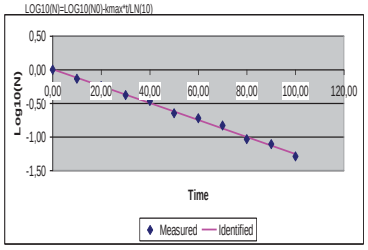


Figure A.1. Wet heat inactivation kinetics at 85 °C fitted by the log–linear model (a) PDA (b) MEA (c) BATA (d) BATB (e) BAA

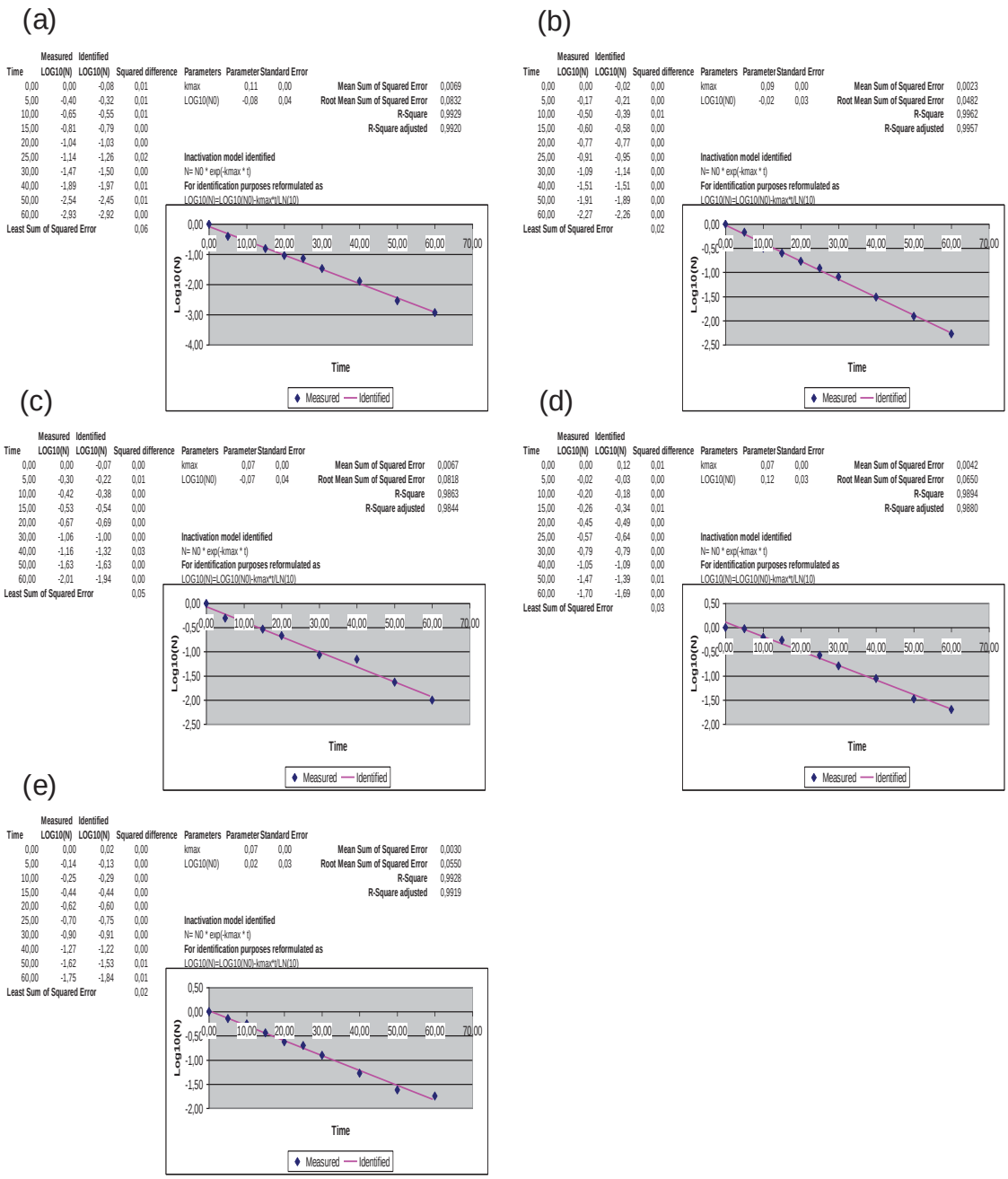


Figure A.2. Wet heat inactivation kinetics at 87.5 °C fitted by the log–linear model (a) PDA (b) MEA (c) BATA (d) BATB (e) BAA

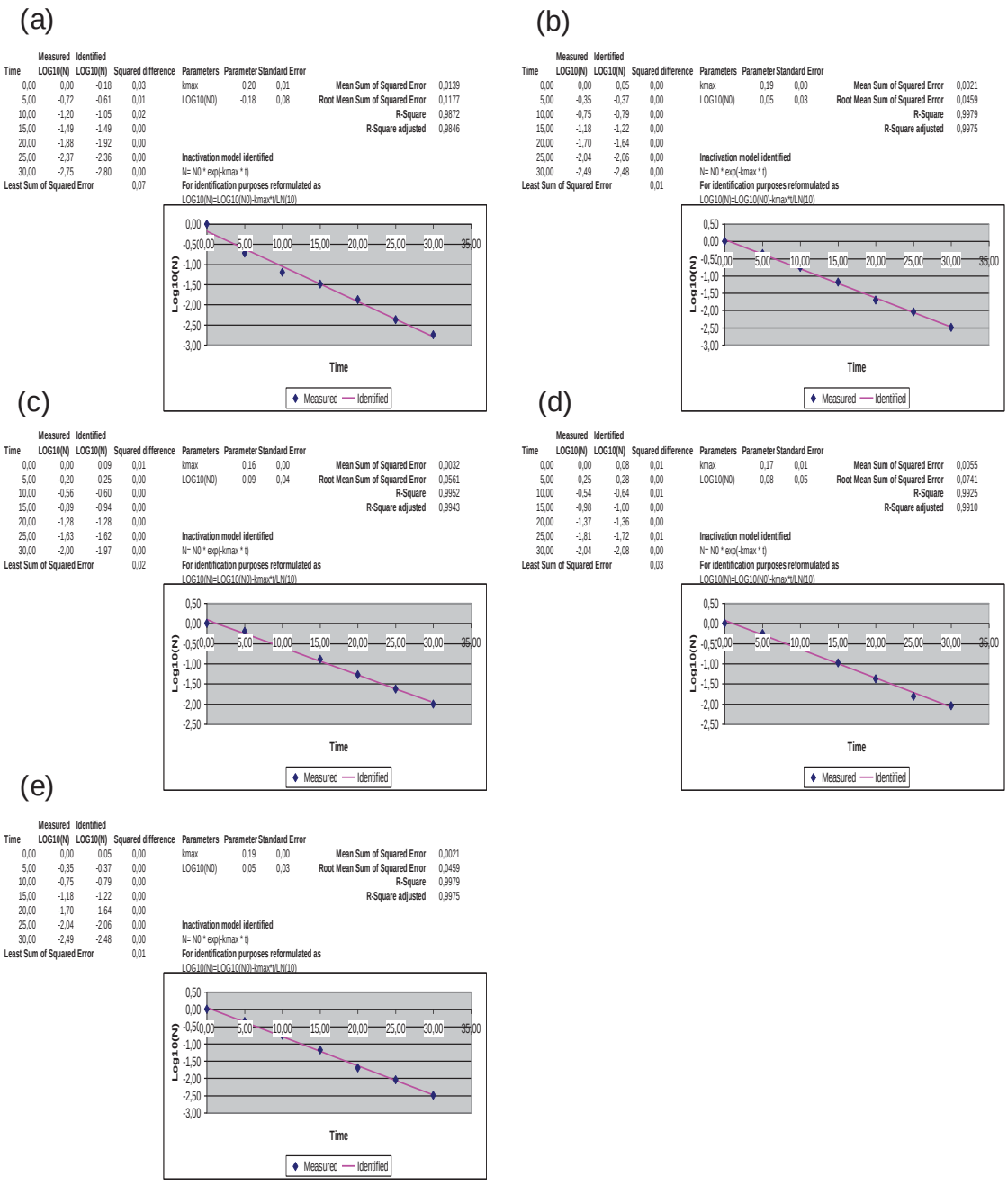


Figure A.3. Wet heat inactivation kinetics at 90 °C fitted by the log–linear model (a) PDA (b) MEA (c) BATA (d) BATB (e) BAA

APPENDIX B

CALIBRATION CURVES

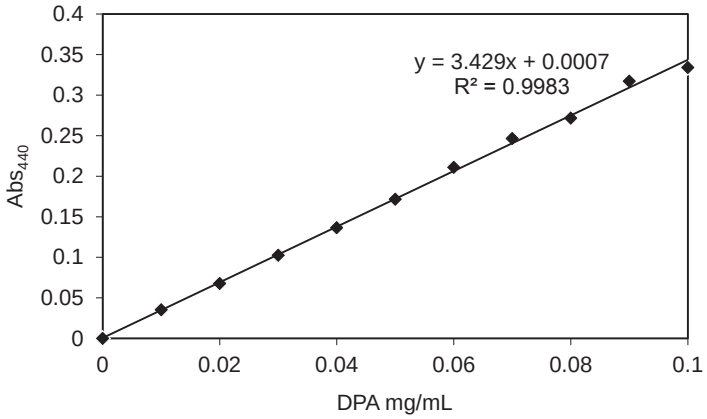


Figure B.1. Calibration curve of dipicolinic acid

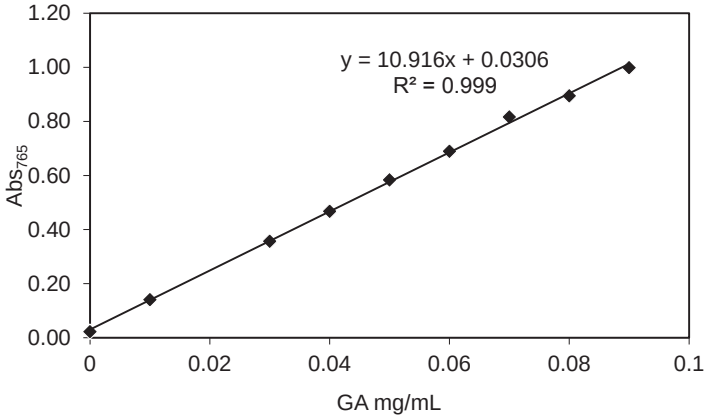


Figure B.2. Calibration curve of gallic acid for total phenol content

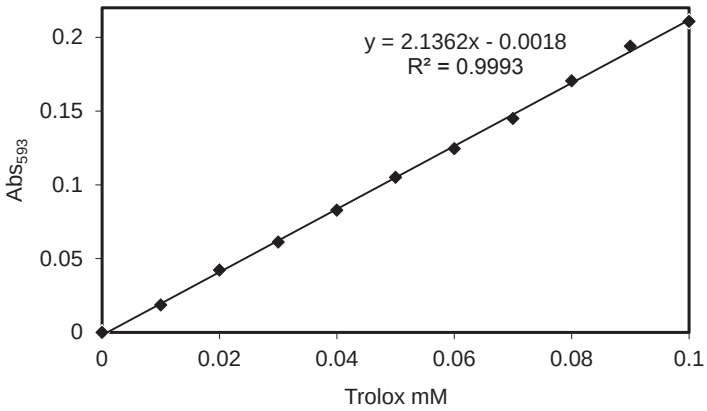


Figure B.3. Calibration curve of Trolox for FRAP assay

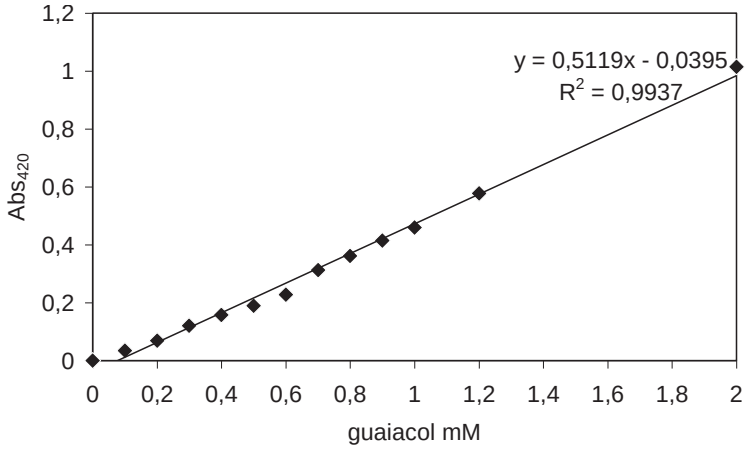


Figure B.4. Calibration curve of guaiacol in apple juice

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- Molva, C., Baysal, A.H. (2014). Effect of sporulation medium on wet–heat resistance and structure of *Alicyclobacillus acidoterrestris* DSM 3922 type strain spores and modeling of the inactivation kinetics in apple juice. *International Journal of Food Microbiology* doi:10.1016/j.ijfoodmicro.2014.07.033.
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