

Antimicrobial Efficacy of Natural Phenolic Compounds against Gram Positive Foodborne Pathogens

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Abstract

Protection of food from pathogens and spoilage organisms has been achieved by a variety of methods. Due to consumer preference, health and economic concerns in recent years, there is considerable interest to employ natural antimicrobials as an alternative to control the growth of microorganisms. This study evaluates the antimicrobial efficacy of natural plant derived phenolic compounds (PDPC) including chlorogenic acid, coumarin, curcumin, ellagic acid, (-) epicatechin, eugenol, rosmarinic acid, rutin, tannic acid, thymol, thymoquinone, and xanthohumol) as preservatives in food products. Several strains of *Bacillus*, *Listeria* and *Clostridium* species were treated with 12 natural PDPCs. Concentrations of 5, 10, 15, and 20 ppm of each compound were evaluated by broth micro-dilution method and the MICs were determined by using optical density after 24 and 60 hours of incubation. Thymoquinone, xanthohumol and ellagic acid demonstrated the highest antimicrobial efficacy (MIC <20 ppm). Structural alterations in treated bacteria were observed via scanning electron microscopy. The results demonstrated that the PDPCs have varying antimicrobial activities against both aerobic and anaerobic Gram-positive foodborne pathogens following 24 hour and 60 hour incubation periods, respectively. Natural sources of phenolic compounds contain major antimicrobial components and have great potential to control the growth of pathogens and be used as natural antimicrobials and food preservatives for extended storage.

This study highlighted the antimicrobial efficacy of some PDPCs which may replace the artificial antimicrobials and preservatives in food industry to partially or completely control or inhibit the growth of harmful bacteria.

Keywords: foodborne pathogen, phenolic compounds, minimum inhibitory concentration (MIC), antimicrobial activity

1. Introduction

Raw and processed foods are vulnerable to contamination during their production, distribution and sale. Unless properly handled and preserved, the growth of spoilage and pathogenic bacteria causes the loss of quality and potential public health problems. The remarkable increase in the number of reported cases of foodborne illnesses requires new solutions and techniques to prevent foodborne illnesses (Vaquero et al., 2011). Foodborne illnesses resulting from the consumption of food contaminated with pathogenic bacteria have been a concern for public health for years. In order to prevent pathogens, extend the storage time, antimicrobial and antioxidant additives, especially of synthetic origin, are added to foods to improve the efficacy of the preservative methods (Gyawali & Ibrahim, 2014).

Since the use of chemical additives is perceived as a health risk by consumers, the food industry has an ever growing interest in using natural antimicrobials to improve food stability and safety against pathogens (Taguri et al., 2004; Santas et al., 2010). Due to the consumers' awareness and demand for natural food products and growing concern about the increased microbial resistance to conventional preservatives (Gyawali & Ibrahim, 2014), the investigation of alternative inhibitors to ensure food safety became the current trend in food industry.

Phenolic compounds (PCs) are widely distributed in foods of plant origin such as fruits, vegetables, nuts, seeds, stems and flowers as well as tea, and barks (Santas et al., 2010). Although the antimicrobial properties of common spice and herb extracts or essential oils, containing PCs, have long been recognized and classified as Generally Recognized As Safe (GRAS) (Lambert et al., 2001) their use as natural preservatives in food is often

limited due to the unacceptable organoleptic changes (Cowan, 1999) they may induce when used in high doses (Friedman, 2014, Weerakkody et al., 2011). Thus, there is an increasing demand for accurate knowledge of the minimum inhibitory (effective) concentrations (MIC) of plant derived phenolic compounds (PDPCs) to enable a balance between the sensory acceptability and antimicrobial efficacy that can be achieved with both *in vitro* and applied studies (Lambert et al., 2001, Phantong et al., 2013).

Although the antimicrobial activity of some PDPCs has been previously studied (Cueva et al., 2010; Kim et al., 2011) the response after long term exposure has not been reported. This study is the first to reveal the antimicrobial efficacy of thymoquinone, xanthohumol and coumarin. Also, antimicrobial activity of chlorogenic acid, curcumin, (-) epicatechin, eugenol, thymol, tannic acid and rutin on pathogenic *Listeria monocytogenes*, *Bacillus* spp. and *Clostridium* spp. needs to be studied more.

The main objectives of this study were (1) to evaluate the antimicrobial efficacy of selected natural PDPCs against Gram-positive foodborne pathogens: *Bacillus subtilis*, *Bacillus cereus*, *Bacillus (Paenibacillus) polymyxa*, *L. monocytogenes*, *Clostridium perfringens*, *Clostridium butyricum* and *Clostridium sporogenes* (2) to determine the MIC of the natural PDPCs and (3) to observe their prolonged antimicrobial activities over the prolonged incubation of 60 h to simulate the long term storage by extending the exposure time of pathogens to PDPCs.

2. Materials and Methods

2.1 Plant Derived Phenolic Compounds

Twelve different natural PDPCs were used in the study including: chlorogenic acid, coumarin, curcumin, ellagic acid, eugenol, (-) epicatechin, rosmarinic acid, rutin, tannic acid, thymol, thymoquinone, and xanthohumol (Sigma-Aldrich, St Louis, MO, USA). All of the compounds are commercially available (powder form) and they were $\geq 95\%$ pure. Each compound was dissolved in ethanol, (95%) (Decon Laboratories, King of Prussia, PA, USA) except for thymoquinone, which was prepared with dimethyl sulfoxide 99.9% (DMSO) (Fisher Scientific, Fair Lawn, NJ, USA). The final phenolic solution was adjusted to approximately pH 5.00 using HCl (15%) to ensure that the pH would not affect the bacterial growth. All solutions were filter sterilized using 0.22 μm filters (Millipore Corporation, Billerica, MA, USA) and stored at 4°C in sterilized sealed glass containers until needed. All experiments were done at ambient temperature.

2.2 Bacterial Strains, Culture Conditions and Preparation of Inoculum

A total of 9 foodborne pathogen strains including *B. subtilis* (ATCC 6051), *B. cereus* (ATCC 11778), *B. polymyxa* (ATCC 842), *C. perfringens* (R. Newsome Research), *C. butyricum* (ATCC 8260), *C. sporogenes* (ATCC 7955), and 3 strains of *L. monocytogenes* (ATCC 7644, UK Animal Diagnostic Lab, and ATCC 49594) were supplied from the American Type Culture Collection and the University of Kentucky. *Listeria* and *Bacillus* were grown and maintained on slants of Brain-Heart Infusion (BHI) agar and *Clostridium* spp. were maintained on thioglycollate medium (TM) anaerobically at 4°C until needed. Prior to each test, the isolates were sub-cultured at least three times before inoculating in BHI for aerobic bacteria and Reinforced Clostridial Broth (RCB) for *Clostridium* spp. Culture growth turbidity, which is indicated by the optical density (OD), was standardized for each bacterium at a wavelength of 660 nm (OD_{660}) by using the spectrophotometer (BioTek Synergy 4, Winooski, VT, USA). For the initial bacterial count approximately 10^7 - 10^8 CFU/ml was targeted and cell counts were confirmed by using the Eddy Jet spiral plater (Neutec Group Inc., Farmingdale, NY, USA) with Plate Count Agar (PCA). The bacterial counts were determined by the Flash and Go plate reader (Neutec Group Inc., Farmingdale). All microbiological media and supplements used in the study were supplied from Difco Laboratories (Sparks, MD, USA) unless otherwise specified.

2.3 Determination of Minimum Inhibitory Concentrations of Phenolic Compounds

Micro broth dilution technique of Antimicrobial Susceptibility testing was performed as outlined in the National Committee for Clinical Laboratory Standards (NCCLS, 2004) and modified for 60 h incubation (Cetin-Karaca & Newman, 2015). Serial dilutions of the compounds (100 μl) were dispensed into 5 ml Mueller Hinton Broth (MHB) and RCB for *Clostridium* to obtain the final concentrations of 5, 10, 15 and 20 ppm (mg l^{-1}). Then, 100 μl of the overnight culture of bacteria was transferred aseptically into MHB/RCB. Compound free inoculated MHB/RCB with and without the solvent were served as growth control and negative control, respectively. One hundred and fifty μl of each sample, including the controls, was dispensed to the wells of a 96-well flat bottom micro-titer plate (Nalge NUNC Int., Corning, NY, USA) and incubated at 37°C. *Clostridium* spp. were incubated anaerobically at 37°C in BBL anaerobe jars with GasPak EZ anaerobe system (Becton and Dickinson, Sparks, MD, USA). All experiments were carried out three times in duplicates.

After inoculation, the micro-titer plates were read immediately to get the initial OD using a calibrated spectrophotometer (BioTek Synergy 4) at 660 nm wavelength. Prior to each incubation process, the samples in the micro-titer plate were shaken automatically for 10 seconds to get a consistent homogeneity. The absorbance was read at 12-hour intervals for a 60 h incubation period. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the compound that visibly inhibited the bacterial growth in comparison with the control (Waško *et al.*, 2014). Antimicrobial activities of PDPCs towards selected pathogens were demonstrated as absorbance difference relative to the control (% treatment/control).

2.4 Investigation of Structural Changes of Cells via Scanning Electron Microscopy

MICs of PDPCs were applied to bacterial cultures and they were incubated for 24 hr at 37°C. Suspensions were filter sterilized through 0.22 µm filters (Thermo Scientific, Nalgene, Rochester, NY, USA) to capture the bacteria and the same filters with bacteria were used for scanning electron microscope (SEM) observations as described by Kalab *et al.* (2008).

The filters containing thin layers of bacterial specimens were fixed with glutaraldehyde fixative (6%) (E.M. Grade, SPI Supplies Inc., West Chester, PA, USA) and then dehydrated using serial dilutions of ethanol; 20%, 40%, 60%, 80%, and 100%, followed by hexamethyldisilazane (Sigma-Aldrich). The specimens were prepared and sputter-coated with carbon using a plasma coating system for SEM (Hummer VI Sputtering System; Technics, Union City, CA, USA). The morphology of bacterial cells was examined in Hitachi S-800 SEM (Tokyo, Japan) and captured images were analyzed by Evex Nano-analysis and Digital Imaging (Evex Analytical Version 2.0.1192, 2006) software.

2.5 Statistical Analysis

All the experiments were repeated three times and the data were calculated as means ± SD. The antimicrobial efficacy of the PDPCs was subjected to General Linear Model procedure of Statistix 9.0 (2008). Differences in the means of *Bacillus* spp., *L. monocytogenes* and *Clostridium* spp. absorbance (OD) influenced by the presence of phenolic compounds were determined by the use of Tukey HSD ($P < 0.05$).

3. Results and Discussion

3.1 Antimicrobial Activity against *Bacillus* spp.

The MICs of selected PDPCs at 60 h are shown in Table 1. Although the antimicrobial activity (for 24 h) of some PCs has been previously reported (Cueva *et al.*, 2010; Jianu *et al.*, 2012; Kim *et al.*, 2011), this study is the first to evaluate the antimicrobial efficacy of selected PDPCs during 60 h of extended incubation. The prolonged antimicrobial activity of PDPCs and the response of the pathogens to long term exposure was observed with the extended 60 h incubation time, which might provide evidence for high potential success in food safety for products stored for extended periods.

Varying degrees of antimicrobial activity was observed with all of PDPCs tested. Growth inhibition rates decreased in the following order; thymoquinone > rutin > ellagic acid > xanthohumol > curcumin > coumarin > thymol > rosmarinic acid > eugenol > chlorogenic acid > (-) epicatechin > tannic acid (Table 1).

Table 1. MICs (ppm) of phenolic compounds against *Bacillus* spp., *Clostridium* spp., and *Listeria* spp. after 60 h of incubation

Compound	Minimum Inhibitory Concentrations (ppm)								
	BS ATCC 6051	BC ATCC 11778	BP ATCC 842	CP RNR	CB ATCC8260	S ATCC 7955	LM1 ATCC 7644	LM2 UK ADL	LM3 ATCC 49594
Chlorogenic acid	< 20.0	< 10.0	< 10.0	< 20.0	< 20.0	< 5.0	< 10.0	< 10.0	< 10.0
Coumarin	< 20.0	< 5.0	< 15.0	> 20.0	< 10.0	< 5.0	< 5.0	< 5.0	< 15.0
Curcumin	< 15.0	< 15.0	< 5.0	< 5.0	< 5.0	< 5.0	> 20.0	< 20.0	> 20.0
Ellagic acid	< 10.0	< 10.0	< 5.0	< 5.0	< 15.0	< 20.0	< 5.0	< 5.0	< 5.0
Eugenol	< 20.0	< 15.0	< 20.0	< 20.0	> 20.0	> 20.0	< 5.0	< 10.0	< 5.0
(-) Epicatechin	< 20.0	< 20.0	< 20.0	< 20.0	< 20.0	< 10.0	< 20.0	< 5.0	< 5.0
Rosmarinic acid	< 15.0	< 10.0	< 15.0	> 20.0	< 20.0	< 10.0	> 20.0	> 20.0	> 20.0
Rutin	< 15.0	< 5.0	< 10.0	< 10.0	> 20.0	> 20.0	< 5.0	< 10.0	< 15.0
Tannic acid	< 20.0	> 20.0	> 20.0	< 20.0	< 20.0	< 10.0	< 10.0	< 15.0	< 20.0
Thymol	< 20.0	< 15.0	< 10.0	< 5.0	< 5.0	< 5.0	> 20.0	> 20.0	> 20.0
Thymoquinone	< 5.0	< 15.0	< 10.0	< 10.0	< 20.0	< 10.0	< 10.0	< 10.0	< 10.0
Xanthohumol	< 5.0	< 20.0	< 5.0	< 5.0	< 5.0	< 5.0	< 10.0	< 5.0	< 10.0

BS: *B. subtilis*, BC: *B. cereus*, BP: *B. polymyxa*, CP: *C. perfringens*, CB: *C. butyricum*, CS: *C. sporogenes*, LM: *L. monocytogenes*.

Thymol consistently inhibited growth of all *Bacillus* tested (MIC < 20 ppm), in good agreement with inhibitory effects of *Zateria multiflora* Boiss extracts containing thymol for *B. subtilis* (MIC < 8 mg/ml) (Saei-Dehkordi *et al.*, 2010). Previously coumarin derivatives were found effective against *B. cereus* (< 25 mg/ml) (Patel *et al.*, 2012) in agreement with our results (coumarin MIC < 20 ppm). Eugenol, rosmarinic acid and tannic acid demonstrated consistent inhibition for *Bacillus* spp. throughout the entire incubation period with MICs, < 20, < 15, and > 20 ppm, respectively.

Figures 1a-7a show the antimicrobial sensitivity of *Bacillus* spp. against selected PDPCs. Thymoquinone, MIC < 15 ppm, exhibited the highest ($P < 0.05$) antimicrobial activity among all tested PDPCs (Figure 1a). However, all strains of *Bacillus* recover and became resistant to thymoquinone after 24 h of incubation ($P < 0.05$). There are no antimicrobial reports for thymoquinone against *Bacillus* except for the inhibitory effect against *Staphylococcus aureus* (< 1g/ml) (Bakathir & Abbas, 2011). Ellagic acid was observed to inhibit the growth of *Bacillus* spp., *B. subtilis* being the most sensitive ($P < 0.05$) with MIC < 10 ppm (Figure 2a). Similarly, rubus honey and pomegranate extracts containing ellagic acid were also highly inhibitory against *B. cereus* with MIC 31.3 mg/ml (Nanasombat & Teckchuen, 2009) and < 18 mm inhibition zone (Hayrapetyan *et al.*, 2012), respectively. There are no previous reports about the antimicrobial activity of xanthohumol for *Bacillus* spp., but hops extracts containing xanthohumol were found effective against gram-positive bacteria including *Micrococcus*, *Mycobacterium*, *Staphylococcus* (MIC < 6.50 μ g/ml) (Zanoli & Zavatti, 2008) and *B. subtilis* (< 31 mm inhibition zone) (Pyla *et al.*, 2010). Although all *Bacillus* spp. in this study were highly sensitive ($P < 0.05$) to xanthohumol with MIC < 20 ppm, *B. polymyxa* and *B. subtilis* showed recovery after 24 h (Figure 3a). The sensitivity of the pathogenic bacteria to PDPCs depends on bacterial species, source, concentration and polyphenol structure of the PCs as well as the methods used for the experiments.

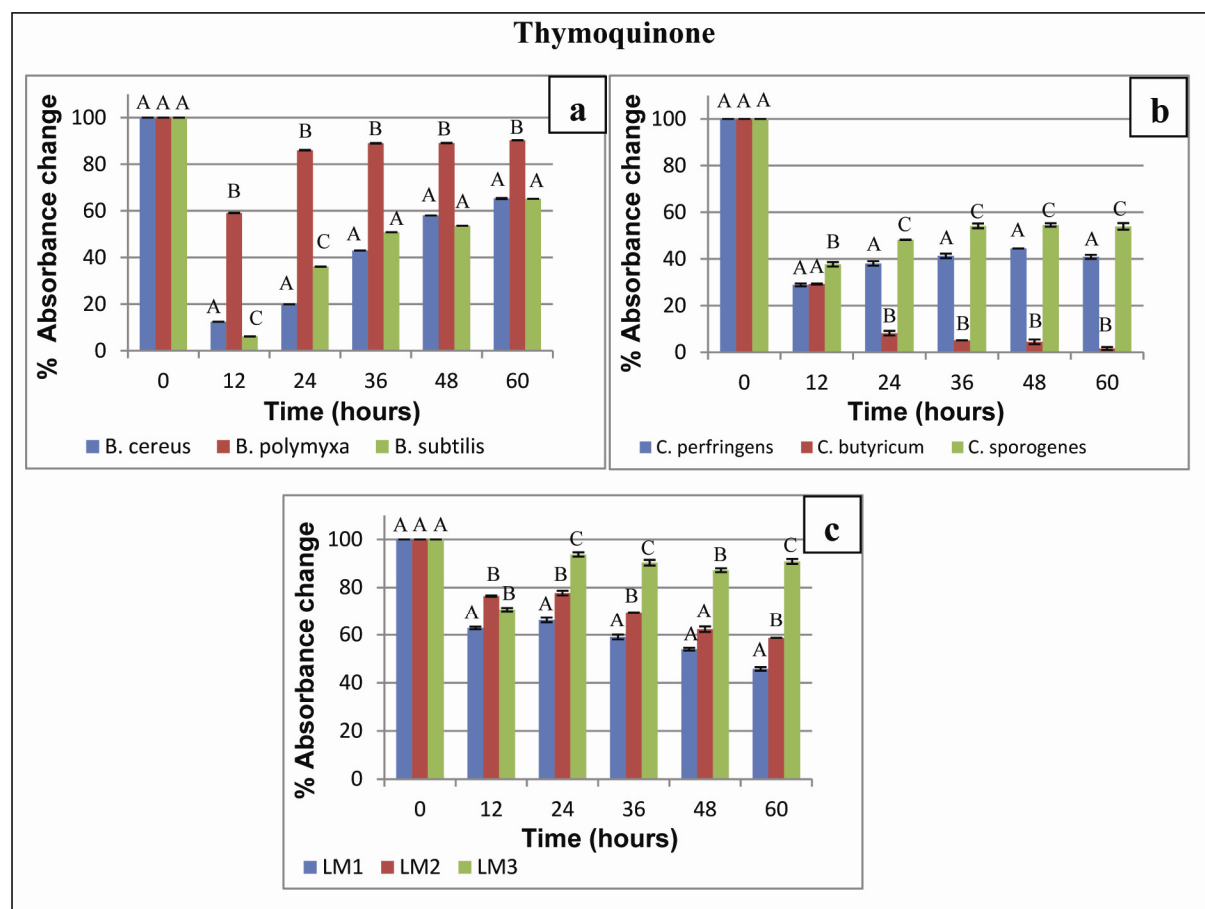


Figure 1. Antimicrobial activity of thymoquinone against a) *Bacillus* spp. (15 ppm); b) *Clostridium* spp. (20 ppm); and c) *Listeria* spp. (*L. monocytogenes*; LM) (10 ppm) are illustrated as relative absorbance difference from the control calculated as: $100 \times \text{treatment/control}$. The data represents the mean values of 3 independent experiments. Error bars denote standard deviation. Different letters denote significant differences between the strains within the same incubation time-points, $P < 0.05$

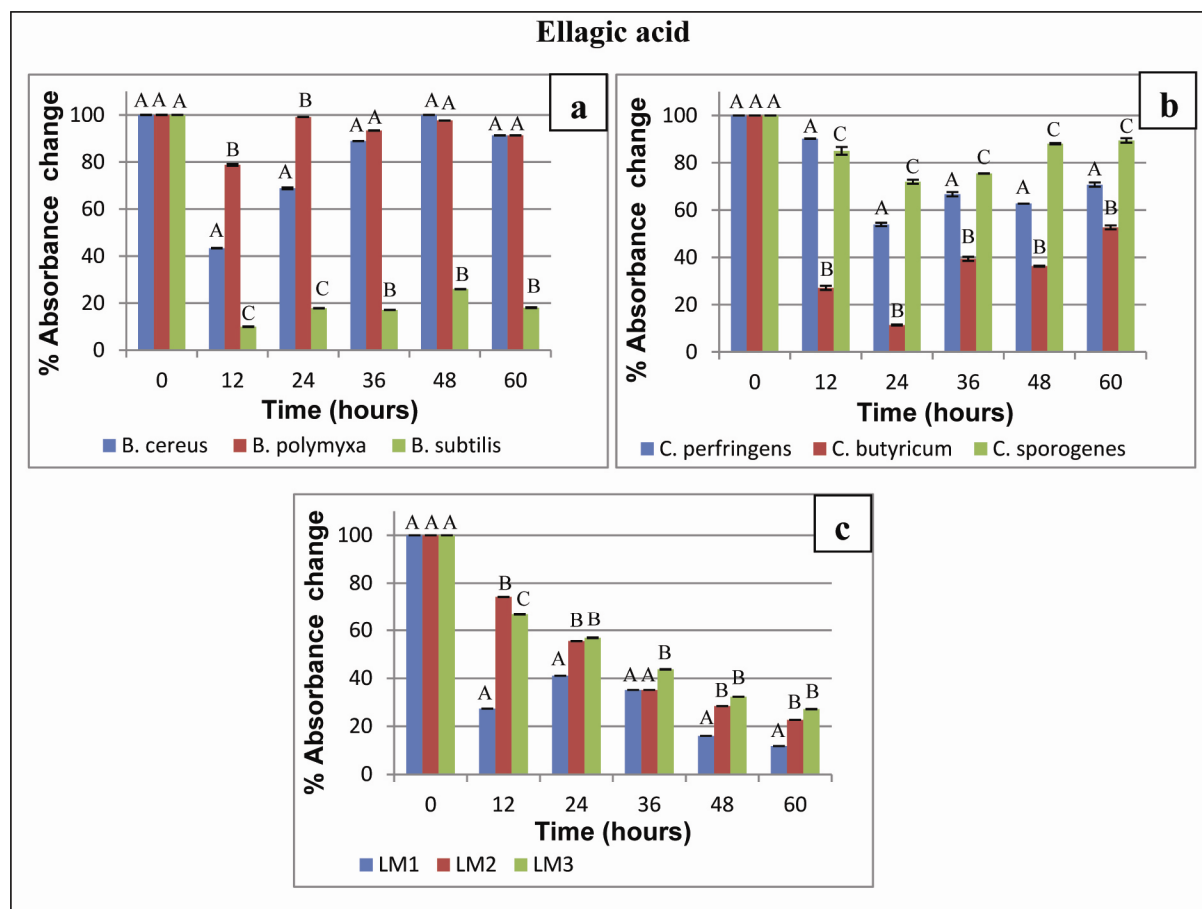


Figure 2. Antimicrobial activity of ellagic acid against a) *Bacillus* spp. (10 ppm); b) *Clostridium* spp. (20 ppm); and c) *Listeria* spp. (*L. monocytogenes*; LM) (5 ppm) are illustrated as relative absorbance difference from the control calculated as: $100 \times \text{treatment/control}$. The data represents the mean values of 3 independent experiments. Error bars denote standard deviation. Different letters denote significant differences between the strains within the same incubation time-points, $P < 0.05$

Curcumin was also highly effective ($P < 0.05$) against all *Bacillus* tested (MIC < 15 ppm) (Figure 4a) in accordance with previous findings of curcumin (MIC 100 $\mu\text{g/ml}$) and nano-curcumin (MIC 75 $\mu\text{g/ml}$) (Bhawana et al., 2011). (-) Epicatechin inhibited ($P < 0.05$) all three species of *Bacillus* (MIC < 20 ppm) (Figure 5a), consistent with Shan et al. (2007) for its (from cinnamon stick extract) efficacy against *B. cereus* (MIC 625 $\mu\text{g/ml}$). Moreover, chlorogenic acid also showed high ($P < 0.05$) antimicrobial activity (MIC < 20 ppm) (Figure 6a) which is in good agreement with previous reports regarding *B. subtilis*, MIC 40 $\mu\text{g/ml}$ (Norajit et al., 2007). Rutin's inhibitory results (Figure 7a) are complying with previous reports of medicinal plants ($> 10,240$ $\mu\text{g/ml}$) and spices (5,120-10,240 $\mu\text{g/ml}$) containing rutin (Askun et al., 2009a; 2009b). *B. cereus* (IC₂₅ < 1 mg/ml) and *B. subtilis* (IC₂₅ 4.12 mg/ml) were also found highly sensitive to rutin extracted from olive leaves (Pereira et al., 2007). However, Lee & Lee (2010) reported rutin (100 $\mu\text{g/ml}$) had no antimicrobial activity against *B. cereus*.

3.2 Antimicrobial Activity against *Clostridium* spp.

MICs of tested PDPCs against *Clostridium* spp. which demonstrated variations in antimicrobial efficacy; curcumin, thymol and xanthohumol being the most and eugenol being the least efficient PDPC (Table 1).

Antimicrobial efficacy of PDPCs against anaerobic bacteria including *Clostridium* spp. haven't been reported broadly, therefore antimicrobial effectiveness of curcumin, coumarin, ellagic acid, (-) epicatechin, tannic acid, thymoquinone and xanthohumol are first being reported in this study.

Previously Jianu et al. (2012) suggested that thymol derived from dill seeds is highly antimicrobial against *C. perfringens* (< 26.06 mm zone inhibition) which is in agreement with our results (MIC < 5 ppm). Eugenol demonstrated antimicrobial activity for all *Clostridia* spp. with MICs > 20 ppm ($P < 0.05$). Consistently, it was

also found highly effective against *C. sporogenes* and *C. perfringens* with MICs <13.5 µg/ml (Brusotti et al., 2012) and <4.50 mg/ml (Candan et al., 2003), respectively.

Figures 1b-7b depict the antimicrobial efficacy of selected PDPCs against *Clostridium* spp. Growth inhibition ($P < 0.05$) was achieved with thymoquinone, MIC <20 ppm (Figure 1b) and ellagic acid, MIC <20 ppm (Figure 2b) during the 60 hours of incubation period. However, *C. perfringens* and *C. sporogenes* showed resistance ($P < 0.05$) to thymoquinone after 24 h. Furthermore, xanthohumol (Figure 3b) and curcumin (Figure 4b) showed very similar and strong antimicrobial efficacy ($P < 0.05$) for all *Clostridium* spp. (MIC <5 ppm) followed by (-) epicatechin (Figure 5b), tannic acid, and coumarin with MICs <20, <20, and >20 ppm, respectively. After 24 h, *C. butyricum* recovered and became resistant to xanthohumol. Recently, neither of chlorogenic acid, rosmarinic acid nor rutin was found inhibitory for *C. sporogenes* (Salawu et al., 2011). However, in this study all three *Clostridium* spp. showed varying degrees of sensitivity ($P < 0.05$) against chlorogenic acid (Figure 6b), rutin (Figure 7b), and rosmarinic acid with MICs of <20 ppm.

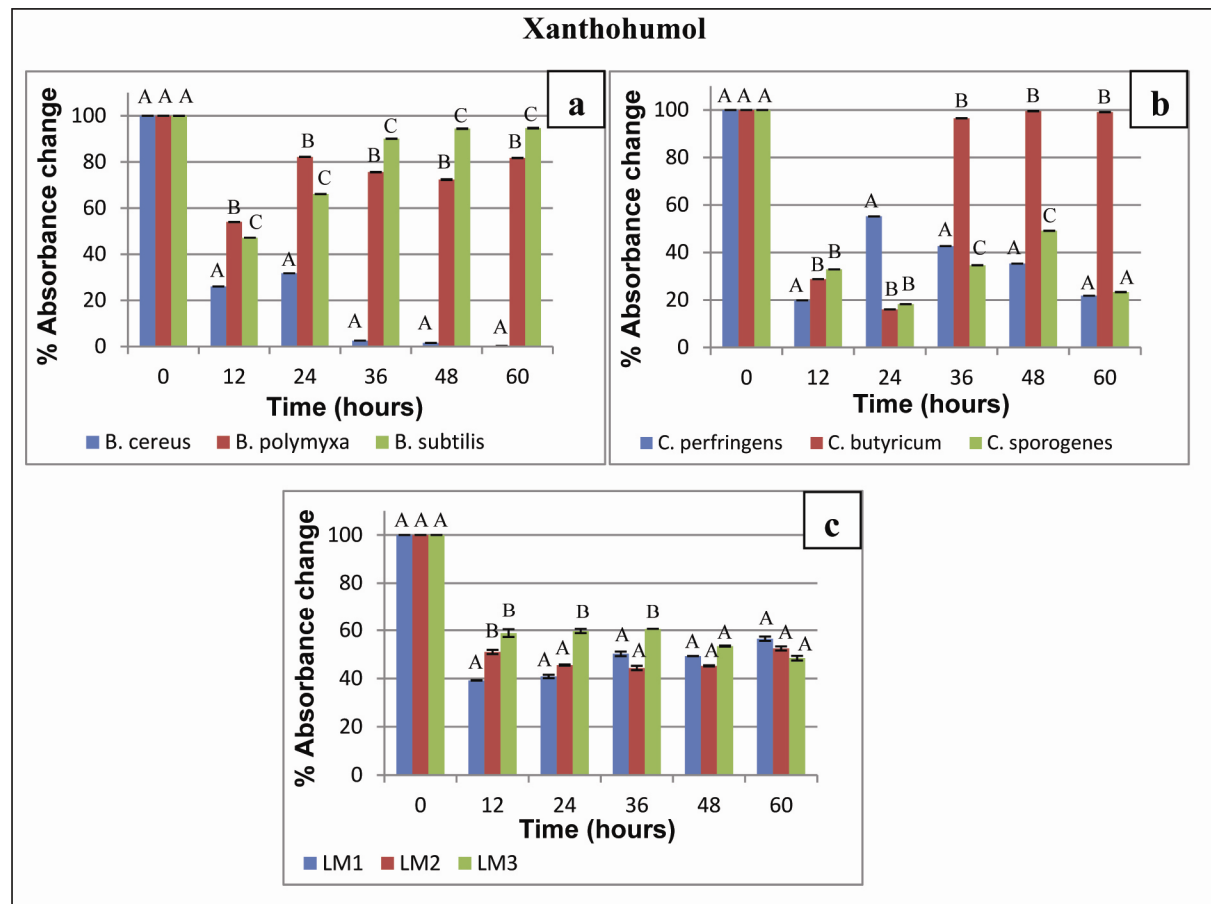


Figure 3. Antimicrobial activity of xanthohumol against a) *Bacillus* spp. (20 ppm); b) *Clostridium* spp. (5 ppm); and c) *Listeria* spp. (*L. monocytogenes*; LM) (10 ppm) are illustrated as relative absorbance difference from the control calculated as: $100 \times \text{treatment/control}$. The data represents the mean values of 3 independent experiments. Error bars denote standard deviation. Different letters denote significant differences between the strains within the same incubation time-points, $P < 0.05$

3.3 Antimicrobial Activity against *L. monocytogenes*

The MICs of selected PDPCs against *L. monocytogenes* (LM1-3) are presented in Table 1. Tannic acid with MIC <20 ppm showed strong antimicrobial activity following 60 hours of incubation. According to Pyla *et al.* (2010) starch-based films impregnated with tannic acid caused a 2.72-log decrease in *L. monocytogenes* in 48 hours incubation period. MIC of eugenol was determined as <10 ppm in accordance with eugenol's efficacy in

reducing the proliferation of *L. monocytogenes* on the surface of fresh lettuce (Kim et al., 2011) and when incorporated into alginate-based edible coatings (Raybaudi-Massilia et al., 2009).

Moreover, thymol was previously found to inhibit *Listeria* (>7 mm inhibition zones) (Tanis et al., 2009), whereas, in this study, neither thymol nor rosmarinic acid showed any antimicrobial activity ($P < 0.05$) against *L. monocytogenes* (20 ppm) at 60 h. This should be due to the different methods (agar diffusion) and higher concentrations used in those studies. Also, the chemical composition of PDPCs may vary due to the variety of plant used, harvesting period and agricultural methods used and the type of extraction method and solvent used.

Figures 1c-7c summarizes the antimicrobial sensitivity of *L. monocytogenes* spp. to selected PDPCs. The highest growth inhibition ($P < 0.05$) was obtained with ellagic acid, MIC <5 ppm (Figure 2c), while curcumin showed the lowest growth inhibition ($P < 0.05$). Similarly Hayrapetyan *et al.* (2012) also found that pomegranate extract (<24.7 mg/ml) containing ellagic acid was effective against *L. monocytogenes*. Being consistent with previous *L. monocytogenes* studies (>7 mm zone inhibition) (Tanis et al., 2009), thymoquinone was highly antimicrobial with MIC <10 ppm (Figure 1c).

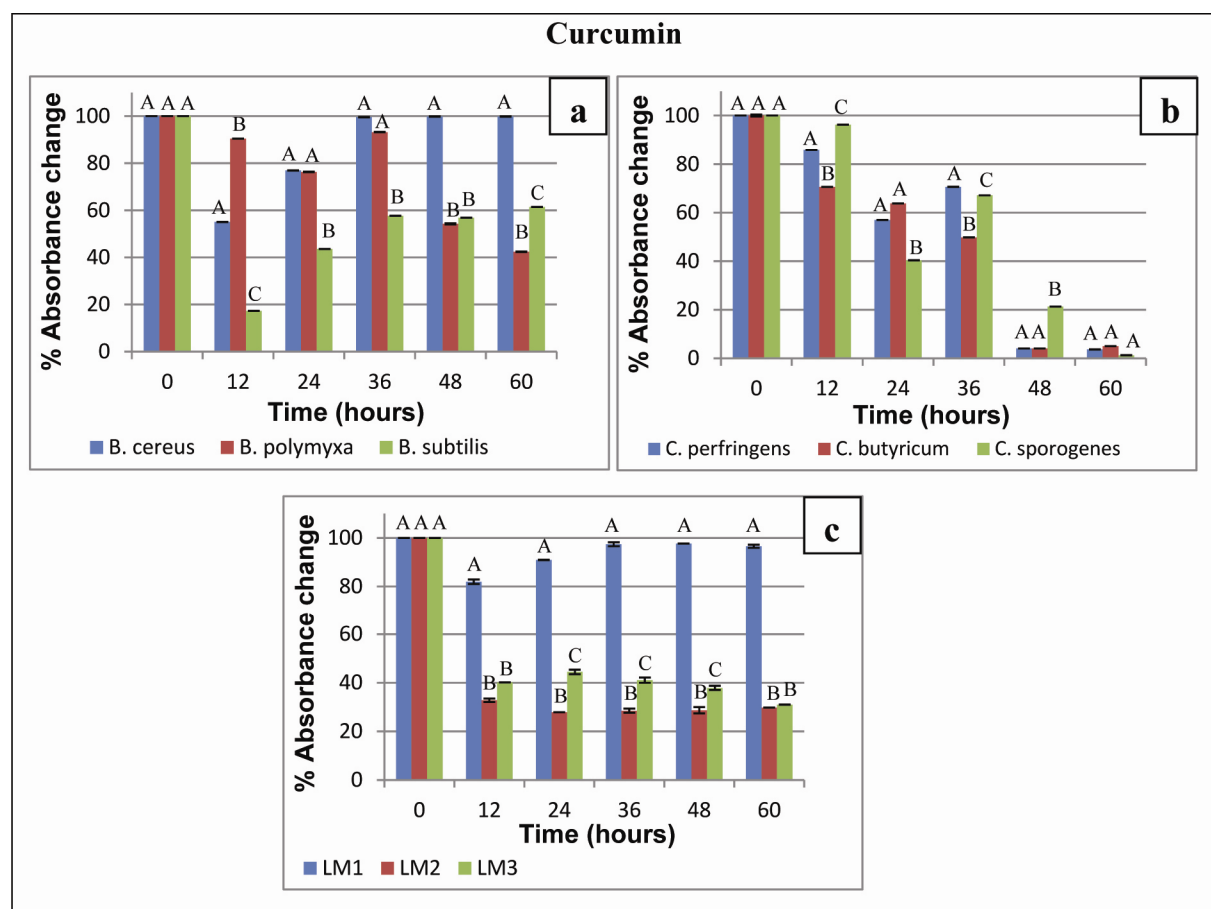


Figure 4. Antimicrobial activity of curcumin against a) *Bacillus* spp. (15 ppm); b) *Clostridium* spp. (5 ppm); and c) *Listeria* spp. (*L. monocytogenes*; LM) (20 ppm) are illustrated as relative absorbance difference from the control calculated as: $100 \times \text{treatment/control}$. The data represents the mean values of 3 independent experiments. Error bars denote standard deviation. Different letters denote significant differences between the strains within the same incubation time-points, $P < 0.05$

All three strains of *L. monocytogenes* were observed to be highly susceptible ($P < 0.05$) when treated with xanthohumol (Figure 3c), curcumin (Figure 4c), and coumarin with MICs <10, >20, and <15 ppm, respectively. After 24 h of incubation, LM3 became resistant to thymoquinone ($P < 0.05$). LM1 showed resistance, while LM2 and LM3 were highly sensitive to curcumin ($P < 0.05$).

Curcumin (<6.25 mg/l) extracted from turmeric (*Curcuma longa*) (Norajit et al., 2007) and coumarin from methanol extracts of *Prangos ferulacea* (<13 mm zone inhibition) (Sagun et al., 2006) were also reported to be highly effective against inhibiting *L. monocytogenes*. When (-) epicatechin (<11.6mg/g) from green tea used on edible film coatings, it was promising as means of controlling the growth and recontamination of *L. monocytogenes* on ready-to-eat meat products (Theivendran et al., 2006). Our results are also in good agreement with these findings with MIC <20 ppm (Figure 5c). LM2 and LM3 were highly sensitive towards (-) epicatechin, while LM1 was resistant ($P < 0.05$). Previously chlorogenic acid obtained from carrot extracts (<55 mg/ml) was reported to be highly antimicrobial against *L. monocytogenes* (Babic et al., 1994) where this study confirmed its antimicrobial efficacy for all three strains of *L. monocytogenes*, MIC <10 ppm (Figure 6c). However, LM2 and LM3 gained resistance ($P < 0.05$) against chlorogenic acid after 36 h. Figure 7c demonstrates the antimicrobial activity of rutin (MIC <15ppm) which was consistent to some extent with previous studies of rutin extracted from Vietnamese coriander (*Polygonum odoratum*) (<83.3 mg/ml) (Nanasombat & Teckchuen, 2009), Greek aromatic plants (10 mm zone inhibition) (Proestos et al., 2006) and Argentinean wines (<500 mg/l) (Rodríguez Vaquero et al., 2007).

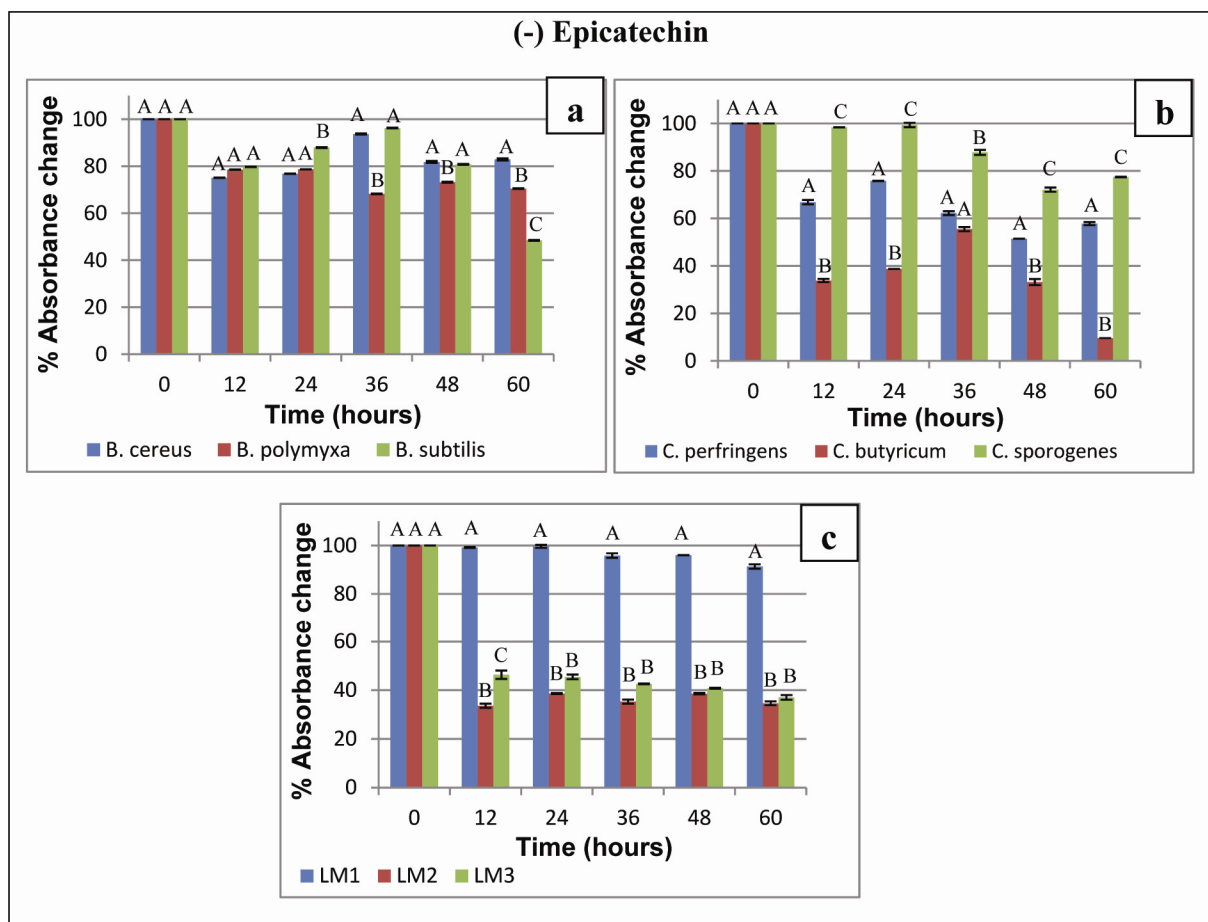


Figure 5. Antimicrobial activity of (-) epicatechin against a) *Bacillus* spp. (20 ppm); b) *Clostridium* spp. (20 ppm); and c) *Listeria* spp. (*L. monocytogenes*; LM) (20 ppm) are illustrated as relative absorbance difference from the control calculated as: $100 \times \text{treatment}/\text{control}$. The data represents the mean values of 3 independent experiments. Error bars denote standard deviation. Different letters denote significant differences between the strains within the same incubation time-points, $P < 0.05$

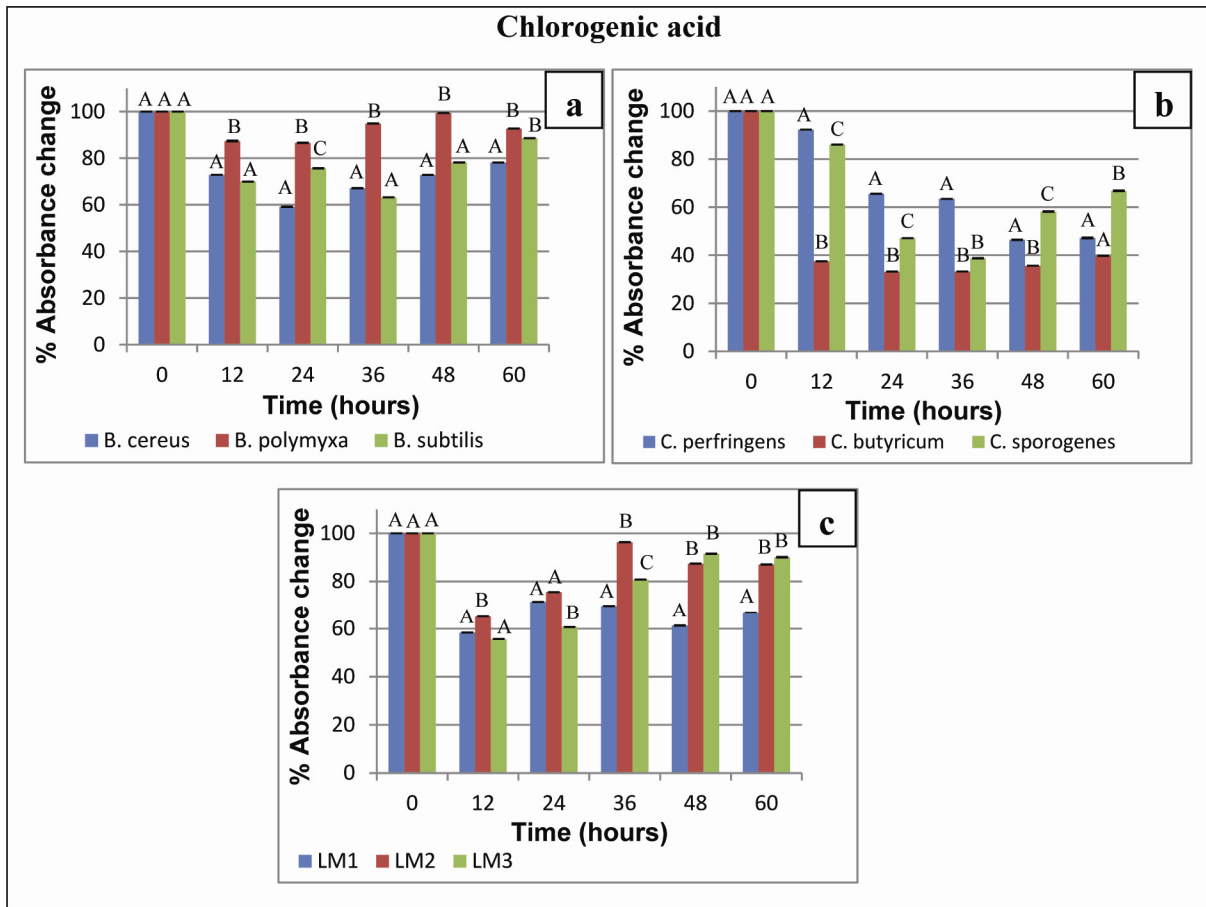


Figure 6. Antimicrobial activity of chlorogenic acid against a) *Bacillus* spp. (20 ppm); b) *Clostridium* spp. (20 ppm); and c) *Listeria* spp. (*L. monocytogenes*; LM) (10 ppm) are illustrated as relative absorbance difference from the control calculated as: $100 \times \text{treatment/control}$. The data represents the mean values of 3 independent experiments. Error bars denote standard deviation. Different letters denote significant differences between the strains within the same incubation time-points, $P < 0.05$

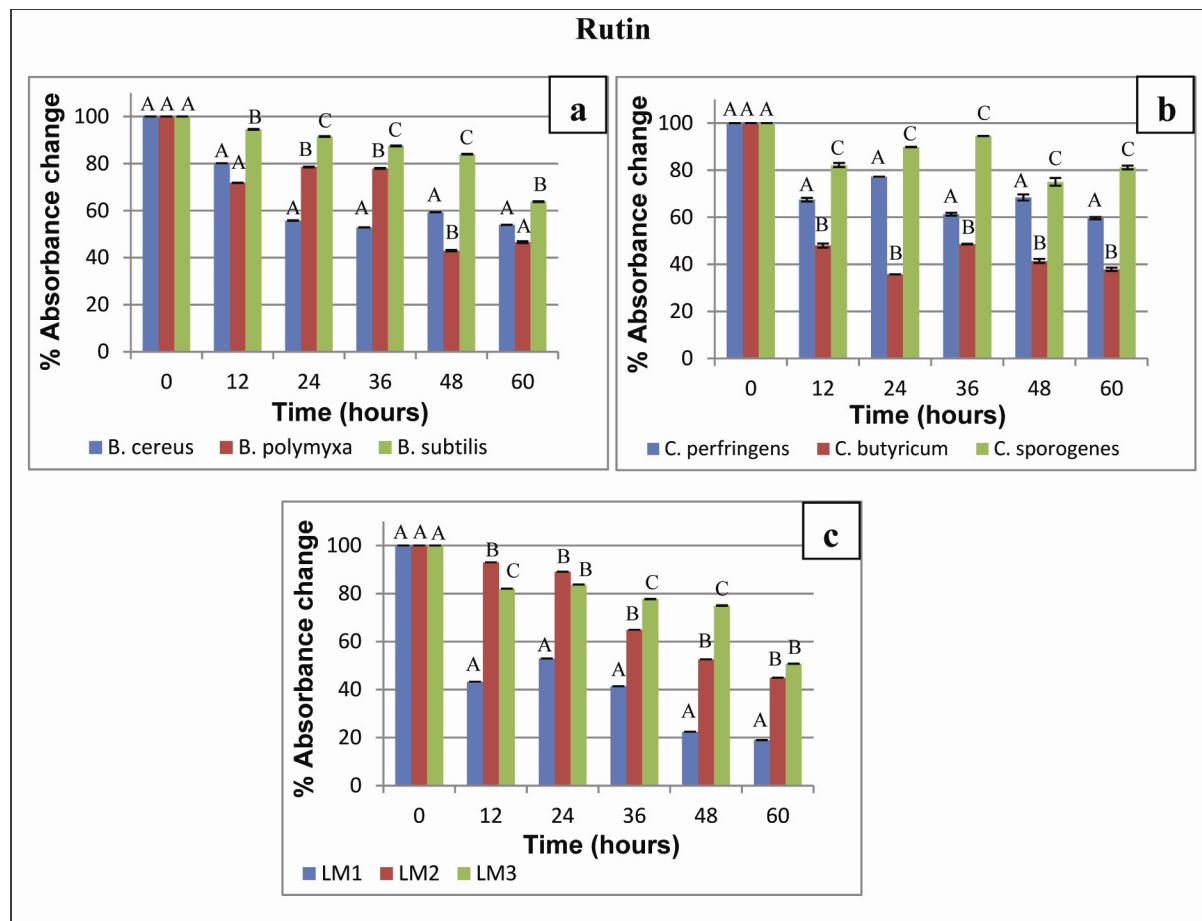


Figure 7. Antimicrobial activity of rutin against a) *Bacillus* spp. (15 ppm); b) *Clostridium* spp. (20 ppm); and c) *Listeria* spp. (*L. monocytogenes*; LM) (15 ppm) are illustrated as relative absorbance difference from the control calculated as: $100 \times \text{treatment}/\text{control}$. The data represents the mean values of 3 independent experiments. Error bars denote standard deviation. Different letters denote significant differences between the strains within the same incubation time-points, $P < 0.05$

3.4 Structural Observations via Scanning Electron Microscope

Treated samples of bacteria were observed by SEM to confirm the antimicrobial effects of the PDPCs along with the morphological changes in the appearance of the cells. Figure 8 illustrates the SEM images of two selected bacteria (*B. subtilis* and *L. monocytogenes*) treated with MICs of chlorogenic acid, thymoquinone and xanthohumol. It is clear that PDPCs caused severe damage to the bacteria via degradation of cell wall followed by the disruption of cytoplasmic membrane and membrane proteins which cause leakage of cell contents. Similar observations along with coagulation of cytoplasm and depletion of proton motive force were also reported by Cetin-Karaca & Newman (2015) and Burt (2004). *B. subtilis* cells were observed to decrease in size (Fig. 8A2, A3) when compared to controls (Fig. 8A1). Rupture of the cell wall and slimy appearance was clearly observed with *L. monocytogenes* (Fig. 8B2, B3). A lot of adhered material around the cells (Fig. 8A3, A4 and B2) and even some empty cells (Fig. 8B2) were observed. Gram-positive bacteria were reported to be more sensitive toward antibacterial substances than gram-negative bacteria (Burt, 2004; Cueva et al., 2010; Shan et al., 2007). This is likely due to hydrophilic surface of gram-negative bacteria on their outer membrane (Burt 2004; Shan et al., 2007) and a unique periplasmic space not found in gram-positive bacteria (Cueva et al., 2010). SEM observations confirmed the severe physical damage and considerable morphological alteration to all tested foodborne pathogens treated with the PDPCs. It was reported that as a part of the mechanism of action, PCs might bind to the cell surface and then penetrate to the target sites, possibly the phospholipid bilayer of the cytoplasmic membrane and membrane-bound enzymes (Cetin-Karaca & Newman, 2015; Gyawali & Ibrahim, 2014). Furthermore, the inhibition of proton motive force, respiratory chain, electron transfer and substrate oxidation could also be observed (Gyawali & Ibrahim, 2014; Shan et al 2007a). Also, the number and position of

substitutions in the benzene ring of the PCs and the saturated side-chain length influence the antimicrobial potential of the PCs against different microorganisms (Gill & Holley, 2006).

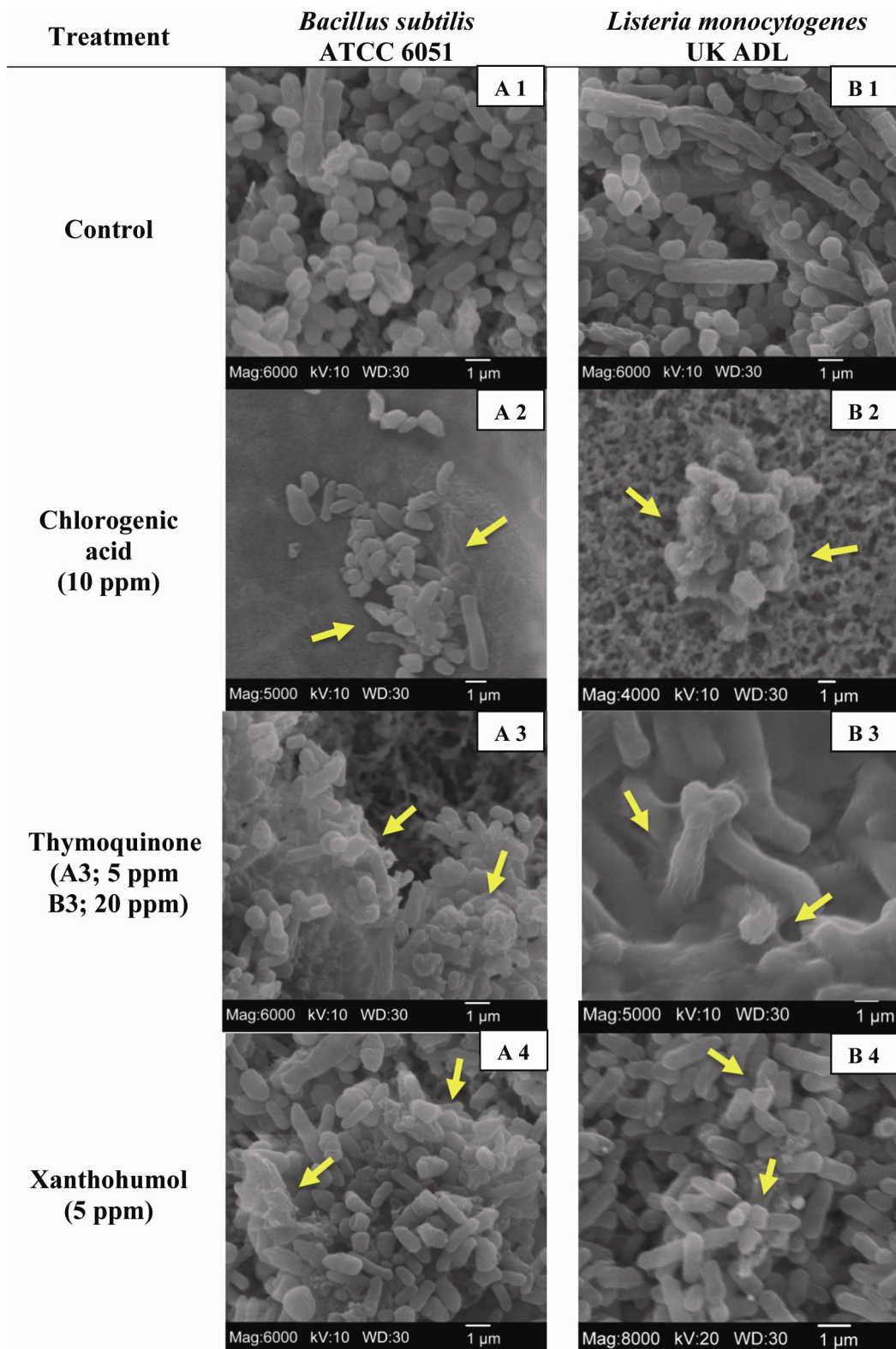


Figure 8. Scanning electron microscope observations of *B. subtilis* (A1-4) and *L. monocytogenes* (B1-4) treated with the natural PDPCs (1, control; 2, chlorogenic acid; 3, thymoquinone; 4, xanthohumol)

4. Conclusion

This study evaluated the antimicrobial activity of PDPCs through 60 hours of incubation, longer than the previous studies established MICs for 24 hours incubation periods. Our findings provide evidence for the high potential success of natural PDPCs in food safety for extended storage time. However it was also found that some of the sensitive pathogens including *Bacillus* and *Clostridium* spp. recover and become resistant with extended incubation periods, while LM1 was resistant from the beginning of the incubation. Thus, long term effects of these bacteria should be investigated carefully, since adaptation of pathogens may lead to resistance to PCs which may be initiated by gene manipulation and interfere with extended storage of foods. It can be concluded that the selected PDPCs exhibit *in vitro* antimicrobial activity against *L. monocytogenes*, *B. subtilis*, *B. cereus*, *B. polymyxa*, *C. perfringens*, *C. sporogenes* and *C. butyricum* even when utilized at low concentrations (5-20 ppm). Moreover, it should be noted that despite being from the same family each individual strain has its own growth and sensitivity characteristics against the PDPCs. In fact, PCs derived from plant extracts have the potential inhibitory activity against pathogenic bacteria and they can be superior alternatives for replacing chemicals used in food preservation. However, to be widely applied in food systems as antimicrobials, more systematic investigations should be done on their organoleptic impact, issues of safety and toxicity.

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