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**Influence of low sonication intensities at different temperatures on acid tolerance, bile tolerance, protease activity and growth of yogurt culture bacteria *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus***

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**INFLUENCE OF LOW SONICATION INTENSITIES AT DIFFERENT  
TEMPERATURES ON ACID TOLERANCE, BILE TOLERANCE, PROTEASE  
ACTIVITY, AND GROWTH OF YOGURT CULTURE BACTERIA *LACTOBACILLUS*  
*DELBRUECKII* SSP. *BULGARICUS* AND *STREPTOCOCCUS SALIVARIUS* SSP.  
*THERMOPHILUS***

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Louisiana State University and  
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in partial fulfillment of the  
requirements for the degree of  
Master of Science  
In  
The Interdepartmental Program in The School of Animal Sciences

By  
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## ABSTRACT

*Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* are dairy cultures widely used in the dairy industry. Low sonication intensity condition is a non-destructive technique that uses sound waves to cause cavitation in aqueous solutions and may improve the permeability of membranes, speed up the transfer of substrates and promote cellular growth and propagation. The objective of this study was to determine the effect of low sonication intensities at different temperatures on acid tolerance, bile tolerance, protease activity and growth of the two dairy cultures. The cultures were freshly thawed and suspended in 0.1% peptone water and 18 ml of sample was sonicated using horn (diameter 13 mm) set at a maximum acoustic power output of 750 W, frequency 24 kHz. The treatments were four sonication intensities of 8.07, 14.68, 19.83 and 23.55 Watts/cm<sup>2</sup> randomized at three different temperatures (4, 22 and 40°C) of inoculated peptone water before sonication. The energy input (1500 Joules) was kept constant in all treatments. Control samples did not receive any sonication treatment. Growth and bile tolerance of samples were determined hourly for 12 hours of incubation. Acid tolerance was determined for *Streptococcus thermophilus* every 5 minutes for 20 minutes of incubation and for *Lactobacillus bulgaricus* every minute for 5 minutes of incubation. Protease activity was determined at 0, 12 and 24 hours of incubation. The experimental design was a completely randomized design (CRD). Three replications were conducted for each experimental condition. Data were analyzed using Proc Mixed Model of Statistical Analysis System (SAS<sup>®</sup>). Differences of least square means were used to determine significant differences at  $P < 0.05$  for main effects (low sonication intensity, time and temperature) two way interaction effect (low sonication intensity \* temperature and low sonication intensity \* time) and three way interaction effects (low sonication intensity \* time \* temperature). Low sonication conditions include a) low

sonication intensities, b) temperatures and c) times, all three of which played a role in influencing the desirable attributes of both microorganisms. Of all the low sonication intensities studied, 14.68 watts /cm<sup>2</sup> had the best overall influence at certain time points for *Streptococcus thermophilus* improving its acid tolerance, bile tolerance and growth at 4°C, growth at 22°C, bile tolerance and growth at 40°C and improving the *Lactobacillus bulgaricus* bile tolerance and growth at 4°C, its acid tolerance and protease activity at 40°C. Low sonication intensity of 19.83 Watts/cm<sup>2</sup> had the overall best influence at certain time points for acid tolerance of both microorganisms at 22°C. Low sonication intensity of 23.55 Watts/cm<sup>2</sup> had the overall best influence at certain time points for protease activity of *Streptococcus thermophilus* at 40°C and *Lactobacillus bulgaricus* at 22°C. Some low sonication conditions improved certain characteristics of culture bacteria.

## CHAPTER 1: INTRODUCTION

### 1.1. Probiotics

Probiotics are living microbial which beneficially affect the host animal in the prevention and treatment of specific pathogen conditions by improving its microbial balance (Fuller, 1991). The beginning of beneficial attributes observed in some selected bacteria is attributed to Eli Metchnikoff, the Russian born Nobel Prize winner in the last century (FAO/WHO, 2006), who discovered that the dependence of the intestinal microorganisms on the food makes it possible to adopt measures to modify the flora in the host's body and to replace the harmful bacteria by beneficial bacteria (Metchnikoff, 1907).

Probiotics play an important role in preventing the growth of potentially pathogenic bacteria and in maintaining the integrity of the gut mucosal barrier (Bengmark, 1998). It has been shown that probiotics are capable of producing similar substance to an antibiotic and of reducing the luminal colonic pH (Fuller, 1991). In vitro some studies have found a stimulation of the intestinal immune system through the enhancement of macrophage and natural killer activities (Salminen, et al., 1993). It is known that certain lactobacilli ssp. adhere to the gut mucosal surface and sterically inhibit the attachment of gram-negative bacteria. Lactobacilli ssp. has been reported to induce the production of growth characteristics and to increase the availability of minerals (Gorbach, 1990).

A commonly used probiotic dairy product is yogurt. According to the definition of yogurt *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are two required microorganisms in yogurt manufacture (Code of federal regulations (21 CFR 131.200, 2010). Dairy Facts (2009) reported 3.44 % increase in sales of yogurt from 2007 to 2008. The global projection predicted

by Global Industry Analysts Inc. (GIA, 2010) indicated that for 2015 yogurt consumption will reach 20.6 million tons, equaling \$67 billion in sales. Granato et al. (2010) and Chandan (1999) explained that the consumption of yogurt was enhanced mainly because of its nutritional value and the beneficial health effects of yogurt cultures. Several studies reported yogurt culture bacteria as probiotics (Guarner et al., 2005).

Probiotics preparation on the market are mainly based on lactic acid bacteria (i.e. lactobacilli, bifidobacteria and streptococci), which are normal components of the gastrointestinal microflora and are all relatively harmless (Fuller, 1991). Recent reports have suggested the important functions of probiotics in the gastrointestinal tract and disease therapy. Kruis et al., (1997) and Rembacken et al., (1999) reported that patients with ulcerative colitis in remission were given oral mesalazine samples containing a non-pathogenic strain of *E. coli* as maintenance treatment and no significant differences were showed between the treatments studied.

Additionally, Shah and Jelen (1990); Gorbach, (1990) reported that some of the health benefits obtained from probiotic bacteria were: 1) the replacement of good intestinal bacteria destroyed by antibiotics, 2) prevention and treatment of diarrhea, 3) prevention of yeast infections and urinary tract infections, 4) the possibility of lowering cholesterol, 5) the improvement of lactose absorption in lactose intolerant people, 6) enhancing the immune system, 7) protection against colon cancer, 8) reducing the symptoms of irritable bowel syndrome.

## 1.2. Gastrointestinal Tract Conditions

One of the most important characteristics of probiotic microorganisms is their ability to survive through the acid in the human stomach and bile in the intestine (Fuller, 1991). According to Soll, (2009) acids are produced in the stomach in three acid phases, called cephalic, gastric and intestinal acid phases. The cephalic phase is activated by the thought, taste, smell and site of food, and swallowing (Soll, 2009) while the gastric acidic phases have the most contribution for acidic conditions in the stomach and are released in response to chemical effects of food and during bloating of stomach (Lloyd & Debas, 1994). Gastric acid is essentially an acidic solution containing 0.5% of hydrochloric acid and abundant amount of potassium chloride and sodium chloride which brings the stomach pH in the range of 1-2 (Lindstrom et al., 2001 and Fuller 1991). The acidic condition in the stomach is essential for protein digestion, suppression of the growth of acid sensitive pathogenic bacteria, and absorption of certain minerals such as calcium and iron and certain vitamins such as B12 (Soll, 2009). Bile juices are secreted by the liver and after are stored in the gall bladder. Bile is a yellow- green solution containing a mixture of bile acids, cholesterol and phospholipids (Carey & Duane, 1994). Bile plays a critical role in the digestion of fat by solubilizing the fat through emulsification process (Begley et al., 2006).

Several investigators have studied the survival of *L. acidophilus* and *Bifidobacterium* spp. in the presence of acid and bile salts (Iwana et al. 1993, Clark and Martin 1993; Conway et al., 1987). Iwana et al. (1993) isolated *Bacillus animalis* from several products available in Europe. Clark et al. (1994) studied the survival of *B. infantis*, *B. adolescentis*, *B. longum*, and *B. bifidum* in acidic conditions and reported that *B. longum* survived the best. Clark and Martin (1993) reported that *B. longum* tolerated bile concentrations of as high as 4.0%, whereas Ibrahim and Bezkorovainy (1993) found *B. longum* to be the least resistant to bile.

### **1.3. Sonication**

Sonication is an acoustic energy or a sound wave which involves the conversion of an electrical signal into a physical vibration, with certain frequency and amplitude that can be directed toward a substance (Riesz, and Kondo, 1992). Low sonication condition is a low intensity and non-destructive technique that provides information about physicochemical properties, such as composition, structure, physical state and flow rate (McClements, J. 1991). One major consequence of sonication is an event called sonoporation, which is the phenomenon that uses sound for modifying the permeability of cell plasma membranes by applying the acoustic cavitation of micro bubbles to enhance delivery. However, it can cause cavitation in aqueous solutions, which is an effective factor in damaging the cell wall of the micro-organisms (Elliott and Winder, 1995). When a bubble collapses, a strong shear rate is generated in the environment that breaks the chemical bonds in the cell wall and membranes (Dubbs, 1996). Depending on the strength and frequency of waves, cell wall structure and sonication environment, the impact of ultrasound will be different. It can be classified into two categories based on its outcome: (i) reparable, or reversible, during which the induction of temporary pores on the cell membrane is followed by pores resealing, leading to cell survival, and (ii) lethal, or irreversibly damaged in which the cell is lysed, leading to cell death (Zhou and Shi, 2006).

According to Earnshaw et al., (1995) some investigations of sonication as a potential microbial inactivation method were reported in the early 1960s. The process of microbial inactivation occur for the thinning of the cell membranes (Butz and Tauscher, 2002; Fellows, 2000). During the sonication process, are generated sound waves that propagate into the liquid media resulting in alternating high-pressure and low-pressure cycles, in consequence are created zones of alternating compression and expansion (Sala et al., 1995; Didenko et al., 1999). These zones of



pressure change causing the formation of small vacuum bubbles (Sala et al., 1995). These bubbles attain certain size and collapse violently during a high pressure cycle, creating shock waves (Sala et al., 1995). Dubbs, (1996) reported that depending on the size and frequency of sound waves, cell wall structure and sonication environment, the impact of sonication will have different effects. Furthermore, the efficiency of a sonication treatment is dependent on the type of bacteria being treated, intensity, temperature, frequency and size sample.

#### **1.4. Factors Affecting Characteristics of Sonicated Microorganisms**

##### **1.4.1. Type of Microorganism**

Microorganisms (especially spores) are relatively resistant to the effects, thus extended periods of sonication would be required to render a product safe. Pitt and Ross, (2003) reported that cells can grow in low sonication ( $<2 \text{ W/cm}^2$ ) due to the following properties of ultrasound: 1) its ability to increase the transport of small molecules (amino acid, peptide, carbon dioxide and water) in solution, and 2) its inability to completely remove cells (or even non-living particles) from surfaces. Although the former aspect is well known, the latter is not; in fact its antithesis is commonly accepted, causing the misconception that ultrasound is efficient at removing cells and particles from surfaces.

Drakopoulou et al. (2008) found that in the disinfection of wastewater using sonoporation, gram-negative bacteria are more readily susceptible to sonication inactivation than the gram-positive bacteria. Usually gram-positive organisms have a thicker and a more tightly adherent layer of peptidoglycans than gram-negative organisms, whereas the latter possess a lipopolysaccharide that contributes greatly to their structural integrity and protects the membrane from certain kinds of chemical attack. Therefore, the target of ultrasound attack may be the lipopolysaccharide or

the inner (cytoplasmic) membrane which consists of a lipoprotein bilayer, since the structure of the peptidoglycan layer does not appear to be a factor (Scherba et al. 1991).

In particular, the dairy industry has attracted attention to investigating probiotic cultures because of the health benefits associated with their consumption. Among these bacteria, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* are important dairy bacteria from a culture and probiotics stand point (Kurmann, 1988). Lactic acid bacteria possess a specific proteolytic activity which degrades the proteins in milk to free amino acids. For this reason, it is suggested that the proteolytic activity could be a good indicator of showing the ability of probiotic microorganisms to improve the nutritional value of milk products (Kurmann, 1988). Probiotic-containing products bring some benefits to consumers and constitute a significant portion of today's emerging functional food sector. They are usually marketed in the form of fermented milk and yogurt products. In the production of fermented milk products, (Kreft and Jelen, 2000; Wang and Sakakibara, 1997) have shown that sonication improves the acidifying activity of lactobacilli, thereby reducing production time and, while accelerating lactose hydrolysis, it induces a sweetening effect in yogurt without increasing the caloric content.

#### **1.4.2. Low Acoustic Intensity (Energy)**

Energy is the amount of joules delivered in the entire batch. This has a close relationship with intensity ( $\text{Watts/cm}^2$ ), depending what amplitude is being used (Sonics Vibracell user manual, 2009). The term amplitude refers to the maximum distance an individual air molecule will move from its starting point as a sound wave passes by (Dubbs, 1996).

The amplitude of a sound wave determines its loudness (Dubbs, 1996). A sound wave with large amplitude will sound louder than a small amplitude wave. This is also true for the measure of the amount of energy in a sound wave. The greater the amplitude wave, the greater the intensity

(Sonics Vibracell user manual, 2009). As the percentages of amplitude increase (from 21% to 39%) the sonication time is reduced.

Ideally the same amount of energy needs to be delivered to all treatments. If the energy is kept constant at 1500 J then at 21% amplitude the sonication time is 140 seconds (while at 39% amplitude the sonication time will be less). So at 21 % amplitude (1500J/ 140 s =) 10.71 Watts are being applied to the sample. The amplitude set up was below 40% because small volumes (18 mL) were processed for the probe size (13 mm) used (Sonics Vibracell user manual, 2009). Temperature settings on the sonicator were set at 4 or 22 or 40°C depending on the appropriate temperature to be used.

Intensity is described as Watts Applied / Probe area. If the probe used is 13 mm in diameter then probe area is ( $\Pi r^2 = 22 / 7 * (6.5 \text{ mm}^2) = 132.7 \text{ mm}^2 =) 1.327 \text{ cm}^2$ .

Hence intensity (i.e. Watts Applied/ Probe area =)  $10.71 \text{ Watts} / 1.327 \text{ cm}^2 = 8.07 \text{ Watts/cm}^2$ .

The sonicator used had a minimum amplitude use of 21%. The sonicator intensity used was considered low sonication intensity because at (all amplitudes (0, 21, 27, 33 and 39%) hence) all intensities (0, 8.07, 14.68, 19.83 and 23.55 Watts/cm<sup>2</sup>) studied there was no increase in sample temperature.

Wang and Sakakibara (1997) reported that milk fermentation with *Lactobacillus delbrueckii* subsp. *bulgaricus* using low intensity sonication (17.2 kW/m<sup>2</sup>), showed higher lactose hydrolysis and viable cells were at higher levels (more than 10<sup>8</sup> cfu/ cm<sup>3</sup>) than conventional fermentation. Furthermore, lactose-hydrolyzed fermented milk is expected to have therapeutic value for people who cannot tolerate the lactose that is contained in normal milk products. To date, lactose-

hydrolyzed fermented milk is produced by fermentation of lactose-hydrolyzed milk or by the simultaneous addition of  $\beta$ -galactosidase and lactic acid bacteria.

According to Shah and Jelen (1990) lactic acid bacteria (LAB) have the highest lactase activity due to its content of lactase or  $\beta$ -D-galactosidase which is an intracellular enzyme. The LAB cells exhibit very little extracellular lactase activity, and it can be increased several times by bacterial cell lysis induced by sonication. It could also hydrolyze a portion of lactose in milk and the products of lactose hydrolysis, glucose, and galactose could be used by slow growing organisms such as *L. acidophilus* and *Bifidobacterium* spp. Additionally Wang et al. (1996) sonicated samples of *Lactobacillus delbrueckii* ssp. *bulgaricus* B-5b for 10 min using a sonicator 300 dismembrator at a frequency of 16 kHz and reported that the highest amount of  $\beta$  - galactosidase released by sonication-fermentation was after 4h of the culture incubation in milk fermentation. This indicates that the intracellular enzyme was not released to the medium during conventional fermentation, but was released during sonicated fermentation.

Wang and Sakakibara (1997) found that in *L. delbrueckii* subsp. *lactis* SBT-2080 the cell viability increased continuously up to 4 h of low sonication (17.2 kW/ m<sup>2</sup>) and then began to decrease after 8 h. Also the static incubation after sonication was important to increase the cell viability, decrease the pH and increase the lactose hydrolysis. This means that the cell propagation was inhibited or the cells were disrupted by prolonged sonication. However, Wang et al., (1996) found that, with the prolongation of the incubation period before sonication, the amount of  $\beta$ -galactosidase released to the medium increased considerably. This is attributed to the fact that  $\beta$ -galactosidase is accumulated intracellularly during the pre-incubation time and that the viable cell count increases.

The factors that affect the microbial inactivation with sonication conditions depend on the process (amplitude, time, temperature, and frequency) and microbial entity (type and growth stage of microorganism).

The lethal effect of ultrasound has been attributed to cavitation due to the growth and subsequent collapse of microscopic bubbles that occurs when ultrasonic waves travel through a liquid. Cavitation can affect a biological system by virtue of the localized temperature rise and mechanical stress (Scherba et al., 1991). Moreover, the dissociation of water molecules into H- and OH- free radicals, as a consequence of the very high temperature and pressures produced by cavitation, could induce adverse chemical changes such as DNA or protein denaturation (Riesz and Kondo 1992).

### **1.4.3 Temperature**

Temperature affects the vapor pressure, surface tension, and viscosity of the liquid medium (Muthukumaran et al., 2006). While increased temperature increases the number of cavitation bubbles, the collapse is cushioned or dampened by the high vapor pressure. Cavitation bubbles form less easily in a highly viscous environment. Increased temperature decreases the viscosity allowing for a more violent collapse (Muthukumaran et al., 2006; Zhou and Shi, 2006). Thus, there is an optimum temperature at which the viscosity is low enough to form enough cavitation bubbles, yet the temperature is low enough to avoid the dampening effect by a high vapor pressure (Muthukumaran et al., 2006).

Temperature is an important influence in the samples used for sonication because recent studies have showed that it has a great influence on the intensity of cavitation. It also modifies several properties of the liquid medium, such as viscosity, surface tension and mainly vapor pressure, which influence cavitation. As the temperature of the liquid increases, its vapor pressure, and

consequently the vapor pressure inside the bubble, also increases when implosion occurs (Raso, et al. 1998). The vapor pressure cushions the collapse of the bubble. Verral and Shegal (1988) reported that the maximum temperature generated during cavitation was inversely proportional to the vapor pressure of the medium. Rooney (1988) reported that the lethality of ultrasonic waves under pressure (Mano-Sonication) treatment at higher temperatures did not decrease, but increased. The converse of this statement might be true for a combination of low sonication intensities and growth favoring temperatures.

The hypothesis was low sonication intensities can stimulate bacteria to improve their probiotic characteristics. The influence of low sonication intensities at various temperatures (refrigeration, room and incubation) on the probiotic characteristics of health beneficial bacteria is not known.

The objectives of this thesis were:

1. To study the influence of low sonication intensities (0, 8.07, 14. 68, 19.83 and 23.55 Watts/cm<sup>2</sup>) on the growth, bile tolerance, acid tolerance, and protease activity of *Streptococcus salivarius* spp. *thermophilus* ST-M5 at refrigeration (4°C), room (22°C) and incubation (40°C) temperatures.
2. To study the influence of low sonication intensities (0, 8.07, 14. 68, 19.83 and 23.55 Watts/cm<sup>2</sup>) on the growth, bile tolerance, acid tolerance, and protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at refrigeration (4°C), room (22°C) and incubation (40°C) temperatures.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Experimental Design

Peptone water (0.1% wt/v) was sterilized by autoclaving at 121°C for 15 minutes. This peptone water was cooled to 4 °C and individually inoculated with 1% (v/v) *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and *Streptococcus salivarius* ssp. *thermophilus* ST-M5 (Chr. Hansen's Laboratory, Milwaukee, WI, USA). The treatments consisted in four low sonication intensities (8.07, 14.68, 19.83 and 23.55 Watts/cm<sup>2</sup>), randomized at three different temperatures (4, 22 and 40 °C) of the peptone water. The treatments had constant frequency (20 kHz) and energy (1500 Joules). The control was the sample that did not receive any sonication treatment at each respective temperature. The control and sonicated samples were tested for acid tolerance, growth, bile tolerance, and protease activity. Acid tolerance was determined by inoculating the control and sonicated samples in the acidified MRS broth and plating for every 5 minutes up to 20 minutes for *Streptococcus salivarius* ssp. *thermophilus* ST-M5 and plating every minute up to 5 minutes for *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12. Growth was determined by plating the control and sonicated samples hourly for 12 hours of incubation of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and *Streptococcus salivarius* ssp. *thermophilus* ST-M5. Bile tolerance of the cultures was determined by growing the treated samples in presence of bile and plating every hour for 12 hours. Protease activity of the control and the sonicated samples was determined by measuring optical density (absorbance units) at 0, 12 and 24 hours of incubation of the samples. The experimental design was a completely randomized design (CRD). Three replications were conducted for each experimental condition.

## 2.2 Sample Preparation

Control and sonicated samples for the growth, acid tolerance, bile tolerance, and protease activity analyses were prepared by inoculating 5 ml of freshly thawed pure frozen concentrated stock solution culture of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and *Streptococcus salivarius* ssp. *thermophilus* ST-M5 (F-DVS LA-K, Chr. Hansen's Laboratory, Milwaukee, WI, USA) into 495 ml of sterile 0.1% peptone water at certain temperatures (4, 22, 40 °C) that make it 1% (v/v) and treated in a pilot plant Sonicator system (750 VCX Sonics, Vibracell). *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and *Streptococcus salivarius* ssp. *thermophilus* ST-M5 in control and sonicated samples for protease activity were inoculated at 10% (v/v) into sterile skim milk (sterilized at 121°C for 15 minutes).

## 2.3 Sonicator Equipment and Treatments

The Sonicator Sonics Vibracell 750 VCX (Sonics & Materials, Inc. 53 Church Hill Road, Newtown, CT 06470-1614 USA) converts 50/60 Hz line voltage to high frequency electrical energy. This high frequency electrical energy is transmitted to the piezoelectric transducer within the converter, where it is changed to mechanical vibrations. The vibrations from the converter are intensified by the probe, creating pressure waves in the liquid (Dubbs, 1996). This action forms millions of microscopic bubbles which expand during the negative pressure excursion and implode violently during the positive excursion (Sonics Vibracell user manual, 2009). It is this phenomenon, referred to as cavitation, which causes considerable amount of energy to be released at the point of implosion, and generates the powerful shearing action at the probe tip. The larger the probe tip, the larger the volume that can be processed but at a lesser intensity. The smaller the probe tip diameter, the greater the intensity (amplitude from 21% to 100%) at the probe tip (Sonics Vibracell user manual, 2009).



The low sonication treatments conditions consisted in a constant frequency (20 kHz) and energy (1500 Joules) using four different low sonication intensities (8.07, 14.68, 19.83 and 23.55 Watts/cm<sup>2</sup> respectively), randomized at three different temperatures (4 , 22 and 40 °C) of the peptone water with the culture before it was sonicated.

## **2.4 Preparation of Media:**

### **2.4.1 *Streptococcus thermophilus* Agar (ST Agar):**

ST agar was prepared by mixing the following ingredients:

10 g of Tryptone, 10 g of Sucrose, 5 g of Yeast extract and 2 g of Di potassium phosphate (K<sub>2</sub>HPO<sub>4</sub>) was dissolved in 1 L distilled water. The pH of the solution was adjusted to  $6.8 \pm 0.1$  using 1 M HCL, 6 ml of 0.5% bromocresol purple and 12 g of agar were added to the medium. The medium was boiled and sterilized at 121°C for 15 min (Dave and Shah, 1996).

### **2.4.2 Lactobacilli MRS Agar:**

MRS agar was prepared according to the manufacturer instructions (Difco™, Dickinson and company, Sparks, MD).

### **2.4.3 pH Modified MRS Agar (pH 5.2):**

The pH of the MRS agar (Difco™, Dickinson and company, Sparks, MD) was adjusted to a pH of 5.2 using 1N HCL (Dave and Shah., 1996).

## **2.5 Analytical Procedures:**

### **2.5.1 Acid Tolerance Test**

The acid tolerance of the two cultures was determined by the method used by Pereira and Gibson, (2002) with slight modifications. The control and sonicated samples were inoculated in acidified MRS broth (Difco™, Dickinson and company, Sparks, MD) previously adjusted to pH 2 (using 1 N HCl. The inoculated acidified MRS broth was incubated at 43°C for *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 for 5 minutes and 37°C for *Streptococcus salivarius* ssp. *thermophilus* for 20 minutes. Every 5 minutes for 20 minutes for *Streptococcus salivarius* ssp. *thermophilus* ST-M5 and every minute for 5 minutes for *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12, 1 ml of the inoculated broth was serially diluted and duplicated in peptone water (0.1% wt/v) and pour plated. The culture *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 was enumerated using Lactobacilli MRS agar and pH modified Lactobacilli MRS agar (Dave and Shah., 1996) and *Streptococcus salivarius* ssp. *thermophilus* ST-M5 was enumerated using ST agar. During the incubation period for *Lactobacillus delbrueckii* ssp. *bulgaricus*, LB-12 plates were kept at 43°C anaerobically for 72 hours and the petriplates for *Streptococcus salivarius* ssp. *thermophilus* ST-M5 were incubated aerobically at 37°C for 24 hours. After the incubation period, the colonies were counted.

### **2.5.2 Growth:**

Growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and *Streptococcus salivarius* ssp. *thermophilus* ST-M5 was determined by the method proposed by Lin and Young (2000) with slight modification. Control and sonicated samples were inoculated (10% [v/v]) into MRS broth (Difco™, Dickinson and company, Sparks, MD) which was previously autoclaved at 121°C for

15 min with pH  $6.5 \pm 0.2$ . Growth of the cultures was determined every two hours for 12 hours of incubation for both cultures at 43°C for *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and 37 °C for *Streptococcus salivarius* ssp. *thermophilus* ST-M5. 1 ml of the inoculated broth was serially diluted and duplicated in peptone water (0.1% wt/v) and pour plated. The culture *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 was enumerated using Lactobacilli MRS agar and pH modified Lactobacilli MRS agar (Dave and Shah., 1996) and *Streptococcus salivarius* ssp. *thermophilus* ST-M5 was enumerated using ST agar. During the incubation period for *Lactobacillus delbrueckii* ssp. *bulgaricus*, LB-12 plates were kept at 43°C anaerobically for 72 hours and the petriplates for *Streptococcus salivarius* ssp. *thermophilus* ST-M5 were incubated aerobically at 37°C for 24 hours (Champagne et al., 2009). After the incubation period the colonies were counted.

### **2.5.3 Bile Tolerance:**

The bile tolerance was determined according to method proposed by Pereira and Gibson, (2002) with slight modifications. The bile tolerance of the two cultures was analyzed in MRS-THIO broth [MRS broth (Difco™, Becton, Dickinson and company, Sparks, MD)] supplemented with 0.3% (wt/v) oxgall (bovine bile) (US Biological, Swampscott, MA) and 0.2 % (wt/v) sodium thioglycolate (Acros Organics, Fair Lawn, NJ). Oxgall was added to test bile tolerance of the bacteria and sodium thioglycolate was used in the broth as oxygen scavenger. Control and sonicated cultures were inoculated 10% (v/v) separately in MRS-THIO broth and incubated at 43°C for *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and 37°C for *Streptococcus salivarius* ssp. *thermophilus* for 12 hours. Each two hours for 12 hours, 1 ml of the inoculated broth was serially diluted in peptone water (0.1% wt/v) and pour plated. The cultures *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and *Streptococcus salivarius* ssp. *thermophilus* ST-M5 were

enumerated using Lactobacilli MRS agar, pH modified Lactobacilli MRS agar, and *Streptococcus thermophilus* agar respectively (Dave and Shah., 1996). The petriplates were incubated anaerobically at 43°C for 72 hours for *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and aerobically at 37°C for 24 hours for *Streptococcus salivarius* ssp. *thermophilus* ST-M5. After the incubation period the colonies were counted.

#### **2.5.4 Protease Activity:**

The extracellular protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and *Streptococcus salivarius* ssp. *thermophilus* ST-M5 was determined using the *o*-phthaldialdehyde (OPA) spectrophotometric method proposed by Oberg et al., (1991) with slight modifications. The control and sonicated samples were inoculated (10% [v/v]) into sterile skim milk (autoclaved at 121 °C for 15 min), and incubated at 40 °C for 0, 12 and 24 hours. After incubation, 2.5 ml from each sample were mixed with 1 ml distilled water and transferred into test tubes containing 5 ml of 0.75N trichloroacetic acid (TCA) (Fisher Scientific) at the same time as the test tubes are being vortexed. After sitting at room temperature for 10 minutes, the acidified samples were filtered through a Whatman Number 2 filter paper (Clifton, NJ). Duplicate aliquots from each TCA filtrate were analyzed by OPA testing using a spectrophotometer. The OPA final solution was prepared by combining the following reagents and diluting to a final volume of 50 ml with distilled water: 25 ml of 100 mM sodium borate (Fisher Scientific); 2.5 ml 20% (wt/wt) SDS (Fisher Scientific); 40 mg of OPA (Alfa Aesar, Ward Hill, MA) dissolved in 1 ml methanol (Sigma); and 100 µl of β-mercaptoethanol (Sigma). One hundred and fifty µl of each TCA filtrate were mixed with 3 ml of OPA reagent in a 3 ml cuvette, and the absorbance at 340 nm was read. Absorbance of the OPA final solution with the

non inoculated sterile skim milk (reference) was subtracted from each sample reading. OPA final solution was used as a blank to calibrate the spectrophotometer.

## **2.6 Statistical Analysis**

Data was analyzed using Proc Mixed model of Statistical Analysis System (SAS<sup>®</sup>). Differences of least square means were used to determine significant differences at  $P < 0.05$  for main effects (low sonication intensity, time and temperature), two ways interaction effects (low sonication intensity \* temperature and low sonication intensity \* time) and three ways interaction effects (low sonication intensity \* time \* temperature). Data are presented as mean  $\pm$  standard deviation of the means. Significant differences were determined at  $\alpha = 0.05$ . Significant difference ( $P < 0.05$ ) among the main effects analyzed using Tukey's adjustment.

## CHAPTER 3: RESULT AND DISCUSSION

### 3.1 Acid Tolerance:

#### 3.1.1 *Streptococcus salivarius* ssp. *thermophilus* ST-M5

The acid tolerance of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 was influenced by various low sonication intensities at different temperatures (4, 22 and 40°C) is shown in Fig 1. There was a significant ( $P<0.05$ ) interaction between sonication intensities \* temperature \* time (Table 1). Also the interaction for treatment \* time exhibited a significant ( $P<0.05$ ) effect (Table 1). Viable counts decreased over time from 0 to 20 minutes (Fig 1). At 4°C, cultures subjected to 14.68 Watts/cm<sup>2</sup> had higher acid tolerance than control over all 20 minutes of incubation (Fig 1A and Table 2). At 22°C, cultures subjected to 19.83 Watts/cm<sup>2</sup> showed significant ( $P<0.05$ ) increase in acid tolerance compared to control (Fig 1B and Table 2) from 0 to 10 minutes. At 40°C, bacterium subjected to 23.55 and 8.07 Watts/cm<sup>2</sup> showed significant ( $P<0.05$ ) increase in acid tolerance compared to control at minute 10 (Fig 1C and Table 2). The viable counts at 40°C when subjected to 23.55 and 8.07 Watts/cm<sup>2</sup> were 5.77 and 5.38 log cfu/ml respectively, whereas the control viable count was 4.26 log cfu/ml.

The treatment \* temperature interaction was significant ( $P<0.05$ ) (Table 1). The influence of temperature and time at a particular sonication intensity is shown in Figure 2. The cultures subjected to control, 8.07 and 14.68 Watts/cm<sup>2</sup> showed better acid tolerance at 40°C compared to 4 and 22°C (Table 4). The temperature had a significant ( $P<0.05$ ) effect (Table 1).

Mean log reduction of the viable counts of the bacterium subjected to various sonication intensities at three different temperatures (4, 22 and 40°C) obtained by subtracting counts at 20 minutes from 0 minute are shown in Table 5. In Table 5 a high number indicates high bacterial

death and a lower number indicates low bacterial death. The log reduction at 4°C exhibited that 23.55 Watts/cm<sup>2</sup> had the lowest bacterial death than the rest of intensities and the control.

Low sonication intensities had a significant ( $P<0.05$ ) effect (Table 1). Some low sonication conditions increased acid tolerance of *Streptococcus thermophilus* (Table 2).

### **3.1.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12**

Acid tolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 was influenced by various sonication intensities at different temperatures (4, 22 and 40°C) is shown in Fig 3. There was a significant ( $P<0.05$ ) interaction between sonication intensity \* temperature \* time (Table 1). The interaction for treatment \* time also was significant ( $P<0.05$ ) (Table 1). The viable counts decreased over time from 0 to 5 minutes (Fig 3). At 22°C, cultures subjected to 19.83 Watts/cm<sup>2</sup> showed significant ( $P<0.05$ ) increase in acid tolerance compared to control at 2 and 3 minutes (Fig 3B and Table 3). Using 19.83 Watts/cm<sup>2</sup> for 2 and 3 minutes the counts were 4.92 and 4.2 log cfu/ml while counts for control were 4.3 and 3.99 log cfu/ml respectively. At 40°C, culture subjected to 14.68 Watts/cm<sup>2</sup> showed significant ( $P<0.05$ ) increase in acid tolerance compared to control at minute 4 and 5 (Fig 3C and Table 3). At minutes 4 and 5, cultures subjected to 14.68 Watts/cm<sup>2</sup> had viable counts of 2.9 and 2.6 log cfu/ml, whereas control viable counts were 2.56 and 2.36 Log cfu/ml respectively.

The treatment \* temperature interaction was significant ( $P<0.05$ ) (Table 1). Control and all sonication intensities showed better acid tolerance at 22°C than 4 and 40°C at minutes 3, 4 and 5 (Fig 4). The temperature had a significant ( $P<0.05$ ) effect (Table 1). All sonication intensities had increased acid tolerance at 22°C than 4 and 40°C (Table 4).

Mean log reduction of the viable counts of the bacterium subjected to various intensities at three different temperatures (4, 22 and 40°C) were obtained by subtracting counts at 5 minutes from 0 minutes are shown in Table 5. In Table 5, a high number indicates high bacterial death and a lower number indicates low bacterial death. The log reduction at 22°C and 40°C, cultures subjected to 14.68 Watts/cm<sup>2</sup> had shown the lowest bacterial death for *Lactobacillus delbrueckii* ssp. *bulgaricus* Lb-12.

Low sonication had a significant ( $P<0.05$ ) effect (Table 1). Some sonication conditions improved acid tolerance of *Streptococcus thermophilus* and comparatively fewer sonication conditions improved acid tolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12. At 5 minutes to *Streptococcus salivarius* ssp. *thermophilus* ST-M5, log values were between 5 to 6 logs (Fig 1), while *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at 5 minutes log values were 2 to 3 logs (Fig 3) respectively. *Streptococcus salivarius* ssp. *thermophilus* ST-M5 was found to be more acid tolerant compared to *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 because after 5 minutes *Lactobacillus delbrueckii* ssp. *bulgaricus* Lb-12 exhibited significant reduction in viable counts. Shah and Jelen (1990) reported that at pH 1.5 *Lactobacillus delbrueckii* ssp. *bulgaricus* proved to be more acid tolerant than *Streptococcus salivarius* ssp. *thermophilus*, probably because they studied different strains of the same bacteria. Among the various lactic acid bacterial strains studied by Pereira and Gibson (2002) the only *Streptococcus thermophilus* strain studied was *Streptococcus thermophilus* DSM 20617 and they reported that this strain was the most acid sensitive losing viability in 15 minutes in acidified MRS broth at pH 2.0. Different lactobacilli strains were studied to grow on MRS broth at pH 2.0 for 120 minutes by Liong and Shah (2005) who reported that *Lactobacillus acidophilus* exhibited decrease in its viable count of 1.72 log cycles than *Lactobacillus casei* which showed reduction of 3.04 log cycles, respectively.



Table 1. Pr > F of low sonication treatment, time, temperature and their interaction for acid tolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12, *Streptococcus salivarius* ssp. *thermophilus* ST-M5

EFFECT	<i>S thermophilus</i>	<i>L bulgaricus</i>
	Pr > F	Pr > F
TRT	<0.0001	<0.0001
TIME	<0.0001	<0.0001
TEMP	<0.0001	<0.0001
TRT*TIME	<0.0001	<0.0001
TRT*TEMP	<0.0001	<0.0002
TRT*TIME*TEMP	<0.0001	<0.0001

Time = Incubation period of 5 minutes for *Lactobacillus delbrueckii* ssp. *bulgaricus* and 20 minutes for *Streptococcus salivarius* ssp. *thermophilus*

Table 2. Pr > F of acid tolerance of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 at various low sonication intensities compared to control (0 Watts/ cm<sup>2</sup>)

Treatment (Watts/cm <sup>2</sup> )	<i>S thermophilus</i>														
	0 min			5 min			10 min			15min			20 min		
	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C
8.07	<0.001	<0.001	0.825	0.955	<0.001	0.015	<0.001	<0.001	<0.001	0.110	<0.001	0.006	0.110	<0.001	0.033
14.68	<0.001	<0.001	0.876	0.007	<0.001	0.012	<0.001	<0.001	0.554	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
19.83	<0.001	<0.001	0.910	0.593	<0.001	<0.001	<0.001	0.013	0.039	<0.001	0.376	0.003	<0.001	0.254	0.004
23.55	0.422	<0.001	0.643	0.018	0.008	<0.001	0.004	0.097	<0.001	0.002	0.284	<0.001	0.110	0.002	0.001

Table 3. Pr > F of acid tolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at various low sonication intensities to control (0 Watts/ cm<sup>2</sup>)

Treatment (Watts/cm <sup>2</sup> )	<i>L bulgaricus</i>																	
	0 min			1 min			2 min			3 min			4 min			5 min		
	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C
8.07	0.002	0.004	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.001
14.68	0.001	0.358	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	0.002	0.007	<0.001	0.028	<0.001	<0.001	0.125	0.001
19.83	0.007	0.109	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	0.005	0.006	<0.001	0.402	0.481	<0.001	0.543	0.001
23.55	0.424	0.019	<0.001	<0.001	0.222	0.065	<0.001	<0.001	<0.001	<0.001	<0.001	0.005	<0.001	0.003	<0.001	<0.001	0.029	0.001

Table 4. Least Square Means for acid tolerance of bacteria as influenced by low sonication intensities

Treatment (Watts/cm <sup>2</sup> )	<i>S thermophilus</i>			<i>L bulgaricus</i>		
	4°C	22°C	40°C	4°C	22°C	40°C
	LSmean	LSmean	LSmean	LSmean	LSmean	LSmean
0	4.75 <sup>B,c</sup>	5.56 <sup>C,b</sup>	5.98 <sup>B,a</sup>	5.02 <sup>A,a</sup>	4.99 <sup>A,a</sup>	4.65 <sup>A,b</sup>
8.07	5.25 <sup>A,c</sup>	5.72 <sup>B,b</sup>	6.15 <sup>A,a</sup>	4.46 <sup>C,b</sup>	4.58 <sup>D,a</sup>	4.1 <sup>C,c</sup>
14.68	5.19 <sup>A,b</sup>	5.41 <sup>D,a</sup>	5.51 <sup>D,a</sup>	4.56 <sup>B,b</sup>	4.88 <sup>B,a</sup>	4.36 <sup>B,c</sup>
19.83	5.30 <sup>A,c</sup>	6.1 <sup>A,a</sup>	5.72 <sup>C,b</sup>	4.52 <sup>B,b</sup>	5.03 <sup>A,a</sup>	4.13 <sup>C,c</sup>
23.55	4.86 <sup>B,b</sup>	5.68 <sup>B,a</sup>	5.77 <sup>C,a</sup>	4.55 <sup>B,b</sup>	4.77 <sup>C,a</sup>	4.33 <sup>B,c</sup>

<sup>ABCD</sup> LSMeans with the same letter within the column are not significantly different

<sup>abcd</sup> LSMeans with the same letter within the row are not significantly different

Table 5. Mean log reduction of the viable counts of the sonicated cultures obtained by subtracting counts at 5 minutes from 0 minutes for *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and 20 minutes from 0 minutes for *Streptococcus salivarius* ssp. *thermophilus* ST-M5

Treatment (Watts/cm <sup>2</sup> )	<i>S thermophilus</i>			<i>L bulgaricus</i>		
	Log cfu/ml			Log cfu/ml		
	4°C	22°C	40°C	4°C	22°C	40°C
0	4.36	3.7	4.85	5.49	3.51	5.71
8.07	5.36	6.21	6.18	6.08	4.09	5.58
14.68	4.76	6.21	5.91	5.67	3.48	4.87
19.83	4.08	4.84	5.22	5.68	3.57	5.31
23.55	4.06	4.96	5.31	6.00	5.28	5.81

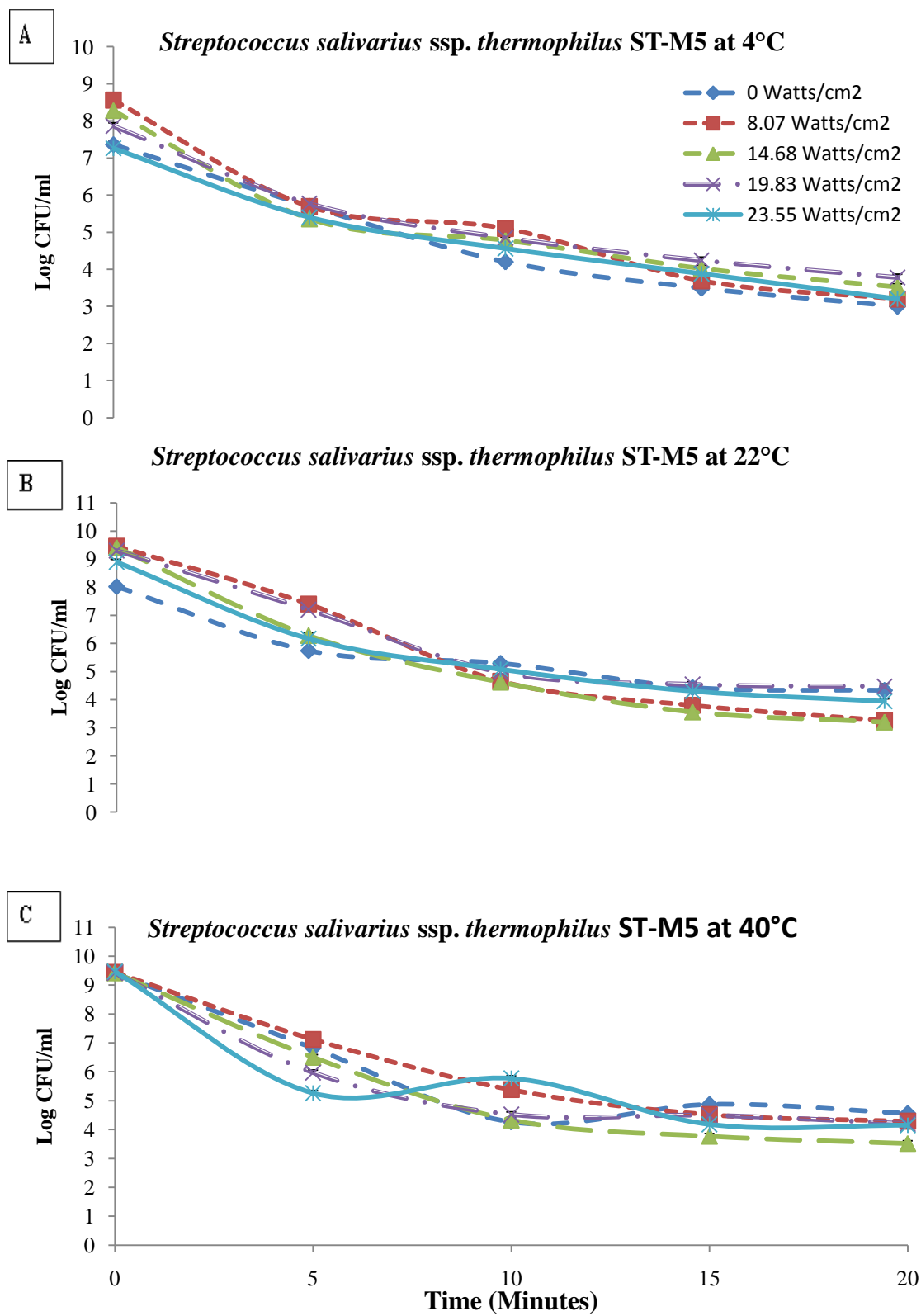


Figure 1. Acid tolerance of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 at 4°C (A), 22°C (B) and 40°C (C)

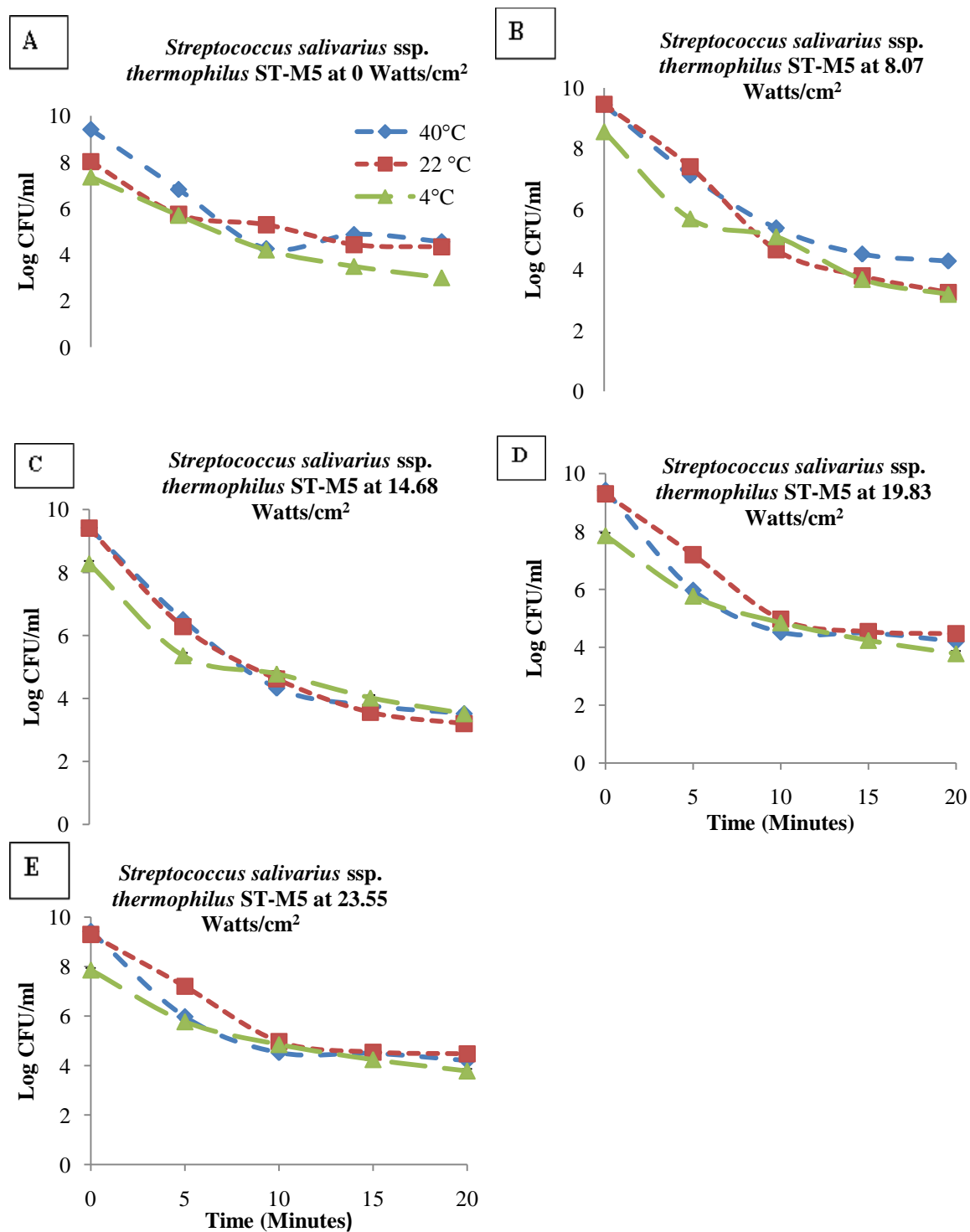


Figure 2. Acid tolerance of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 at 0 (A), 8.07 (B), 14.68 (C), 19.83 (D) and 23.55 Watts/cm² (E) at 4, 22 and 40°C

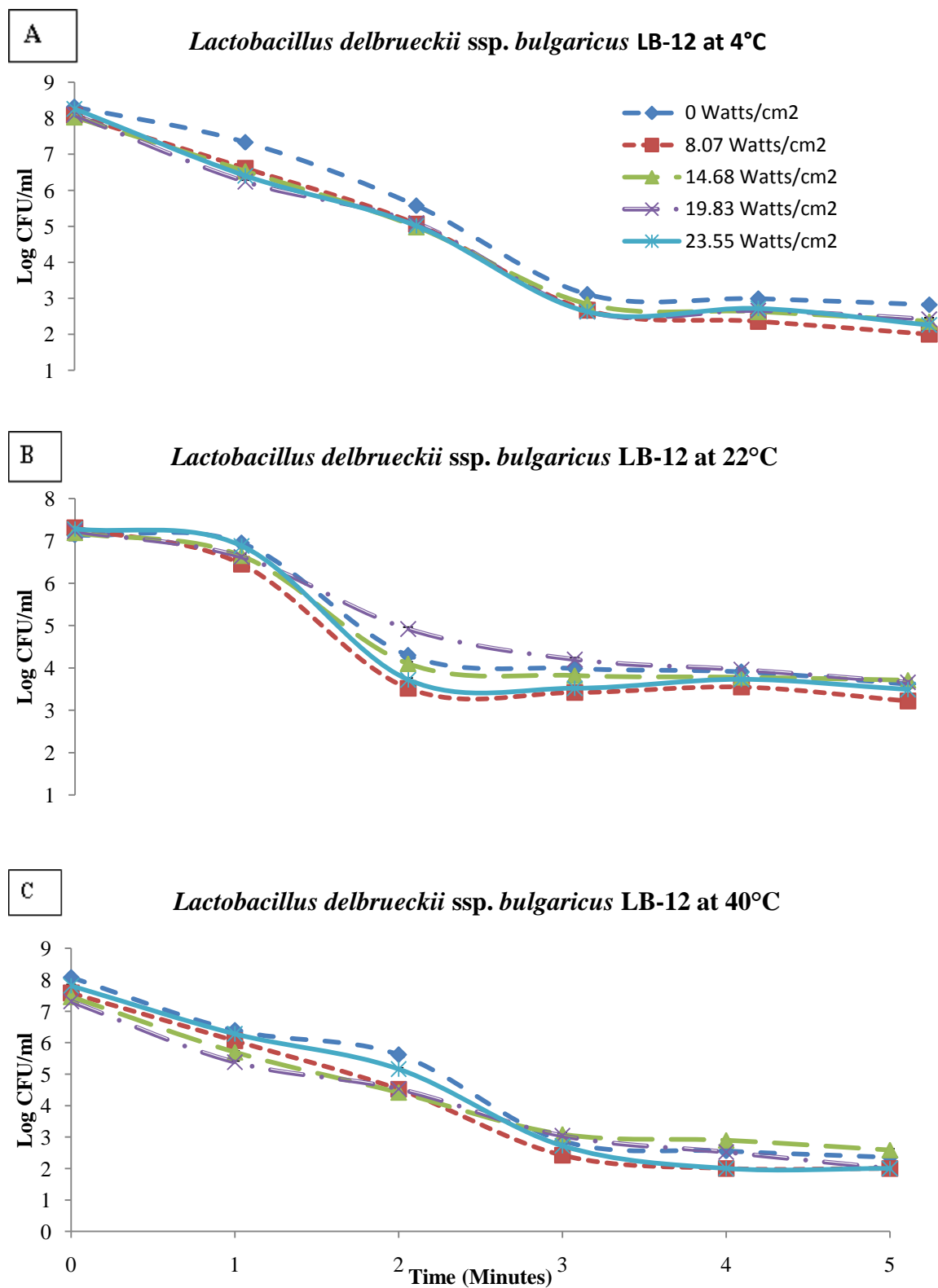


Figure 3. Acid tolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at 4°C (A), 22°C (B) and 40°C (C)

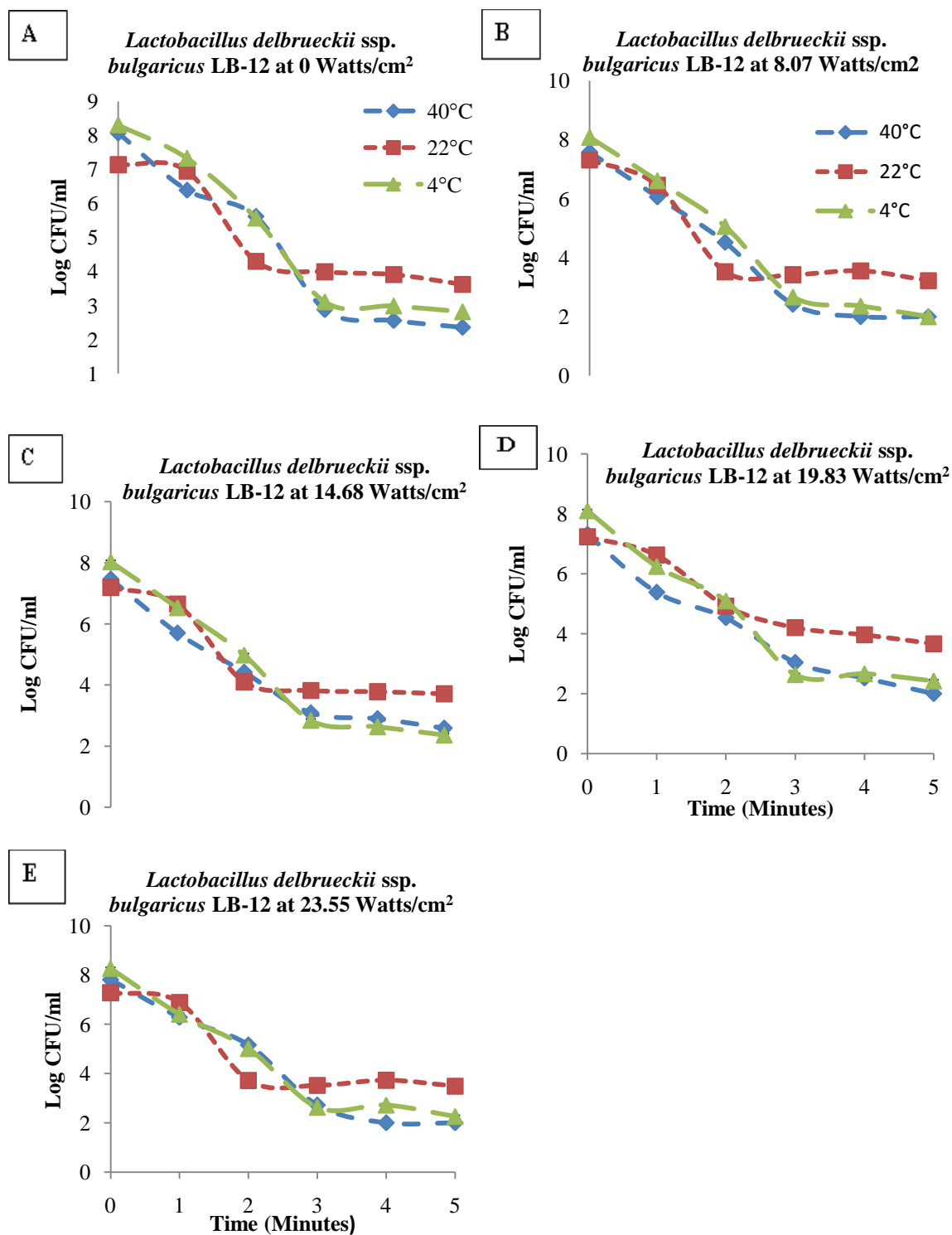


Figure 4. Acid tolerance of *Lactobacillus delbrueckii ssp. bulgaricus* LB-12 at 0 (A), 8.07 (B), 14.68 (C), 19.83 (D) and 23.55 Watts/cm<sup>2</sup> (E) at 4, 22 and 40°C

## 3.2 Bile Tolerance:

### 3.2.1 *Streptococcus salivarius* ssp. *thermophilus* ST-M5

The bile tolerance of *Streptococcus Salivarius* ssp. *thermophilus* ST-M5 was influenced by various low sonication intensities at three different temperatures (4, 22 and 40°C) is shown in the Figure 5. There was a significant ( $P<0.05$ ) interaction for low sonication intensities \* temperature \* time (Table 6). The interaction for low sonication intensities \* time was also significant ( $P<0.05$ ) (Table 6). Viable counts decreased from 0 to 12 hours (Fig 5). At 4°C, bile tolerance of cultures subjected to 14.68 Watts/cm<sup>2</sup> was significantly ( $P<0.05$ ) than the control at 10 hours (Fig 5A Table 7). At 22°C, the control and cultures subjected to 19.83 Watts/cm<sup>2</sup> showed significantly ( $P<0.05$ ) higher bile tolerance compared to the rest (Table 9). At 40°C bile tolerance of cultures subjected to 14.68 and 19.83 Watts/cm<sup>2</sup> was significantly ( $P<0.05$ ) higher in bile tolerance compared to the control at 8 to 12 hours (Fig 5C and Table 7).

The treatment \* temperature interaction was significant ( $P<0.05$ ) (Table 6). Control and all sonication intensities showed better bile tolerance at 40°C compared to 4 and 22°C during 12 hours of incubation (Fig 6 and Table 9). The temperature had a significant ( $P<0.05$ ) effect (Table 6). The cultures sonicated at 40°C exhibited higher bile tolerance for 14.68 Watts/cm<sup>2</sup> than the rest (Table 9).

Mean log reduction of the viable counts of the bacterium subjected to various intensities at three different temperatures (4, 22 and 40°C) was obtained by subtracting counts at 12 hours from 0 hour is exhibited in Table 10. In Table 10, a high number indicates high bacterial death and a lower number indicates low bacterial death. At 22 °C, cultures subjected to 14.68 Watts/cm<sup>2</sup> had the lowest bacterial death for *Streptococcus salivarius* ssp. *thermophilus* ST-M5 (Table 10). At



40°C, the control (0 Watts/cm<sup>2</sup>) had the highest bacterial death compared to the low sonication treatments (Table 10). The low sonication treatment of 8.07 Watts/cm<sup>2</sup> showed the lowest bacterial death. Using 8.07 Watts/cm<sup>2</sup> the log reduction was 0.23, while control showed a log reduction of 0.64 indicating that on applying 8.07 Watts/cm<sup>2</sup> increase the rate of survivability of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 and was almost 3 times higher than control during the incubation time (Table 10).

Low sonication had a significant ( $P<0.05$ ) effect (Table 6). Some low sonication conditions improved bile tolerance of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 (Table 10).

### **3.2.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12:**

The bile tolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 was influenced by various low sonication intensities at three different temperatures (4, 22 and 40°C) is shown in the Figure 7. There was no significant ( $P = 0.6765$ ) interaction for low sonication intensities \* temperature \* time (Table 6). The interaction for low sonication intensities \* time was not significant ( $P = 0.2318$ ) (Table 6). Viable counts decreased from 0 to 12 hours (Fig 7). At 4°C, bile tolerance of cultures subjected to control and 14.68 Watts/cm<sup>2</sup> was significantly ( $P<0.05$ ) higher compared to the rest of low sonicated intensities (Table 9).

The treatment \* temperature interaction was significant ( $P<0.05$ ) (Table 6). The influence of temperature and time at a particular sonication intensity is shown in Figure 8. Control and all sonication intensities showed better ( $P<0.05$ ) bile tolerance at 22°C than 4 and 40°C during 12 hours of incubation (Table 9). The temperature had a significant ( $P<0.05$ ) effect (Table 6).

Mean log reduction of the viable counts of the bacterium subjected to various intensities at three different temperatures (4, 22 and 40°C) that was obtained by subtracting counts at 12 hours from

0 hour is exhibited in Table 10. In Table 10, a high number indicates high bacterial death and a lower number indicates low bacterial death. The log reduction at 4, 22 and 40°C showed that control had the highest bacterial death for *Lactobacillus bulgaricus* compared to the rest of low sonication intensities.

Low sonication had a significant ( $P<0.05$ ) effect (Table 6). Taking log reduction values over 12 hours into consideration, the treated samples performed better than control (Table 10). Clark and Martin (1994) reported that in the presence or absence of bile acid (Oxgall) *B. longum* and *L. bulgaricus* tolerated bile concentrations as high as 4.0%. Furthermore Shah and Jelen (1990) have shown attributes to increase bile tolerance of Lactobacilli strains to their rigid cell wall. There could be other factors responsible for increased bile tolerance of *L. bulgaricus* and *Streptococcus thermophilus* when subjected to low sonication conditions. Bile salts offer antibacterial activity due to the fact that all bacteria have a cell membrane consisting of lipids and fatty acids which are susceptible to being dissolved and destructed by bile salts (Begley et al., 2006). Lick S. et al., (2001) showed that *S. thermophilus* and *Lactobacillus bulgaricus* strains are able to survive gastrointestinal passage in vivo and detected viable *S. thermophilus* in human duodenal samples after fresh yogurt ingestion. *S. thermophilus* showed no significant differences in their growth in MRS broth containing 0%, 0.2% and 0.4% (wt/v) Oxgall for 12 hours of incubation at 37°C and monitored hourly for the growth spectrophotometrically at 650 nm (Pereira and Gibson, 2002). Overall, the data of Begley et al., (2006) strongly supported the hypothesis that microbial Bile Salt Hydrolase (BSH) functions in the detoxification of bile salts, and in doing so, increases the intestinal survival and persistence of producing strains in the hostile environment of the gastrointestinal tract. The precise mechanism by which BSH enzyme plays a role in the tolerance of bile is not yet fully understood.

Table 6. Pr > F of low sonication treatment, time, and temperature their interaction for bile tolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12, *Streptococcus salivarius* ssp. *thermophilus* ST-M5

EFFECT	<i>S thermophilus</i>	<i>L bulgaricus</i>
	Pr > F	Pr > F
TRT	<0.0001	<0.0001
TIME	<0.0001	<0.0001
TEMP	<0.0001	<0.0001
TRT*TIME	<0.0001	0.2318
TRT*TEMP	<0.0001	<0.0002
TRT*TIME*TEMP	<0.0001	0.6765

Table 7. Pr > F of bile tolerance of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 at various low sonication intensities compared to control (0 Watts/ cm<sup>2</sup>)

Treatment (Watts/cm <sup>2</sup> )	<i>S thermophilus</i>																				
	0 hrs			2 hrs			4 hrs			6 hrs			8 hrs			10 hrs			12 hrs		
	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C
8.07	0.481	0.381	0.001	0.001	0.065	0.001	0.001	0.296	0.009	0.107	0.148	0.983	0.764	0.160	0.263	0.077	0.268	0.022	0.396	0.343	0.059
14.68	0.044	0.349	0.049	0.001	0.030	0.048	0.018	0.038	0.692	0.581	0.100	0.065	0.068	0.101	0.006	0.002	0.307	0.001	0.001	0.444	0.034
19.83	0.918	0.823	0.491	0.481	0.615	0.011	0.132	0.302	0.487	0.049	0.168	0.417	0.456	0.393	0.016	0.148	0.472	0.006	0.001	0.745	0.021
23.55	0.198	0.762	0.062	0.481	0.511	0.014	0.699	0.003	0.734	0.821	0.009	0.291	0.112	0.043	0.432	0.001	0.092	0.100	0.001	0.097	0.193

Time = Incubation period of 12 hours

Table 8. Pr > F of bile tolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at various low sonication intensities compared to control (0 Watts/ cm<sup>2</sup>)

Treatment (Watts/cm <sup>2</sup> )	<i>L bulgaricus</i>																				
	0 hrs			2 hrs			4 hrs			6 hrs			8 hrs			10 hrs			12 hrs		
	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C
8.07	0.064	0.001	0.001	0.855	0.035	0.001	0.957	0.219	0.001	0.985	0.106	0.024	0.814	0.044	0.138	1.00	0.001	0.006	0.687	0.060	0.195
14.68	0.001	0.001	0.001	0.567	0.297	0.001	0.814	0.001	0.001	0.820	0.074	0.129	0.521	0.015	0.018	0.742	0.021	0.009	0.127	0.060	0.068
19.83	0.001	0.001	0.001	0.001	0.848	0.001	0.451	0.078	0.026	0.474	0.052	0.203	0.444	0.053	0.194	0.071	0.252	0.079	0.979	0.904	0.884
23.55	0.001	0.001	0.001	0.717	0.309	0.016	0.003	0.010	0.007	0.432	0.032	0.058	0.628	0.357	0.030	0.108	0.392	0.031	0.395	0.957	0.596

Table 9. Least Square Means for bile tolerance of bacteria as influenced by low sonication intensities

Treatment (Watt/cm <sup>2</sup> )	<i>S thermophilus</i>			<i>L bulgaricus</i>		
	4°C	22°C	40°C	4°C	22°C	40°C
	LSmean	LSmean	LSmean	LSmean	LSmean	LSmean
0	8.52 <sup>A,c</sup>	10.22 <sup>A,b</sup>	10.51 <sup>B,a</sup>	7.73 <sup>A,c</sup>	8.43 <sup>A,a</sup>	8.15 <sup>A,b</sup>
8.07	8.54 <sup>A,c</sup>	10.13 <sup>C,b</sup>	10.31 <sup>C,a</sup>	7.68 <sup>B,b</sup>	7.96 <sup>C,a</sup>	7.6 <sup>D,c</sup>
14.68	8.48 <sup>A,c</sup>	10.12 <sup>C,b</sup>	10.57 <sup>A,a</sup>	7.71 <sup>A,b</sup>	7.95 <sup>C,a</sup>	7.59 <sup>D,c</sup>
19.83	8.42 <sup>B,c</sup>	10.18 <sup>A,b</sup>	10.55 <sup>B,a</sup>	7.51 <sup>C,c</sup>	8.13 <sup>B,a</sup>	7.74 <sup>B,b</sup>
23.55	8.44 <sup>B,c</sup>	10.10 <sup>D,b</sup>	10.52 <sup>B,a</sup>	7.52 <sup>C,c</sup>	8.10 <sup>B,a</sup>	7.66 <sup>C,b</sup>

<sup>ABCD</sup> LSMeans with the same letter within the column are not significantly different

<sup>abcd</sup> LSMeans with the same letter within the row are not significantly different

Table 10. Mean log difference in the viable counts of the sonicated cultures obtained by subtracting viable log cfu/ml between 0 hour and 12 hours of incubation in the presence of bile acid (Oxgall)

Treatment (Watts/cm <sup>2</sup> )	<i>S thermophilus</i>			<i>L bulgaricus</i>		
	Log cfu/ml			Log cfu/ml		
	4°C	22°C	40°C	4°C	22°C	40°C
0	2.35	0.4	0.64	1.51	1.27	1.72
8.07	2.46	0.41	0.23	1.03	0.96	1.18
14.68	2.47	0.39	0.35	0.62	0.85	1.28
19.83	2.67	0.44	0.44	0.93	0.68	0.90
23.55	2.7	0.5	0.42	1.12	0.55	0.86

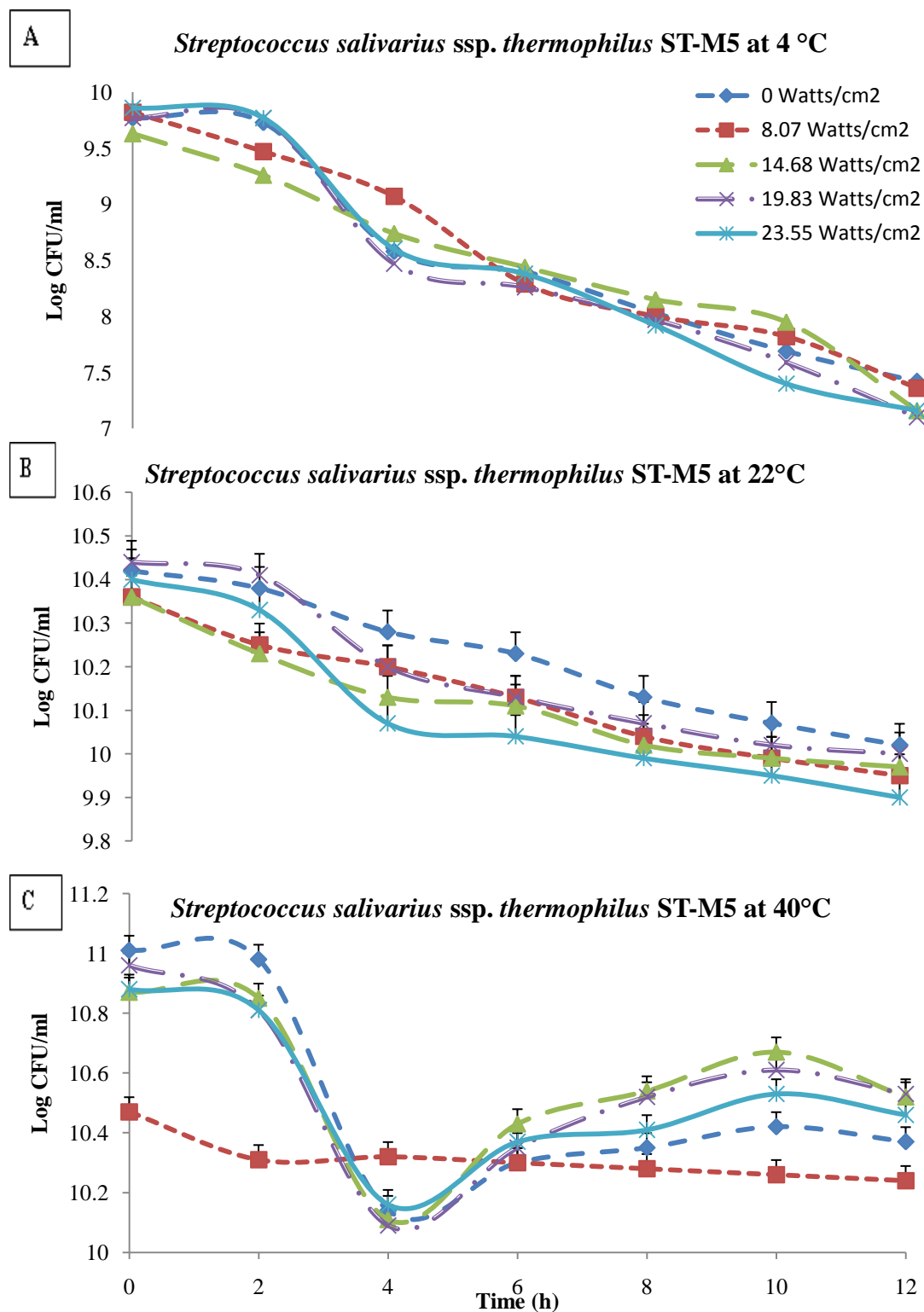


Figure 5. Bile tolerance of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 at 4°C (A), 22°C (B) and 40°C (C)

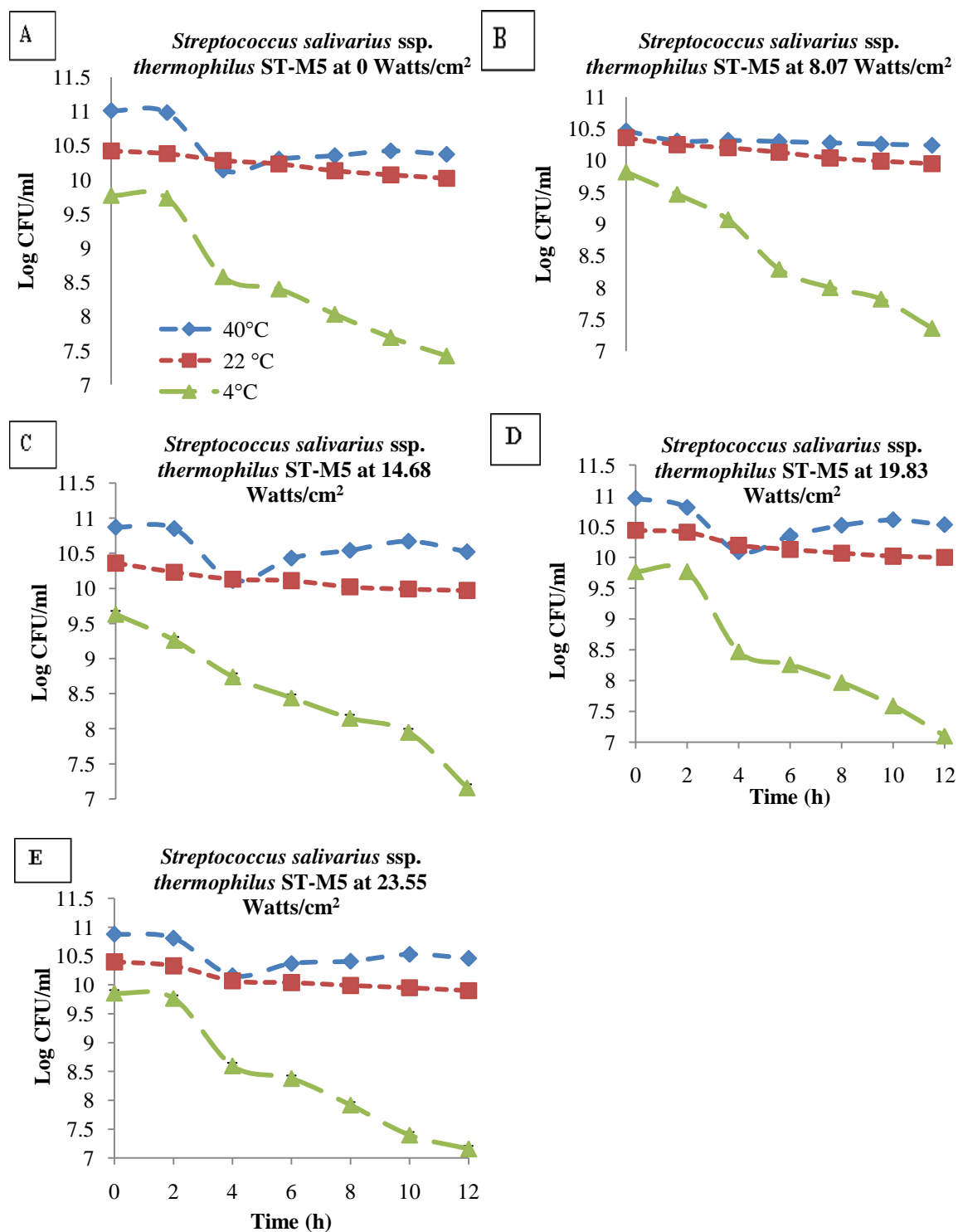


Figure 6. Bile tolerance of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 at 0 (A), 8.07 (B), 14.68 (C), 19.83 (D) and 23.55 Watts/cm<sup>2</sup> (E) at 4, 22 and 40°C

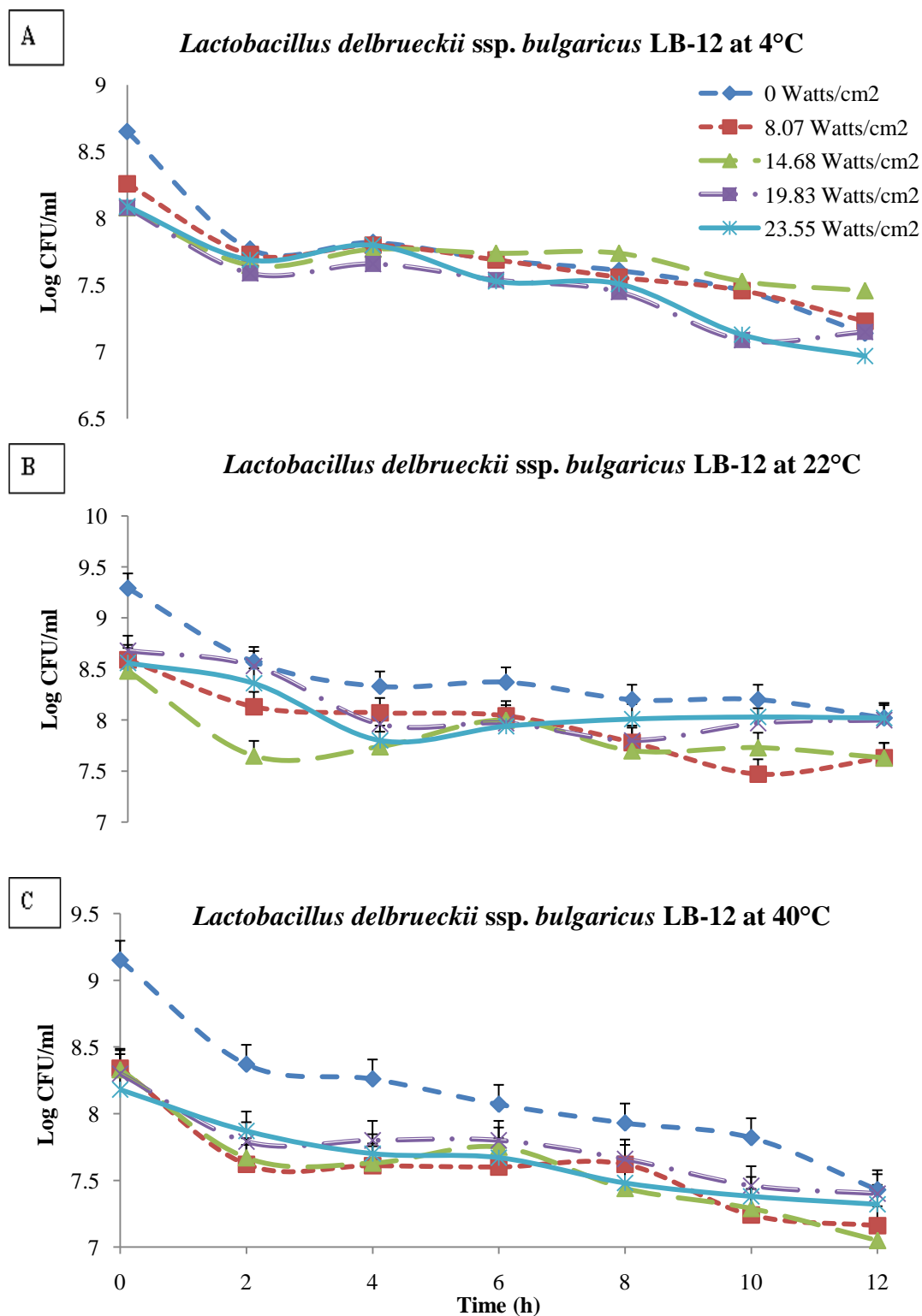


Figure 7. Bile tolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at 4°C (A), 22°C (B) and 40°C (C)



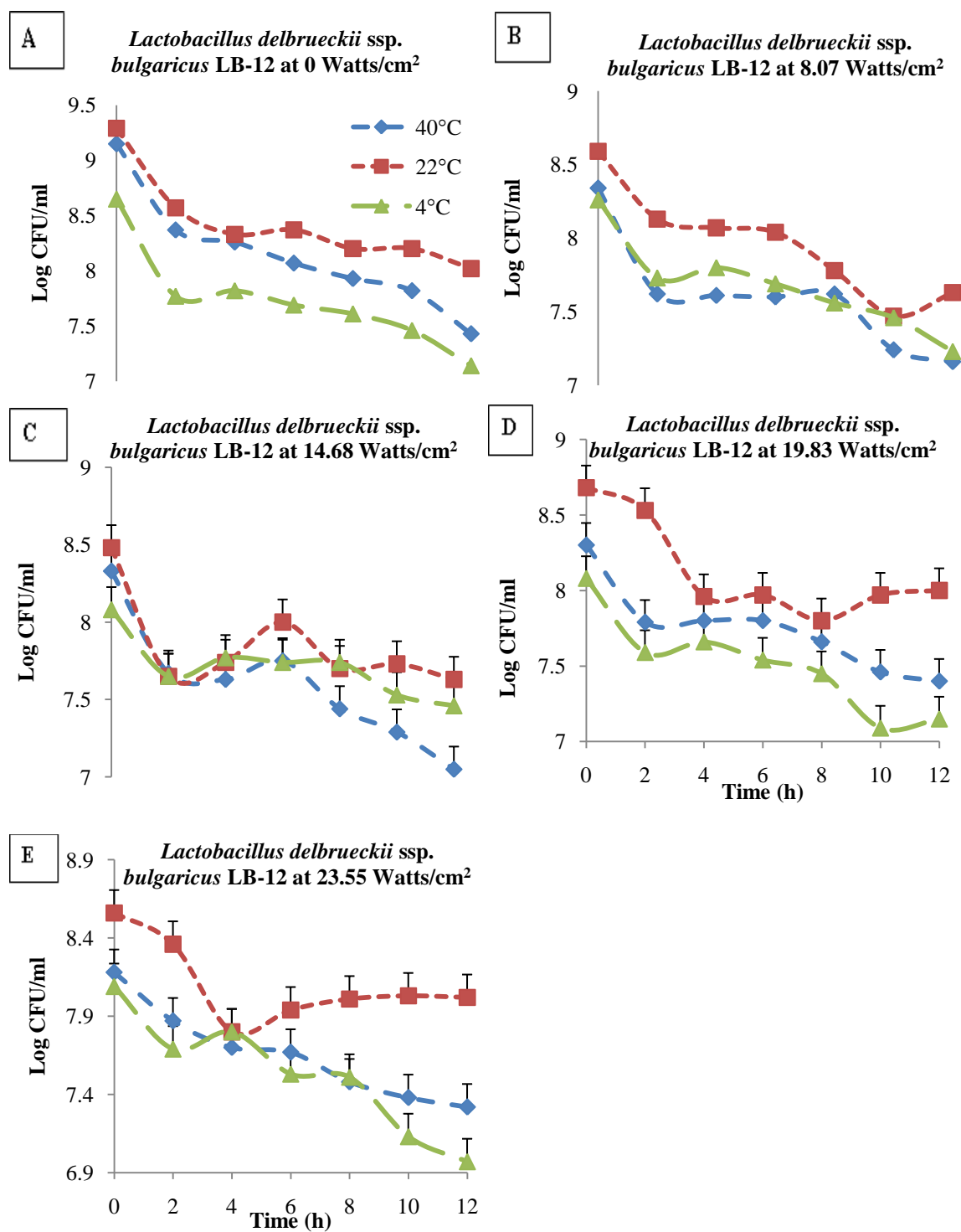


Figure 8. Bile tolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at 0 (A), 8.07 (B), 14.68 (C), 19.83 (D) and 23.55 Watts/cm<sup>2</sup> (E) at 4, 22 and 40°C

### 3.3 Growth

#### 3.3.1 *Streptococcus salivarius* ssp. *thermophilus* ST-M5

The growth characteristics of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 influenced by various low sonication intensities at different temperatures (4, 22 and 40°C) over 12 hours is shown in the Figure 9. There was significant ( $P<0.05$ ) interaction between low sonication intensities \* temperature \* time (Table 11). The interaction for low sonication intensity \* time was significant ( $P<0.05$ ) (Table 11). Viable counts increased from 0 to 12 hours (Fig 9). At 4, 22 and 40°C, all the low sonication intensities showed significant ( $P<0.05$ ) increase in viable counts compared to the control from 6 to 12 hours of incubation (Fig 9A, B, C and Table 12).

The low sonication intensity \* temperature was significant ( $P<0.05$ ) (Table 11). The influence of temperature and time at a particular sonication intensity is shown in Figure 10. Cultures subjected to 8.07, 14.68 and 23.55 Watts/cm<sup>2</sup> showed better growth at 4°C compared to 22 and 40°C (Table 14). The temperature had a significant ( $P<0.05$ ) effect (Table 11). The cultures sonicated at 4°C showed higher ( $P<0.05$ ) viable counts compared to control (Table 14).

The low sonication intensities had a significant ( $P<0.05$ ) effect (Table 11). Some low sonication conditions increased growth of *Streptococcus salivarius* ssp. *thermophilus* ST-M5.

#### 3.3.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12

The growth characteristics of *Lactobacillus delbrueckii* ssp. *bulgaricus* as influenced by various low sonication intensities at different temperatures (4, 22 and 40°C) over 12 hours is shown in the Figure 11. There was significant ( $P<0.05$ ) interaction between low sonication intensities \* temperature \* time (Table 11). The interaction for low sonication intensity \* time was significant ( $P<0.05$ ) (Table 11). Viable counts increased over time from 0 to 12 hours (Fig 11). The low sonication intensity \* temperature interaction was significant ( $P<0.05$ ) (Table 11). The influence

of temperature and time at a particular sonication intensity is shown in Figure 12. All low sonication intensities and control showed better ( $P<0.05$ ) viable counts at 4°C compared to 22 and 40°C (Table 14). The temperature had a significant ( $P<0.05$ ) effect (Table 11). The low sonication intensities also was significant ( $P<0.05$ ) (Table 11). Low sonication conditions adversely influenced growth of *Lactobacillus delbrueckii* ssp. *thermophilus* LB-12.

According to Aronsson et al., (2001), cell physiology could be affected for the application of electrical treatments. The growth of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 subjected to low sonication intensities at different temperatures enhanced exponential growth phase after 2 hours and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 also reach exponential growth phase after 2 hours of incubation. Liong and Shah (2005) reported growth of *L. bulgaricus* and *L. acidophilus* to be predominant in the first 9-15 hours after which it reached a stationary phase. Furthermore, the behavior of both cultures was different; both reached the highest viable count in the logarithmic phase at 10 -12 hours of incubation.

Simova et al., (2006) analyzed the growth characteristics of *S. thermophilus* T15 and *L. bulgaricus* HP1 pre-incubating both cultures for 5.5 hours before inoculation and reported that growth reached exponential phase in the first 5 hours and reached stationary phase in 8-12 hours. Hülshager et al., (1983) reported that the application of electric fields to the bacterial cells of *E. coli* resulted in the lag and stationary phase growth to be more resistant to the negative effect of electric fields and survive more than the cells in the exponential phase of growth. In addition, Kobayashi, Y. et al., (2009) has demonstrated that low intensity of pulse ultrasound treatments stimulates cell proliferation and production of proteoglycan in human nucleus pulposus cell line, possibly by enhancement of growth factor-related genes.

Table 11. Pr > F of low sonication treatment, time, and temperature their interaction for growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and *Streptococcus salivarius* ssp. *thermophilus* ST-M5

EFFECT	<i>S thermophilus</i>	<i>L bulgaricus</i>
	Pr > F	Pr > F
TRT	<0.0001	<0.0001
TIME	<0.0001	<0.0001
TEMP	<0.0001	<0.0001
TRT*TIME	<0.0001	<0.0001
TRT*TEMP	<0.0001	<0.0002
TRT*TIME*TEMP	<0.0001	<0.0001

Time = Incubation period of 12 hours

Table 12. Pr > F of growth of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 at various low sonication intensities compared to control (0 Watts/ cm<sup>2</sup>)

Treatment (Watts/cm <sup>2</sup> )	<i>S thermophilus</i>																				
	0 hrs			2 hrs			4 hrs			6 hrs			8 hrs			10 hrs			12 hrs		
	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C
8.07	0.004	0.001	0.001	0.158	0.001	0.006	0.001	0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
14.68	0.001	0.001	0.001	0.001	0.001	0.142	0.001	0.233	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
19.83	0.001	0.038	0.001	0.001	0.001	0.002	0.001	0.035	0.055	0.001	0.015	0.007	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
23.55	0.001	0.001	0.001	0.001	0.001	0.003	0.001	0.662	0.001	0.001	0.001	0.001	0.001	0.018	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Table 13. Pr > F of growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at various low sonication intensities compared to control (0 Watts/ cm<sup>2</sup>)

Treatment (Watts/cm <sup>2</sup> )	<i>L. bulgaricus</i>																				
	0 hrs			2 hrs			4 hrs			6 hrs			8 hrs			10 hrs			12 hrs		
	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C
8.07	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
14.68	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
19.83	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
23.55	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Table 14. Least Square Means for growth of bacteria as influenced by low sonication intensities

Treatment (Watt/cm <sup>2</sup> )	<i>S. thermophilus</i>			<i>L. bulgaricus</i>		
	4°C	22°C	40°C	4°C	22°C	40°C
	LSmean	LSmean	LSmean	LSmean	LSmean	LSmean
0	11.19 <sup>C,b</sup>	11.22 <sup>C,a</sup>	11.05 <sup>C,c</sup>	10.15 <sup>A,a</sup>	9.99 <sup>A,b</sup>	9.86 <sup>A,c</sup>
8.07	11.28 <sup>B,a</sup>	11.25 <sup>B,b</sup>	11.14 <sup>A,c</sup>	9.69 <sup>B,a</sup>	9.54 <sup>B,b</sup>	9.28 <sup>B,c</sup>
14.68	11.30 <sup>A,a</sup>	11.28 <sup>A,b</sup>	11.13 <sup>A,c</sup>	9.68 <sup>B,a</sup>	9.43 <sup>D,b</sup>	9.22 <sup>CD,c</sup>
19.83	11.28 <sup>B,a</sup>	11.27 <sup>AB,a</sup>	11.13 <sup>A,b</sup>	9.65 <sup>B,a</sup>	9.47 <sup>C,b</sup>	9.18 <sup>D,c</sup>
23.55	11.31 <sup>A,a</sup>	11.23 <sup>C,b</sup>	11.09 <sup>B,c</sup>	9.67 <sup>B,a</sup>	9.47 <sup>C,b</sup>	9.25 <sup>BC,c</sup>

<sup>ABCD</sup> LSMeans with the same letter within the column are not significantly different

<sup>abcd</sup> LSMeans with the same letter within the row are not significantly different

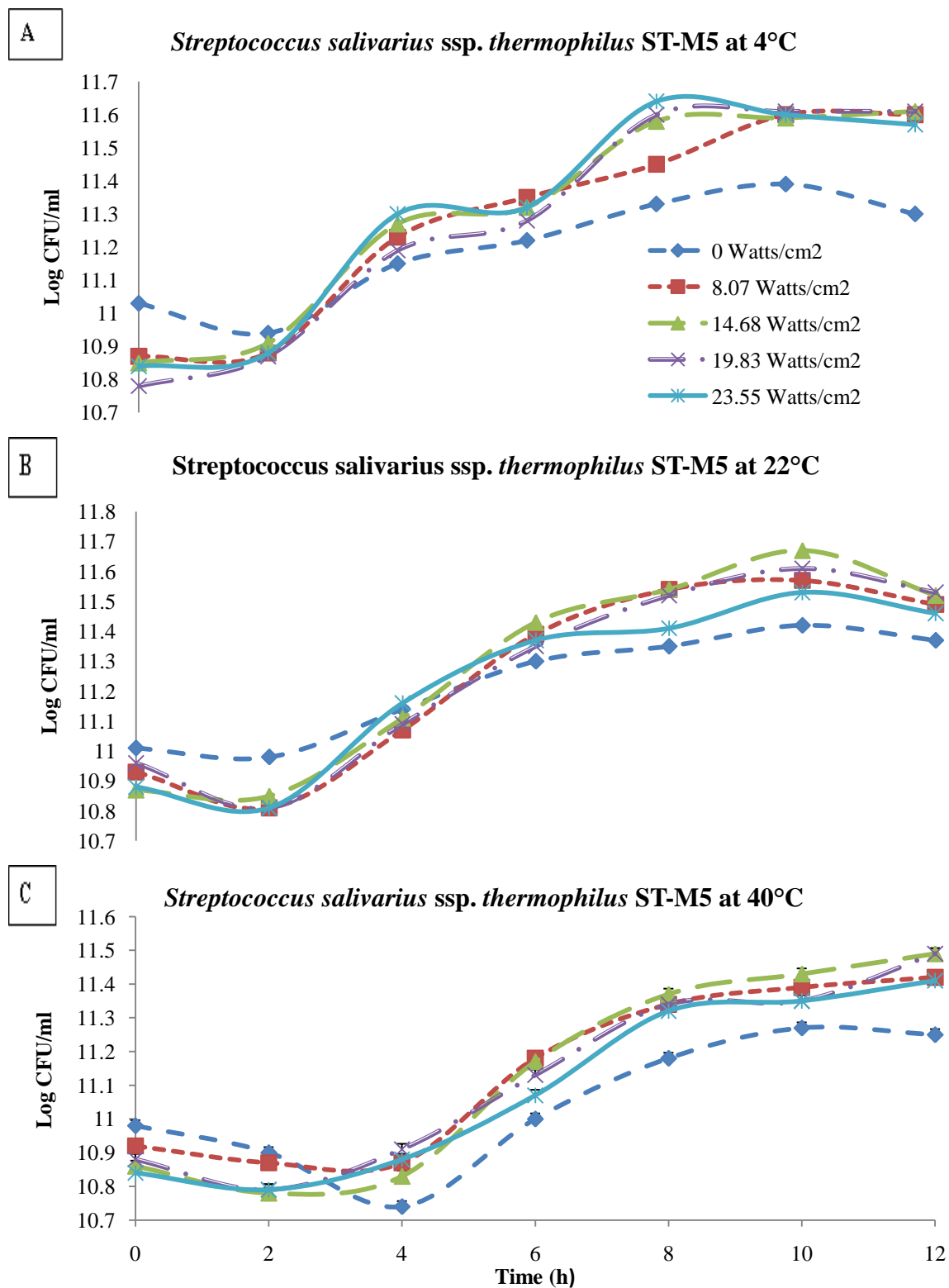


Figure 9. Growth of *Streptococcus salivarius ssp. thermophilus* ST-M5 at 4°C (A), 22°C (B) and 40°C (C)

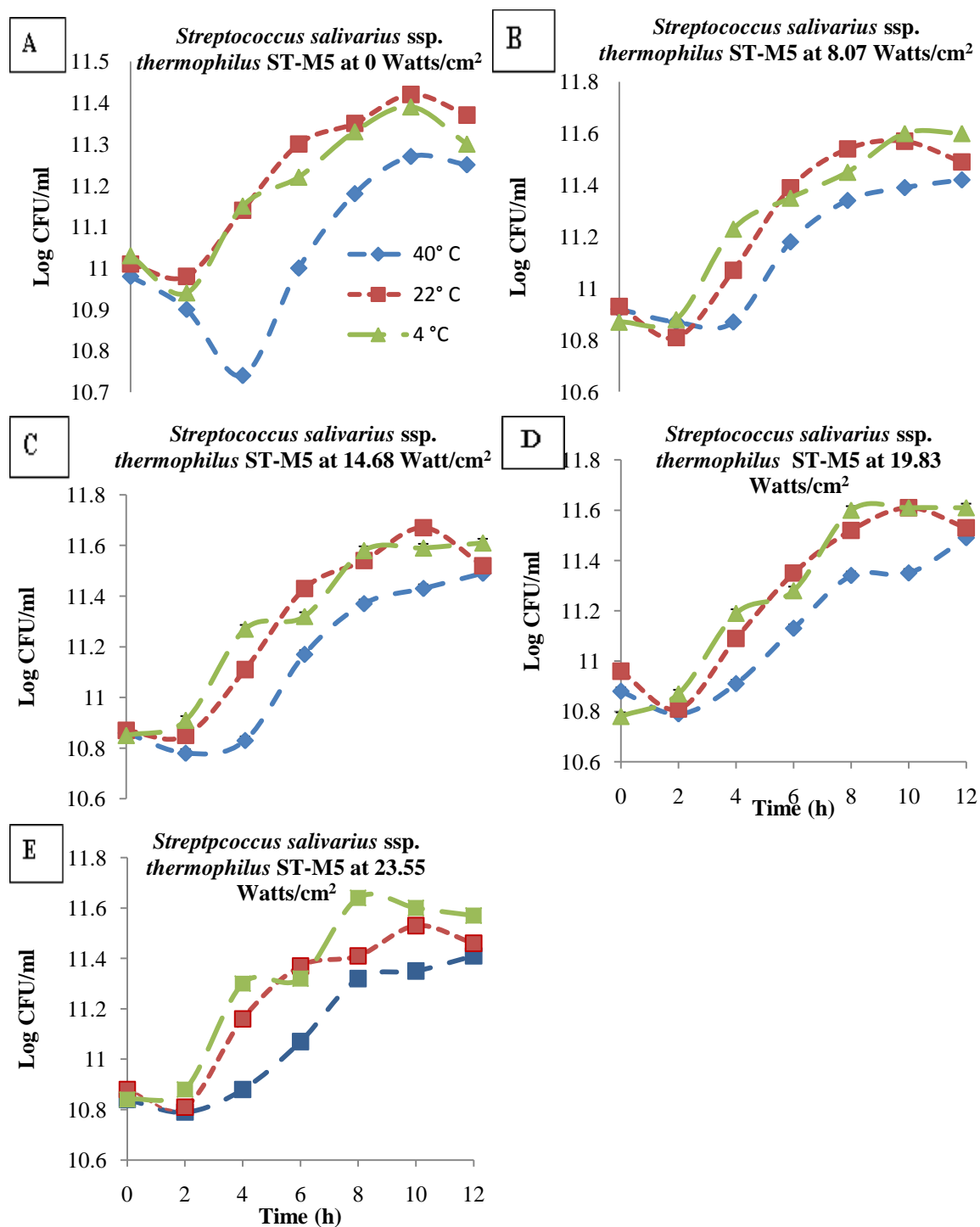


Figure 10. Growth of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 at 0 (A), 8.07 (B), 14.68 (C), 19.83 (D) and 23.55Watts/cm<sup>2</sup> (E) at 4, 22 and 40°C

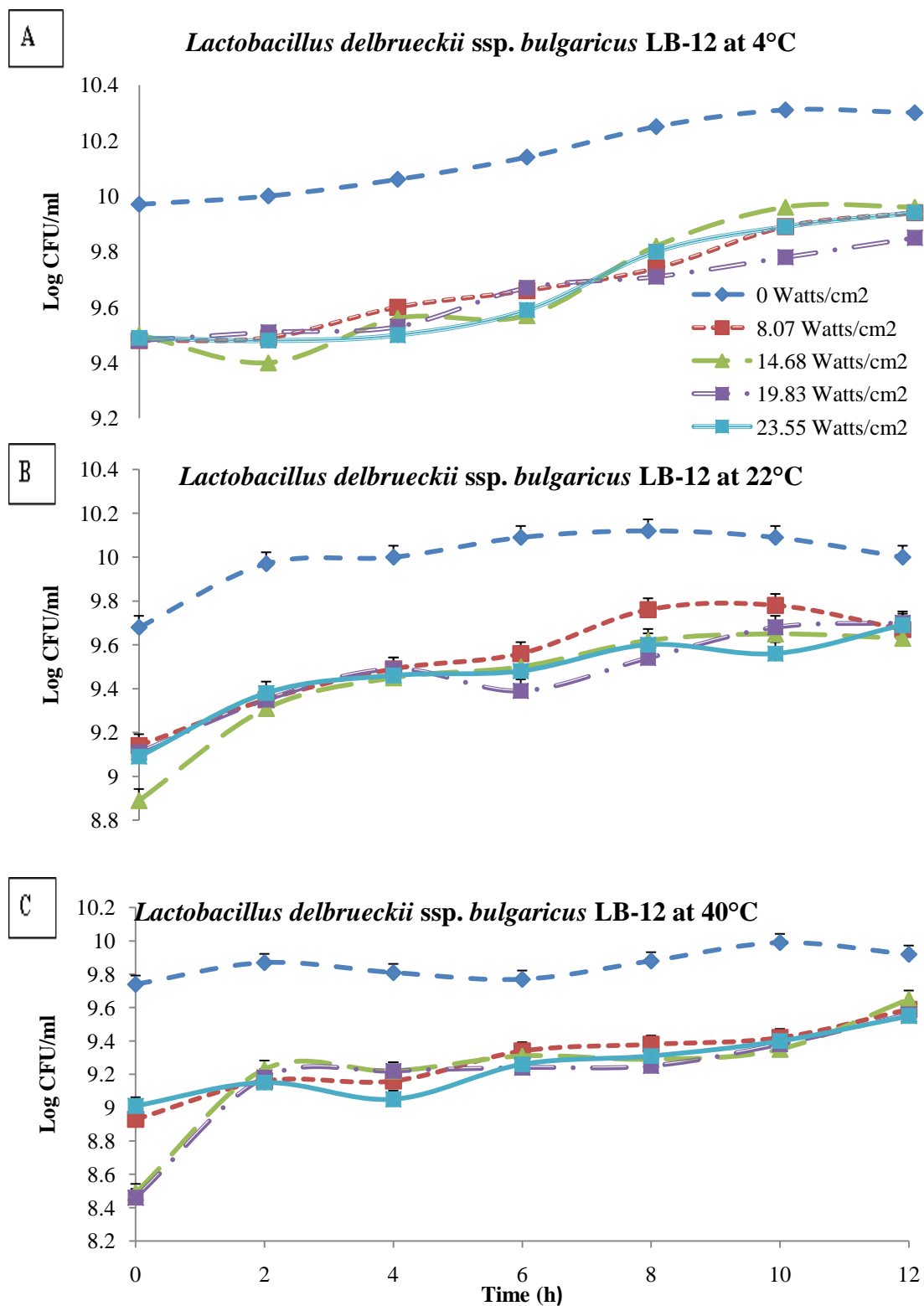


Figure 11. Growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at 4°C (A), 22°C (B) and 40°C (C)



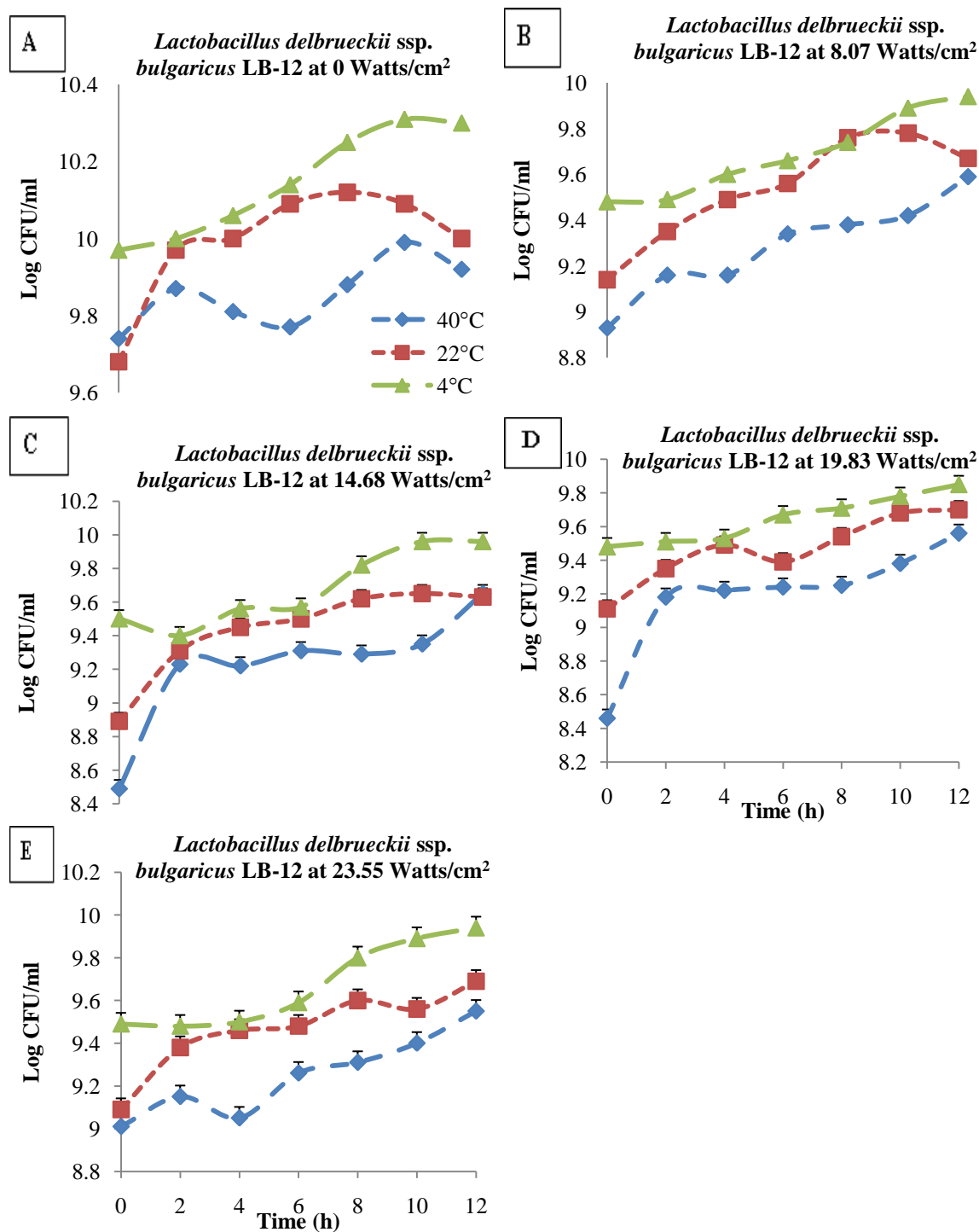


Figure 12. Growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at 0 (A), 8.07 (B), 14.68 (C), 19.83 (D) and 23.55 Watts/cm<sup>2</sup> (E) at 4, 22 and 40°C

### 3.4 Protease Activity

#### 3.4.1 *Streptococcus salivarius* ssp. *thermophilus* ST-M5

The protease activity of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 was influenced by various low sonication intensities at different temperatures (4, 22 and 40°C) is shown in the Figure 13. There was significant ( $P<0.05$ ) interaction between low sonication intensities \* temperature \* time (Table 15). The interaction for low sonication intensity \* time was not significant ( $P = 0.5350$ ) (Table 15). Absorbance units increased over time from 0 to 24 hours (Fig 13). At 4°C, protease activity of cultures subjected to 8.07 Watts/cm<sup>2</sup> was significant ( $P<0.05$ ) higher than the control from 12 to 24 hours (Fig 13A and Table 16 and 18). At 40°C, 23.55 Watts/cm<sup>2</sup> showed significant ( $P<0.05$ ) increase in protease activity compared to control at 0, 12 and 24 hours (Fig 13C and Table 16). Using 23.55 Watts/cm<sup>2</sup> for 0, 12 and 24 hours the Optical Density (OD) values were 0.11, 0.16 and 0.17 absorbance units while OD values for control were 0.09, 0.12 and 0.15 absorbance units respectively.

The low sonication intensity \* temperature interaction was significant ( $P<0.05$ ) (Table 15). The influence of temperature and time at a particular sonication intensity is shown in Figure 14. Cultures subjected to 19.83 and 23.55 Watts/cm<sup>2</sup> showed better protease activity at 40°C compared to cultures subjected to 4 and 22°C (Fig 14 C, D and Table 18). The temperature had a significant ( $P<0.05$ ) effect (Table 15). The cultures subjected to 23.55 and 19.83 Watts/cm<sup>2</sup> at 40°C showed higher protease activity compared to control (Table 18).

The low sonication intensities had significant ( $P<0.05$ ) effect (Table 15). Some low sonication conditions increased protease activity of *Streptococcus salivarius* ssp. *thermophilus* ST-M5.

### 3.4.2 *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12

The protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 as influenced by various low sonication intensities at different temperatures (4, 22 and 40°C) is shown in the Figure 15. There was significant ( $P<0.05$ ) interaction between low sonication intensities \* temperature \* time (Table 15). The interaction for low sonication intensity \* time was significant ( $P<0.05$ ) (Table 15). Absorbance units increased over time from 0 to 24 hours (Fig 15). At 4°C, OD values of cultures subjected to 14.68 Watts/cm<sup>2</sup> were significantly ( $P<0.05$ ) higher in protease activity than the control from 12 to 24 hours (Fig 15A and Table 17). At 22°C, cultures subjected to 23.55 Watts/cm<sup>2</sup> had significantly ( $P<0.05$ ) the highest protease activity compared to control at 12 and 24 hours (Fig 15B and Table 17). Using 23.55 Watts/cm<sup>2</sup> for 12 and 24 hours the OD values were 0.45 and 0.86 absorbance units while OD values for control were 0.37 and 0.57 absorbance units respectively. At 40°C, cultures subjected to 8.07, 14.68 and 23.55 Watts/cm<sup>2</sup> showed significant ( $P<0.05$ ) increase in protease activity compared to control from 12 to 24 hours (Fig 15C, Table 17 and 18).

The low sonication intensity \* temperature interaction was significant ( $P<0.05$ ) (Table 15). Cultures subjected to 8.07 and 14.68 Watts/cm<sup>2</sup> showed better protease activity at 40°C than cultures subjected to 4 and 22°C (Fig 16 B, C and Table 18). The temperature had a significant ( $P<0.05$ ) effect (Table 15). The cultures sonicated at 40°C showed higher protease activity compared to control (Table 18). The low sonication intensities had significant ( $P<0.05$ ) effect (Table 15). Some low sonication conditions increased protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12.

This study of low sonication intensities at three different temperatures (4, 22 and 40°C) showed that *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 exhibited higher OD values, hence high protease activity compared to *S. thermophilus*. Shah and Jelen, (1990) reported that *L. bulgaricus* exhibited higher  $\beta$ -galactosidase activity compared to *S. thermophilus* and *L. acidophilus*. Additionally, Wang et al. (1996) sonicated samples of *Lactobacillus delbrueckii* ssp. *bulgaricus* B-5b inoculated in sterile non fat dried milk for 10 min using a sonicator 300 dismembrator at a frequency of 16 kHz and reported that the highest amount of  $\beta$ -galactosidase released by sonication-fermentation was after 4h of the culture incubation in milk fermentation. This indicated that the intracellular enzyme was not released to the medium during conventional fermentation, but was released during sonicated fermentation.

Table 15. Pr > F of low sonication treatment, time, and temperature their interaction for protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12, *Streptococcus salivarius* ssp. *thermophilus* ST-M5

EFFECT	S thermophilus	L bulgaricus
	Pr > F	Pr > F
TRT	<0.0001	<0.0001
TIME	<0.0001	<0.0001
TEMP	<0.0001	<0.0001
TRT*TIME	0.5350	<0.0001
TRT*TEMP	<0.0001	0.0368
TRT*TIME*TEMP	<0.0001	<0.0001

Time = Incubation period of 24 hours

Table 16. Pr > F of protease activity of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 at various low sonication intensities compared to control (0 Watts/ cm<sup>2</sup>)

Treatment (Watts/cm <sup>2</sup> )	<i>S thermophilus</i>								
	0 hr			12 hrs			24 hrs		
	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C
8.07	0.345	0.329	0.001	0.034	0.008	0.001	0.042	0.776	0.469
14.68	0.592	0.132	0.117	0.103	0.001	0.924	0.037	0.117	0.097
19.83	0.117	0.034	0.329	0.140	0.097	0.091	0.080	0.285	0.057
23.55	0.469	0.011	0.049	0.001	0.001	0.001	0.080	0.075	0.007

Table 17. Pr > F of protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at various low sonication intensities compared to control (0 Watts/ cm<sup>2</sup>)

Treatment (Watts/cm <sup>2</sup> )	<i>L bulgaricus</i>								
	0 hr			12 hrs			24 hrs		
	4°C	22°C	40°C	4°C	22°C	40°C	4°C	22°C	40°C
8.07	0.639	0.778	0.953	0.104	0.851	0.003	0.117	0.001	<0.001
14.68	0.742	0.971	0.664	0.001	0.566	0.001	0.002	0.005	<0.001
19.83	0.783	0.751	0.920	0.012	0.158	0.163	0.257	0.001	<0.001
23.55	0.751	0.846	0.586	0.127	0.001	0.001	0.011	<0.001	<0.001

Table 18. Least Square Means for protease activity of bacteria as influenced by low sonication intensities

Treatment (Watts/cm <sup>2</sup> )	<i>S. thermophilus</i>			<i>L. bulgaricus</i>		
	4°C	22°C	40°C	4°C	22°C	40°C
0	0.05 <sup>B,b</sup>	0.11 <sup>A,a</sup>	0.12 <sup>C,a</sup>	0.27 <sup>C,b</sup>	0.34 <sup>C,a</sup>	0.37 <sup>B,a</sup>
8.07	0.07 <sup>A,b</sup>	0.09 <sup>AB,a</sup>	0.09 <sup>D,a</sup>	0.32 <sup>BC,c</sup>	0.40 <sup>BC,b</sup>	0.52 <sup>A,a</sup>
14.68	0.06 <sup>AB,b</sup>	0.09 <sup>B,a</sup>	0.11 <sup>C,a</sup>	0.40 <sup>A,b</sup>	0.38 <sup>BC,b</sup>	0.52 <sup>A,a</sup>
19.83	0.07 <sup>A,c</sup>	0.10 <sup>A,b</sup>	0.13 <sup>B,a</sup>	0.34 <sup>AB,b</sup>	0.44 <sup>AB,a</sup>	0.47 <sup>A,a</sup>
23.55	0.06 <sup>AB,c</sup>	0.08 <sup>B,b</sup>	0.15 <sup>A,a</sup>	0.35 <sup>AB,b</sup>	0.46 <sup>A,a</sup>	0.52 <sup>A,a</sup>

<sup>ABCD</sup> LSMeans with the same letter within the column are not significantly different

<sup>abcd</sup> LSMeans with the same letter within the row are not significantly different

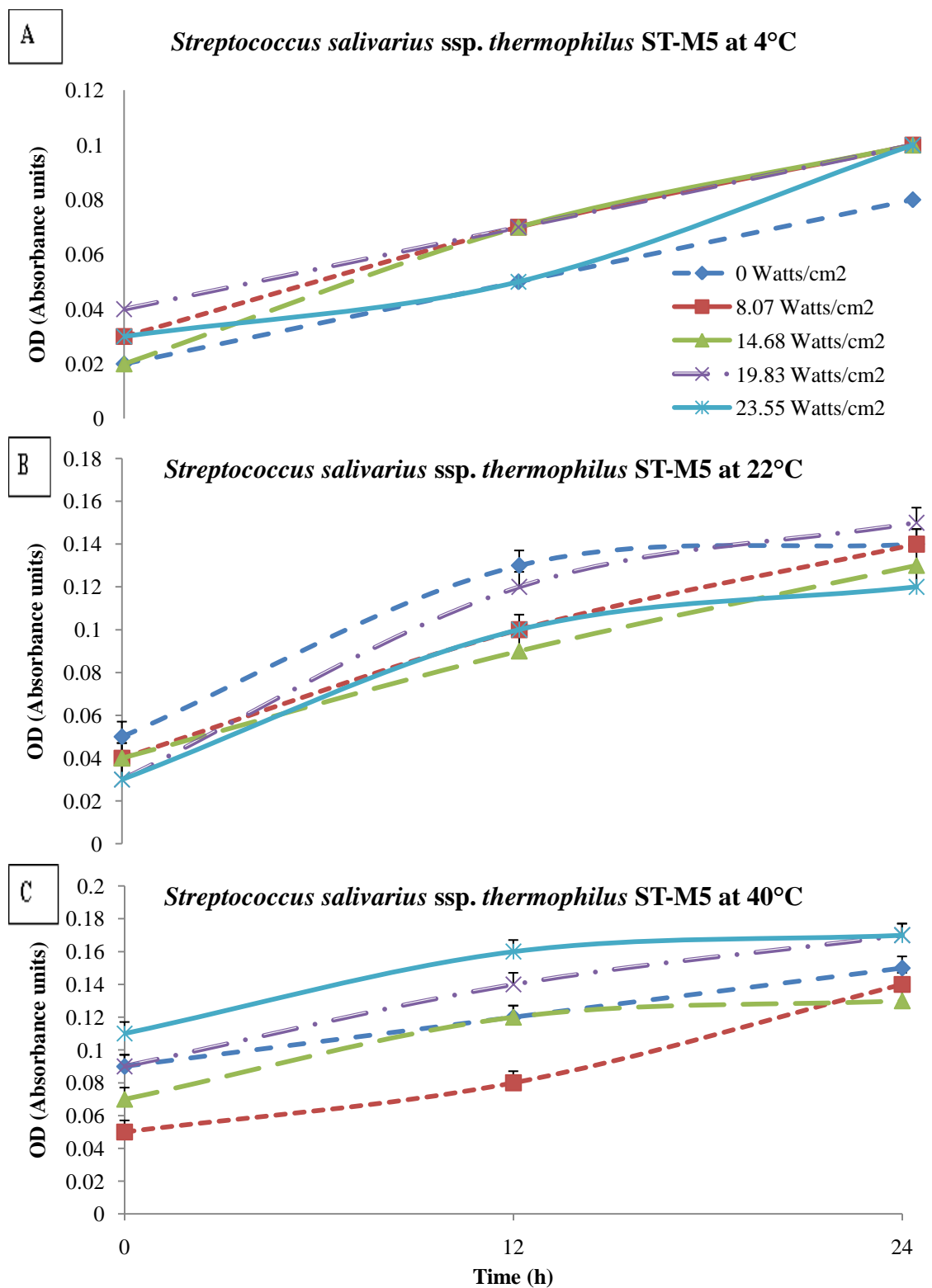


Figure 13. Protease activity of *Streptococcus salivarius ssp. thermophilus* ST-M5 at 4°C (A), 22°C (B) and 40°C (C)

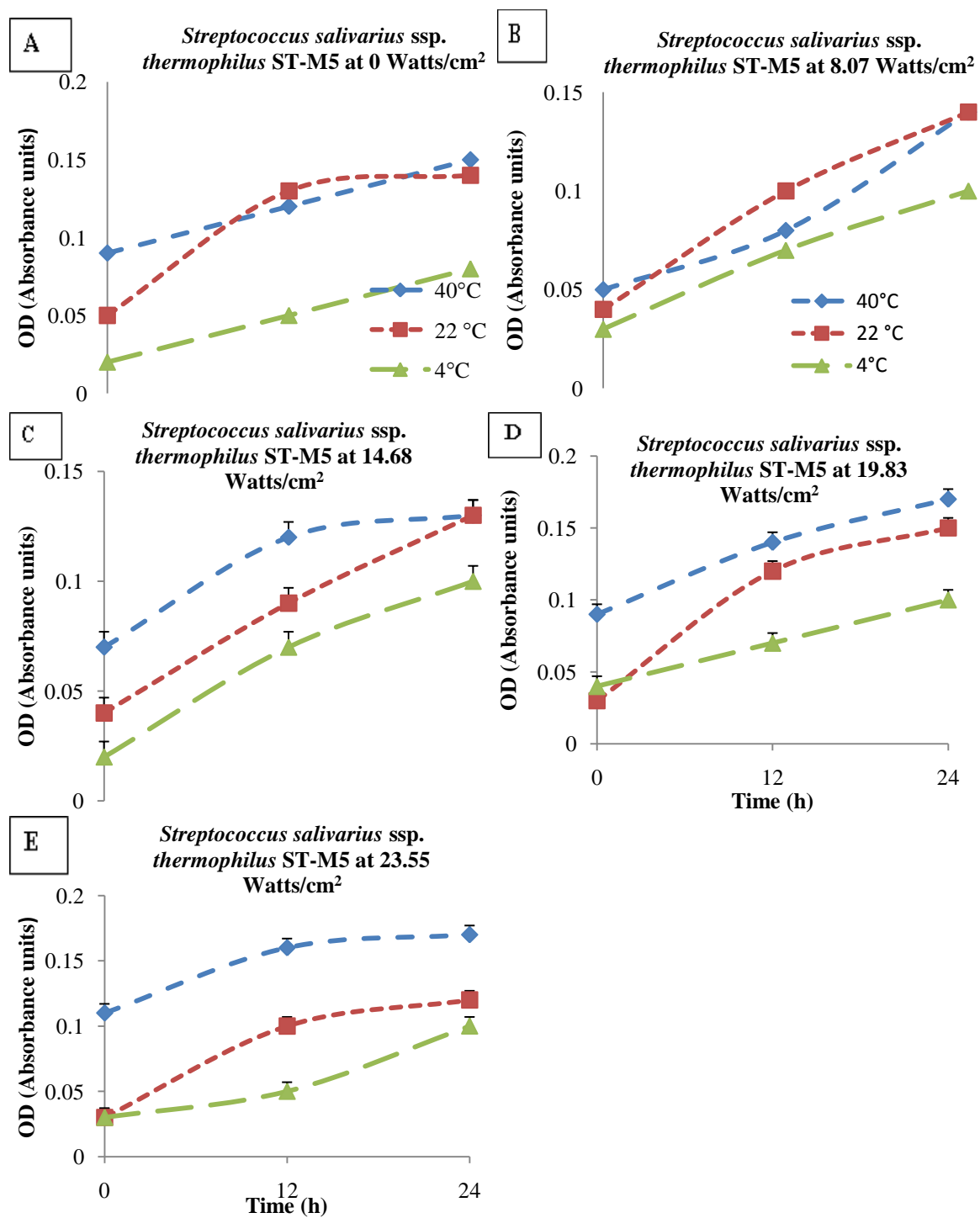


Figure 14. Protease activity of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 at 0 (A), 8.07 (B), 14.68 (C), 19.83 (D) and 23.55 (E) at 4, 22 and 40°C



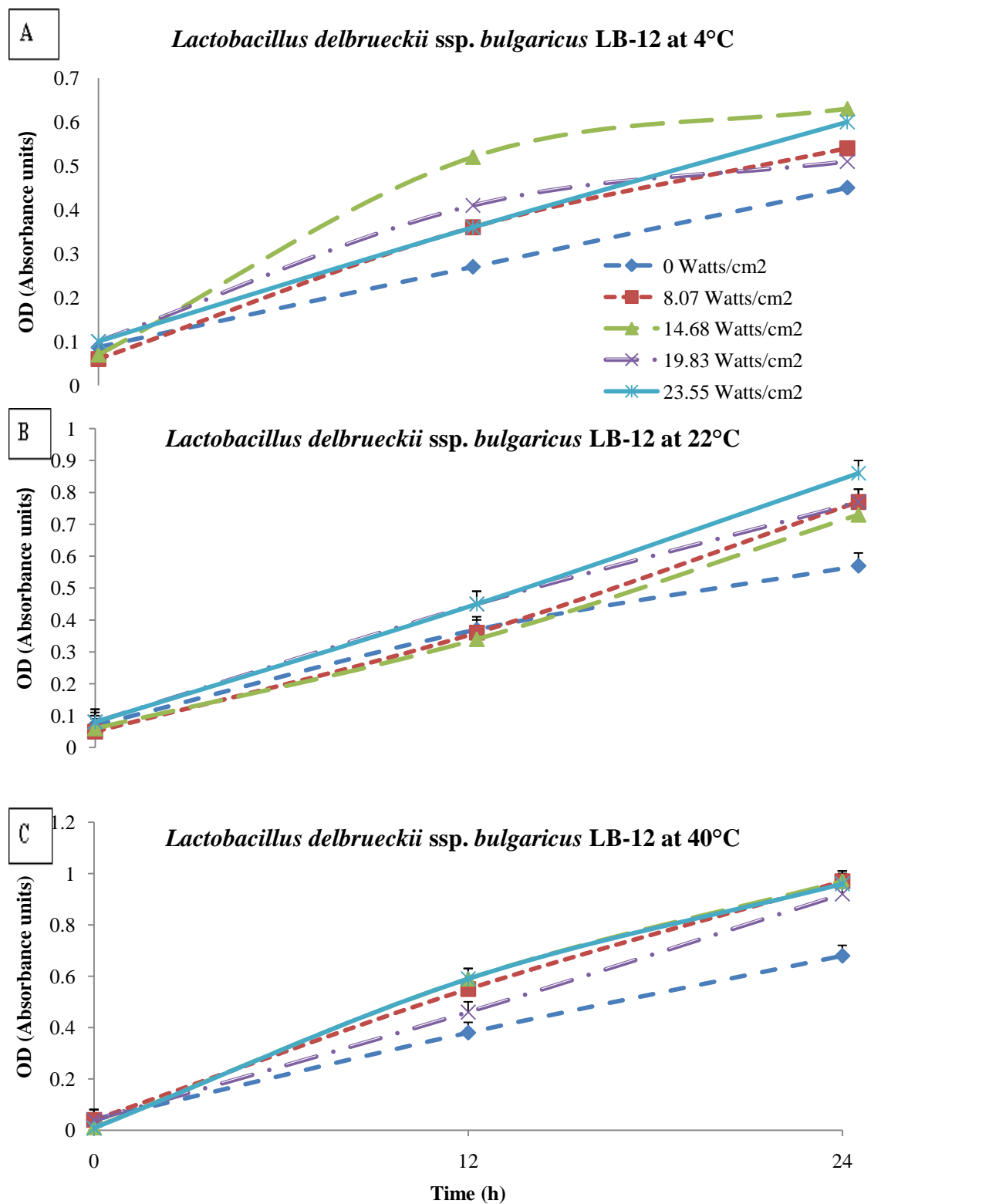


Figure 15. Protease activity of *Lactobacillus delbrueckii ssp. bulgaricus* LB-12 at 4°C (A), 22°C (B) and 40°C (C)

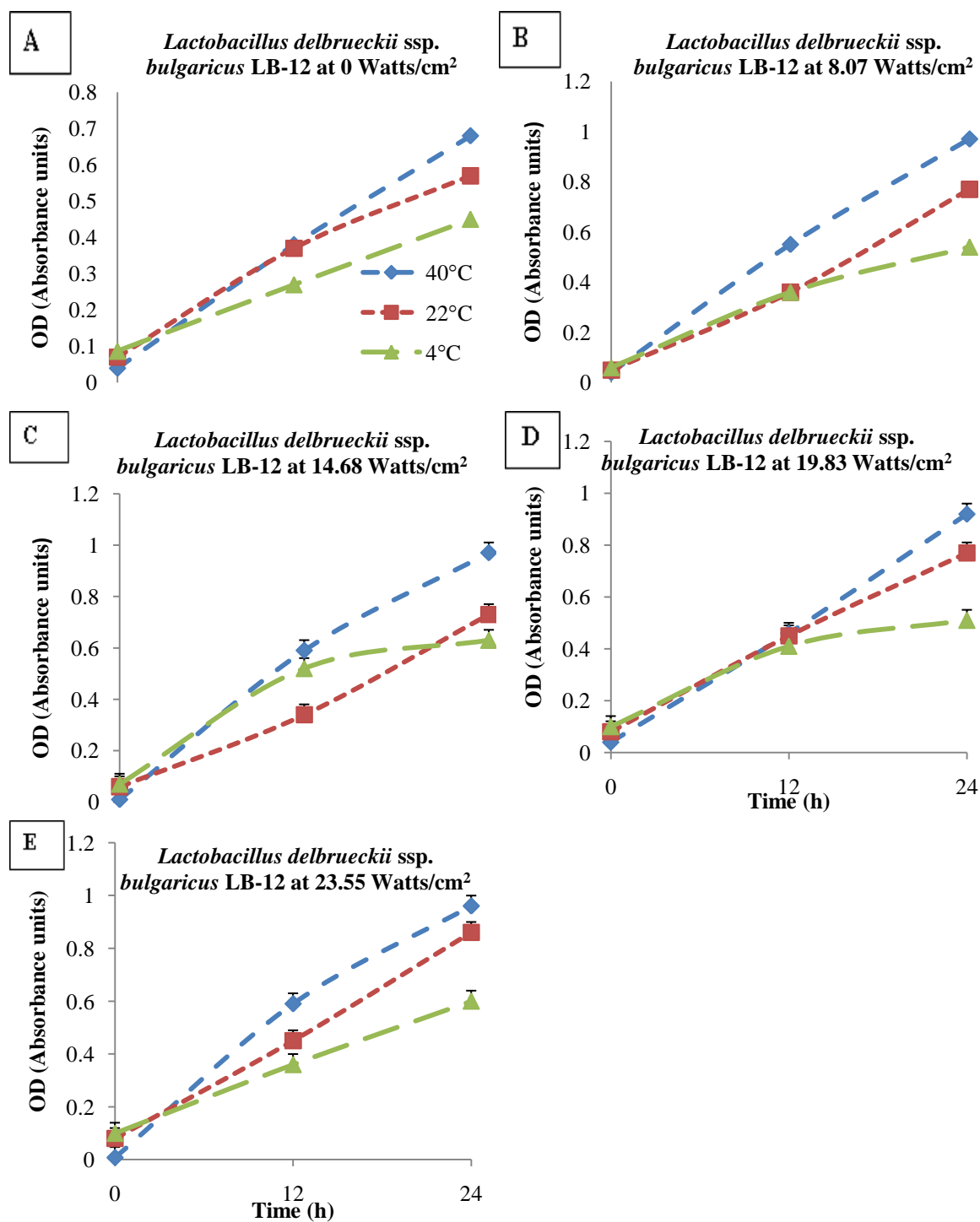


Figure 16. Protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at 0 (A), 8.07 (B), 14.68 (C), 19.83 (D) and 23.55 Watts/cm<sup>2</sup> (E) at 4, 22 and 40°C

## CHAPTER 4: CONCLUSIONS

Low sonication conditions include a) low sonication intensities, b) temperatures and c) times all three of which played a role in influencing the desirable attributes of both microorganisms. Of all the low sonication intensities studied, 14.68 watts /cm<sup>2</sup> had the best overall influence at certain time points for *Streptococcus salivarius* ssp. *thermophilus* ST-M5 improving its acid tolerance, bile tolerance and growth at 4°C, growth at 22°C, bile tolerance at 40°C, growth at 40°C and improving the *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 bile tolerance and growth at 4°C, its acid tolerance and protease activity at 40°C. Low sonication intensity of 19.83 Watts/cm<sup>2</sup> had the overall best influence at certain time points for acid tolerance of both microorganisms at 22°C. Low sonication intensity of 23.55 Watts/cm<sup>2</sup> had the overall best influence at certain time points for protease activity of *Streptococcus salivarius* ssp. *thermophilus* ST-M5 at 40°C and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 at 22°C. Some low sonication conditions improved certain characteristics of culture bacteria.

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