Variation of microbial load and biochemical activity of ready-to-eat salads in Cyprus as affected by vegetable type, season, and producer

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ABSTRACT

Fresh vegetables are important components of an everyday balanced diet making ready to-eat-salads (RTE) a commodity widely consumed. However, in the past few years these products have been linked with outbreaks of salmonellosis and listeriosis; thus the continuous investigation of their safety is an essential requirement. A total of 216 samples of ready-to-eat salads from the Cypriot market were analysed to determine the microbiological quality and safety, along with physicochemical attributes of the salads and identify possible correlations between them. The samples were randomly collected from four retail outlets and correspond to five different salad producing companies. Furthermore, the effects of season, salad producer and type of salad and/or their interactions with the tested parameters were investigated. The results revealed that the higher microbial load among seasons was observed in samples collected during spring. *Escherichia coli* was found in 11.57% of samples and 2.62% of isolates were found to be able to produce extended spectrum β-lactamase (ESBL). All samples were found negative for *Salmonella enterica*, whereas *Listeria monocytogenes* was present in 3.70% of samples. Higher levels of spoilage bacteria (lactic acid bacteria and *Pseudomonas* spp.) were detected during winter and spring. Additionally, the %CO_2 production was affected by the type of salad, while the interaction between producer and type of salad, affected total phenolic content and antioxidant activity of samples. A positive correlation of phenols and antioxidants with the presence of *Staphylococcus* spp., *Pseudomonas* spp., *E. coli* and *Bacillus cereus* was observed, suggesting that excessive handling increases microbial load and plant stress.

1. Introduction

Dietary guidelines suggest the daily consumption of vegetables as an important source of vitamins, mineral, dietary fibre and phytonutrients. There is a general consensus that a diet rich in vegetables may reduce the risk of heart disease and protect against certain types of cancer (FAO, 2015). More and more people are changing their eating habits; increasing their daily intake of vegetables. Ready-to-eat salads are a healthy, low calorie and convenient option for a contemporary and busy lifestyle.

Ready-to-eat salads are characterised minimally processed foods as their processing includes washing, peeling, cutting, drying and packaging and not any heat treatment (de Oliveira et al., 2011). Minimally processed fruits and vegetables are more susceptible to microbial contamination and proliferation, due to procedures such as cutting and peeling that can damage their outer natural protection, release plant juices favouring microbial growth (Abadias et al., 2008). Additionally spoilage bacteria, including several species of *Pseudomonas* and mesophilic organisms (e.g., lactic acid bacteria and *Enterobacteriaceae*) may be present in fresh produce and their activity (i.e., production of enzymes with lytic activity) may lead to great losses of quality, due to texture break down, or release of off-odours (Remenant et al., 2015). Vegetables have also been considered as vehicles of foodborne pathogens including enterohaemorrhagic strains of *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp. and *Campylobacter* spp. (Park et al., 2012). These pathogens may contaminate vegetables throughout the food chain either pre- (water, soil, manure, insects, handling) and/or post-harvest (water, peeling, cutting, packaging, handling) (EFSA, 2011).

Consumption of fresh produce and minimally processed fruits and vegetables has been linked to an increasing number of outbreaks of foodborne illnesses (Jeddi et al., 2014). It is noteworthy that the

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presence of extended spectrum β-lactamase (ESBL)- producing bacteria, especially from *Enterobacteriaceae* family (*E. coli* and *Klebsiella pneumonia*) have been reported in leafy vegetables (Blaak et al., 2014; Kim et al., 2015). It is thus important to monitor the microbiological quality of fresh-cut packaged salads.

Health Protection Agency (HPA; United Kingdom) has given some guidelines regarding the assessment of the microbiological safety of ready-to-eat foods. According to these guidelines total aerobic counts of ready-to-eat salads usually range between 6 and 8 log cfu/g; *Enterobacteriaceae* counts are separated in three categories (> 4 log cfu/g: unsatisfactory, 2–4 log cfu/g: borderline and < 2 log cfu/g: satisfactory) and similarly *E. coli* presence is categorised (> 2 log cfu/g: unsatisfactory, 1.3–2 log cfu/g: borderline and < 1.3 log cfu/g: satisfactory) (HPA, 2009).

Previous microbiological surveys of ready-to eat salads have investigated the presence of total aerobic counts, coliforms, *E. coli*, *Salmonella* spp., *Listeria* spp., yeasts and moulds (Tournas, 2005; Caponigro et al., 2010; Santos et al., 2012; Gurler et al., 2015; Losio et al., 2015). The occurrence of main foodborne pathogens such as *E. coli* O157:H7, *Listeria* spp. and *Salmonella* spp. has only been occasionally reported (Jeddi et al., 2014). Despite the extensive focus on hygiene and safety of fresh cut salads, to our knowledge, there are a few studies that have related the variability in the microbial load of ready-to-eat salads with their visual quality and %CO_2 production, which directly affect the acceptability of the products by the consumers (Caponigro et al., 2010; Santos et al., 2012; Nousiainen et al., 2016). For instance, Caponigro et al. (2010) studied the microbial populations and visual quality of ready-to-eat salads in Italy. Another study conducted in Portugal by Santos et al. (2012) assessed the microbial quality and sensory attributes (differences in taste) of ready-to-eat salads; while Nousiainen et al. (2016) studied the bacterial quality and safety as well as the O_2/CO_2 composition of ready-to-eat salads in Finland. Moreover, none of them have studied the possible correlations between the microbial load, quality attributes and plant tissue response to stress (both biotic and abiotic), including the non-proper handling and aversive storage conditions of ready-to-eat salads.

The aims of this study were: (i) to assess the variability in the microbiological quality of ready-to-eat salads in Cyprus, capturing the combined effect of collecting season, salad producer and type of salad and (ii) to assess the correlation between microbial load, antioxidant activity and respiration (in terms of %CO_2 production) of the samples tested. Furthermore, the possible relation between the antioxidant activity of salads and their microbial load will be investigated.

2. Materials and methods

2.1. Sampling

In this study, a total of 216 samples (ready-to-eat salads) were randomly collected from different supermarkets in four cities of Cyprus (Larnaca, Limassol, Nicosia, Paphos) during one year period, in four sampling periods, namely autumn (October–November 2016), winter (January-February 2017), spring (April-May 2017) and summer (July-August 2017). For each period, samples were collected once a week, transported to the laboratory within 2 h, in a cool box, and stored at 7 °C for further analysis. Samples were examined for their production of CO_2 due to the respiration process, total phenolic content and antioxidant activity as well as for the presence of *Listeria* spp. and *Salmonella* spp. and the levels of the following microbial groups (as described in Section 2.2): total viable count, *Enterobacteriaceae*, coliforms, *E. coli*, *Staphylococcus* spp., *B. cereus*, lactic acid bacteria, *Pseudomonas* spp., yeasts and moulds.

In each sample, appropriate amount of fresh tissue for microbiological analysis (*Salmonella* spp., *Listeria* spp. and others) and extraction of polyphenols and antioxidants was collected and stored at −20 °C. The majority of salad producers used modified atmosphere packaging (MAP) with single-layer orientated polypropylene (OPP) or double-layer polyethelene (PE) material. Fresh produce sanitation was taken place during the washing steps with chlorine-based products (~2–3 ppm of free chlorine on the washing water), while treatment with sanitation prior to processing was not a common application.

2.2. Microbiological analyses

For total viable count (TVC), *Enterobacteriaceae*, coliforms, *E. coli*, *Staphylococcus* spp., *B. cereus*, LAB, *Pseudomonas* spp. and yeast and moulds sampling 1 g of salad (the sampling weight was based on preliminary tests and previous reports of Xylia et al., 2017) was homogenized in stomacher for 1 min with 9 mL of Maximum Recovery Diluent (MRD)(Merck, Darmstadt, Germany). Serial dilutions were prepared using MRD and a volume of 0.1 mL was plated onto appropriate culture media to determine TVC in Plate Count agar (PCA) (Merck, Darmstadt, Germany) at 30 °C for 48 h; *Enterobacteriaceae* in Violet Red Bile Dextrose agar (VRBDA) (Merck, Darmstadt, Germany) at 37 °C for 24 h; coliforms and *E. coli* in selective and differential chromogenic Coliforms agar (Biolab, Hungary) at 37 °C for 24 h, for the simultaneous detection of coliforms (pink colonies) and *E. coli* (blue colonies); staphylococci in Baird-Parker agar (BPA) (Merck, Darmstadt, Germany) supplemented with egg yolk tellurite emulsion (Merck, Darmstadt, Germany) at 37 °C for 24 h; *B. cereus* in Cereus Selective agar acc. to MOSSEL (MYP agar) (Merck, Darmstadt, Germany) supplemented with egg yolk (Merck, Darmstadt, Germany) and selective supplement (Merck, Darmstadt, Germany) at 30 °C for 48 h; *Pseudomonas* spp. in cetrimide-nalidixicacid (CN) agar for Pseudomonas (Biokar diagnostics, France) at 37 °C for 48 h; lactic acid bacteria (LAB) in De Man, Rogosa and Sharpe agar (MRS agar) (Liofilchems.r.l, Italy) and yeast and moulds on Rose Bengal Chloramphenicol (CAF) agar (Liofilchems.r.l, Italy) at 25 °C for 5 days.

Typical *E. coli* (blue) colonies from Coliforms agar (Biolab, Hungary) were subcultured on Tryptone Bile Glucuronic agar (TBX agar) (Himedia, India) and incubated at 37 °C for 24 h. Blue colonies from TBX were streaked on Chromatic Extended Spectrum β-lactamase agar (ESBL agar) (Liofilchems.r.l, Italy) for the detection of antibiotic resistance. After incubation at 37 °C for 24 h, pink or purple colonies on ESBL agar were identified as ESBL producing *E. coli*.

Assessment of the microbiological safety of ready-to-eat salads were made according to HPA guidelines (HPA, 2009) while total aerobic counts were further categorised in three levels as i) satisfactory (< 6 log cfu/g), ii) borderline (6–8 log cfu/g; based on HPA 2009) and iii) unsatisfactory (> 8 log cfu/g).

2.2.1. Isolation and identification of Salmonella spp. and Listeria spp.

Standard cultivation method was carried out for the isolation of *Salmonella* spp. as recommended by ISO 6579 (ISO 6579, 2002) with modifications. Briefly, 5 g of each sample, representative portion from different parts of the salad, were homogenized with 45 mL Buffered peptone water (BPW) (Merck, Darmstadt, Germany) and incubated at 37 °C for 24 h. Following incubation, a volume of 0.1 mL was transferred into 10 mL of Rappaport Vassiliadis broth (RVS) (Merck, Darmstadt, Germany) and incubated at 41.5 °C for 24 h. Then, a loopful of RVS was streaked on Xylose Lysine Deoxycholate agar (XLD agar) (Scharlau, Spain) and incubated at 37 °C for 24 h. Red colonies with black centre were isolated, subcultured on Brain Heart Infusion Agar (BHI agar) (Biolab, Hungary) and incubated at 37 °C for 24 h.

For the isolation of *Listeria* spp., was conducted using the ISO (11290-1, 2004) procedure, with modifications. Briefly, an independent 5 g of each sample (as above) were homogenized with 45 mL of half Fraser broth (Merck, Darmstadt, Germany) and incubated at 30 °C for 24 h. Then, 0.1 mL from half Fraser broth was transferred into 10 mL of full Fraser broth (Merck, Darmstadt, Germany) and incubated at 37 °C for 48 h. After that, a loopful of full Fraser broth was streaked on Augusti Ottaviani Listeria Selective Agar (ALOA agar) (Merck, Darmstadt,

Germany) and incubated at 37 °C for 24 h. Blue-green colonies surrounded by an opaque halo (typical Listeria-like colonies) were isolated, subcultured on BHI agar (Biolab, Hungary) and incubated at 37 °C for 24 h.

2.2.2. Molecular confirmation of Salmonella enterica and Listeria monocytogenes

DNA extraction from isolated colonies was performed with heat lysis, where one presumptive colony was diluted in 100 μL of sterile distilled water, heated at 100 °C for 20 min, centrifuged at 13000 g and supernatant was stored at −20 °C until use.

Salmonella spp. isolates were identified with real-time polymerase chain reaction (real-time PCR) using iCycler (Bio-Rad, USA) and a set of forward primer *inv*139 (5′-GTGAAATTATCGCCACGTTCGGGCAA-3′) and reverse primer *inv*141 (5′-TCATCGCACCGTCAAAGGAACC-3′) for the amplification of a 284 bp fragment of the *inv*A gene (Malorny et al., 2003). *Listeria* spp. isolates were identified using real-time PCR for the amplification of a 274 bp fragment of the *prf*A gene with forward primer *prf*A LIP1 (5′-GATACAGAAACATCGGTTGGC-3′) and reverse primer *prf*A LIP2 (5′-GTGTAATCTTGATGCCATCAGG-3′) (Rossmanith et al., 2006). In both protocols, the template for real-time PCR assays was genomic DNA from heat lysed cells (2 μL) and KAPA CYBR FAST qPCR Master mix (KAPA Biosystems, USA).

2.3. Respiration production

For the determination of the metabolic respiration process of salads, the package atmosphere of each salad was sucked out (prior opening each bag) by a dual gas analyser (International Control Analyser Ltd) for 60 s. Results were expressed as percentage of CO_2 production (the outcome of the initially flushed CO_2 and that produced by respiration).

2.4. Polyphenol content and antioxidant activity of ready-to-eat salads

Pooled plant tissue (1 g) from each sample was homogenized with 50% (v/v) methanol for 60 s and extraction was assisted with ultrasound water bath (35 kHz frequency and 325 W power output) for 30 min. After that, the samples were centrifuged at 4600 g at 4 °C for 15 min and the supernatants were stored at −20 °C until used for the determination of total phenols and the antioxidant activity.

Total phenols was determined with the Folin-Ciocalteu method at 755 nm as previously described by Tzortzakis et al. (2007) and results were expressed as equivalents of gallic acid (Scharlau, Spain) per g of fresh weight (mg of GAE/g Fwt) through a calibration curve with gallic acid.

The antioxidant activity of each sample was evaluated with three different methods as 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, ferric reducing antioxidant power (FRAP) assay and 2.2′-azinobis-(ethylbenzothiazoline-6-sulfonic acid) radical cation discoloration (ABTS) assay. The ability of the samples to scavenge the DPPH (Sigma-Aldrich, Germany) radical was evaluated as previously described by Chrysargyris et al. (2016). Absorbance at 517 nm was measured and results were expressed as mg of trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid] per g of fresh weight (mg of trolox/g Fwt).The capacity of the extracts to reduce Fe^{3+} was evaluated by measuring the absorbance of the [Fe (TPTZ)$_2$]$^{2+}$ complex at 593 nm, as previously described by Chrysargyris et al. (2016). Results were expressed ass mg of trolox per g of fresh weight (mg of trolox/g Fwt). The capacity of the extracts to scavenge the ABTS (Sigma-Aldrich, Germany) radical at 734 nm was determined according to Wojdylo et al. (2007). Results were expressed as mg of trolox per g of fresh tissue (mg of trolox/g Fwt).

2.5. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version

22 where the effect of season, salad producer and type of salad as well as their interactions on the phenolic content, antioxidant activity and %CO_2 production of samples was assessed with three way ANOVA. Data means were also compared with one way analysis of variance (ANOVA) and Tukey's multiple range tests were calculated for the significant data $P < 0.05$. All measurements were done in duplicates. The confidence intervals (CI) for the proportions were also calculated using the adjusted Wald confidence interval (Agresti and Coull, 1998). The association between total viable counts and the presence of other microorganisms tested was assessed by Pearson's correlation coefficient (Cohen, 1988). Differences between positive and negative samples were analysed by the independent-samples *t*-test and the magnitude of size effect was evaluated the based on Eta squared (η^2) (Cohen, 1988).

3. Results

3.1. Microbiological analysis

3.1.1. Variability in microbial load associated with sampling period

Samples collected during spring were found to have higher microbial load, in most of the microorganisms, tested compared to samples from other seasons. Total viable counts ranged between 5.12 and 9.75 log cfu/g with an average of 7.73 log cfu/g (Fig. 1). The majority of the samples 113 out of 216 (52.31%) had a viable count between 6 and 8 log cfu/g, which categorizes them as borderline, according to HPA guidelines (see Fig. 1). Spring showed the highest value (8.18 log cfu/g). During autumn, 7 out of 72 samples (9.72%) were characterised as satisfactory (< 6 log cfu/g), whereas in summer 53 out of 72 samples (73.61%) were characterised as unsatisfactory (> 8 log cfu/g). Spring also showed the highest value for *Enterobacteriaceae* (7.05 log cfu/g). Only a small fraction of the samples falls into the satisfactory category (< 2 log cfu/g) and these were observed only during autumn and winter (1.38 and 2.77%, respectively).

The majority of samples, 191 out of 216 (88.43%) were negative for *E. coli*, whereas counts for positive samples (11.57%; 95% CI: 7.91–16.6%) ranged between 1.48 and 4.88 log cfu/g. A total of 15 samples (6.94%), were characterised as borderline (cfu/g range: 1.3–2 log), whereas 10 (4.63%) were unsatisfactory, with cfu/g numbers exceeding 2 log. When comparing seasons, the highest average value (3.17 log cfu/g) was recorded during autumn. All positive *E coli* samples were tested for antibiotic resistance. ESBL testing on the positive *E. coli* isolates revealed that five samples (2.62%) were positive with three of them were isolated during summer and two over spring.

The lowest value for *Staphylococcus* spp. was recorded in autumn (3.01 log cfu/g) and the highest in spring and summer (4.21 and 4.02 log cfu/g, respectively). Twenty samples (9.26%) were found positive for *B. cereus* and with values ranging from 1.48 to 4.28 log cfu/g. Positive samples were found in all seasons [9 (12.5%), 1 (2.78%), 6 (8.33%) and 4 (11.11%) in autumn, winter, spring and summer, respectively]. Additionally, lactic acid bacteria were present in 198 samples (91.67%) and values ranged between 1.48 and 7.39 log cfu/g. The lowest average value was observed during summer (4.52 log cfu/g), whereas the highest values during winter and spring (5.61 and 5.66 log cfu/g, respectively). The presence of *Pseudomonas* spp. was observed in 165 samples (76.39%) and values ranged between 1.48 and 9.04, log cfu/g. The lowest values were observed during autumn and summer (5.15 and 5.76 log cfu/g, respectively), whereas the highest values during the winter and spring (6.85and 7.34 log cfu/g). Yeasts and moulds were present in 197 samples (91.20%) and values ranged between 1.63 and 6.68 log cfu/g. The lowest values were observed during summer and spring (4.66 and 4.87 log cfu/g, respectively), whereas the highest value during winter (5.30 log cfu/g).

The majority of samples, 212 out of 216 (98.15%) were found negative for *Salmonella* spp. via the enrichment and culture-based steps of the detection, whereas four samples (collected during autumn) which were showed presumptive positive colonies after enrichment were
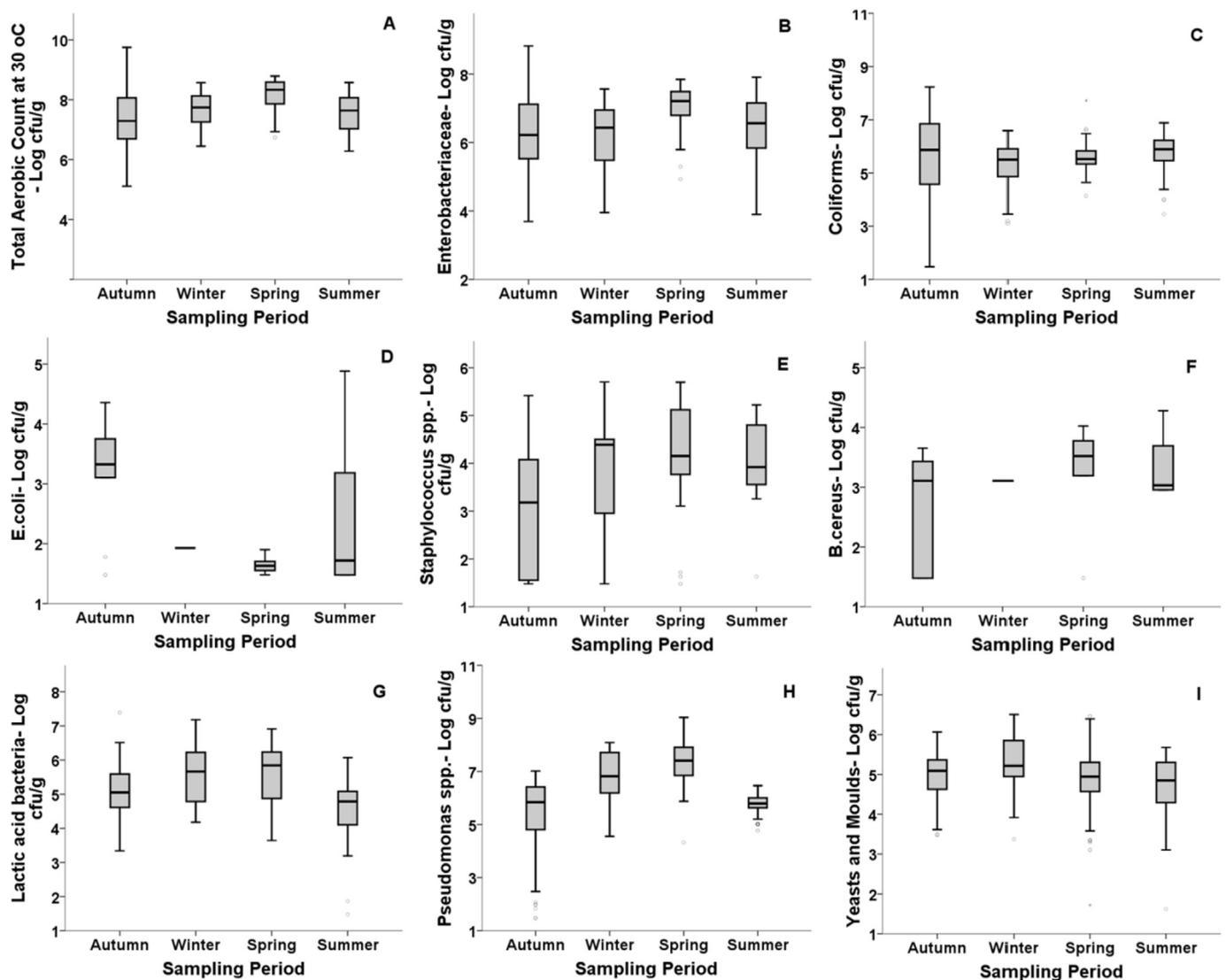
Fig. 1. Microbiological quality of ready to eat salads collected during one year period from supermarkets in Cyprus as affected by season. Results include only positive samples for each microorganism tested and are the mean value ± standard deviation. Each box contains 50 per cent of cases and whiskers represent the rest. The line across the inside of the box represents the median value.

negative for *S. enterica,* according to the PCR-tested samples for the presence of *inv*A gene. The presence of *L. monocytogenes* was detected in eight samples (3.70%; 95% CI: 1.8–7.3%) that were collected during spring.

3.1.2. Variability in microbial load associated with salad producer

The highest average value for total viable count was observed in samples from producer B (8.32 log cfu/g), whereas samples from producer E showed the lowest TVC levels (7.10 log cfu/g). *Enterobacteriaceae* family counts ranged between 1.48 and 8.82 log cfu/g, whereas only one sample (combination of lettuce with two or more ingredients) was found negative and came from salad producer A on the second sampling period. Totally 208 out of 216 (96.30%) samples were characterised as unsatisfactory (> 4 log cfu/g), according to HPA guidelines (HPA, 2009). The lowest average value was observed by salad producer E (5.46 log cfu/g), whereas salad producer B showed the highest (7.27 log cfu/g) levels in their samples (Fig. 2). The majority of samples tested (207 out of 216) (95.8%) were also positive for coliforms. During spring, the lowest value for coliforms was observed in samples by salad producer E (4.63 log cfu/g), whereas samples of salad producer B showed the highest (6.47 log cfu/g) value. Five out of the nine negative samples (55.56%) that did not harbour coliforms came

from salad producer A, whereas the rest came from producer D (4 samples). Additionally, the nine negative samples were found in autumn (7 samples) and in winter (2 samples) sampling period.

Salad producer E was the only one with negative for *E. coli* samples throughout the four sampling periods. *Staphylococcus* spp. was present in almost half of the samples (105 out of 216) (48.11%; 95% CI: 42.03–55.24%) and *S. aureus* was isolated from only two samples (0.93%; 95% CI: 0.04–3.53%) (1.48 log cfu/g each) that came from salad producer A (plain rocket) and C (combination of lettuce and chives). Only salad producer E did not had any positive samples for *B. cereus,* whereas samples from salad producer A, B, C, D were found positive (7 (7.61%), 1 (4.17%), 10 (17.86%) and 2 (7.69%), respectively). Furthermore, among salad producers, producer E had the lowest average value for lactic acid bacteria counts and producer B the highest (4.67 and 5.63 log cfu/g, respectively). Similarly, salad producer E and D had the lowest values for *Pseudomonas* spp. (5.63 and 6.09 log cfu/g, respectively), whereas producer B the highest (7.36 log cfu/g). No significant difference was observed among yeasts and moulds counts of the tested samples from the five salad producers (*P* = 0.831). Samples found positive for *L. monocytogenes* derived from salad producer C (2.78%), A and D (0.46%, respectively).
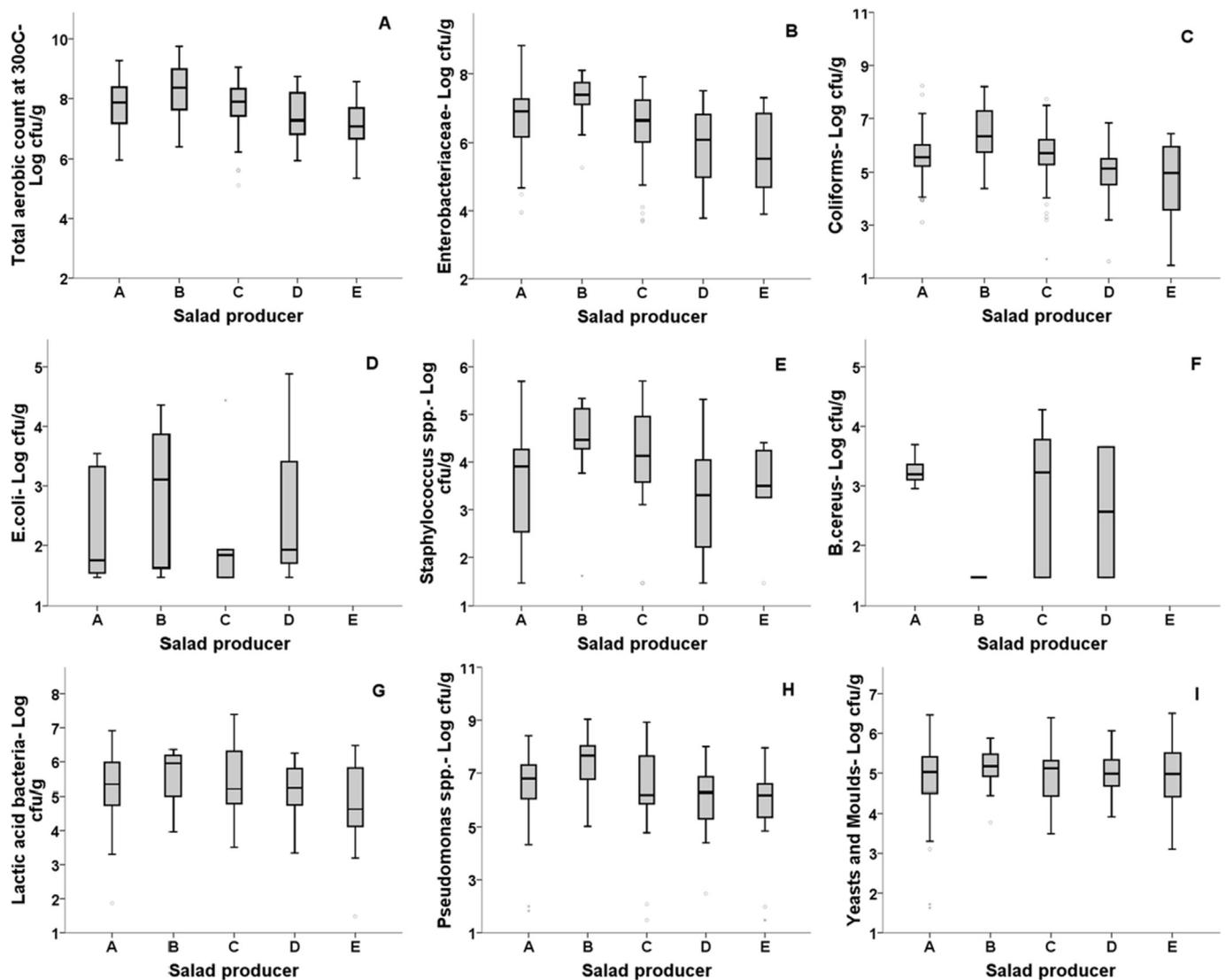
Fig. 2. Effect of salad producer on the microbiological quality of ready to eat salads collected during one year period from supermarkets in Cyprus. Results include only positive samples for each microorganism tested and are the mean value $\pm$ standard deviation. Each box contains 50 per cent of cases and whiskers represent the rest. The line across the inside of the box represents the median value.

3.1.3. Variability in microbial load associated with the type of salad

Salads with combination of lettuce and two or more ingredients were found to have the highest average counts of *E. coli* (3.37 log cfu/g) (Fig. 3). Additionally, the combination of lettuce and cabbage had the highest value for *Staphylococcus* spp. (5.22 log cfu/g) among the different types of salad. The combination of lettuce with rocket had the lowest average value for *Pseudomonas* spp. (5.35 log cfu/g), contrary to the combination of lettuce cabbage or endive/radicchio which showed the highest levels (i.e., 6.70 and 6.71 log cfu/g, respectively). No significant differences between types of salads was observed for total viable counts ($P = 0.277$), *Enterobacteriaceae* ($P = 0.235$), coliforms ($P = 0.189$), lactic acid bacteria ($P = 0.405$) and yeasts and moulds counts ($P = 0.218$). The majority of samples positive for *L. monocytogenes* came from salads containing plain lettuce and the combination of lettuce with cabbage (1.38% and 0.92%, respectively).

3.2. Total phenolic content, antioxidants and CO_2

3.2.1. Sampling period

Total phenolic content ranged between 0.73 and 1.40 mg GAE/g Fwt (Table 1). Among sampling periods, the highest value was observed during autumn (1.40 mg GAE/g Fwt), whereas spring and summer

showed the lowest values (1.04 and 1.05 mg GAE/g Fwt, respectively). The antioxidant levels of the samples were tested with three different assays (DPPH, FRAP and ABTS). All three assays showed that the lowest value was observed during summer ($P < 0.05$). The production of CO_2 ranged between 0.25 and 20.49%. Fresh cut salad respiration was increased (8.22% CO_2) during spring whereas autumn and winter showed the lowest values (4.79 and 5.10% of CO_2, respectively).

3.2.2. Salad producer

The content of phenolics was varied among producers as the highest values were observed by salad producer E and A (1.33 and 1.34 mg GAE/g Fwt, respectively) and the lowest by salad producer C (0.85 mg GAE/g Fwt) (Table 1). All three antioxidant assays showed that the lowest antioxidant levels were observed by salad producer C ($P < 0.05$). Among salad producers, producer E had the lowest %CO_2 value (3.46%) and producer B the highest (9.83%). No differences were found on CO_2 production among producers A, E, and D.

3.2.3. Type of salad

Salads with rocket, lettuce and the combination of lettuce with two or more ingredients were found to have the highest phenolic content (1.40 and 1.37 mg GAE/g Fwt, respectively), whereas the combination
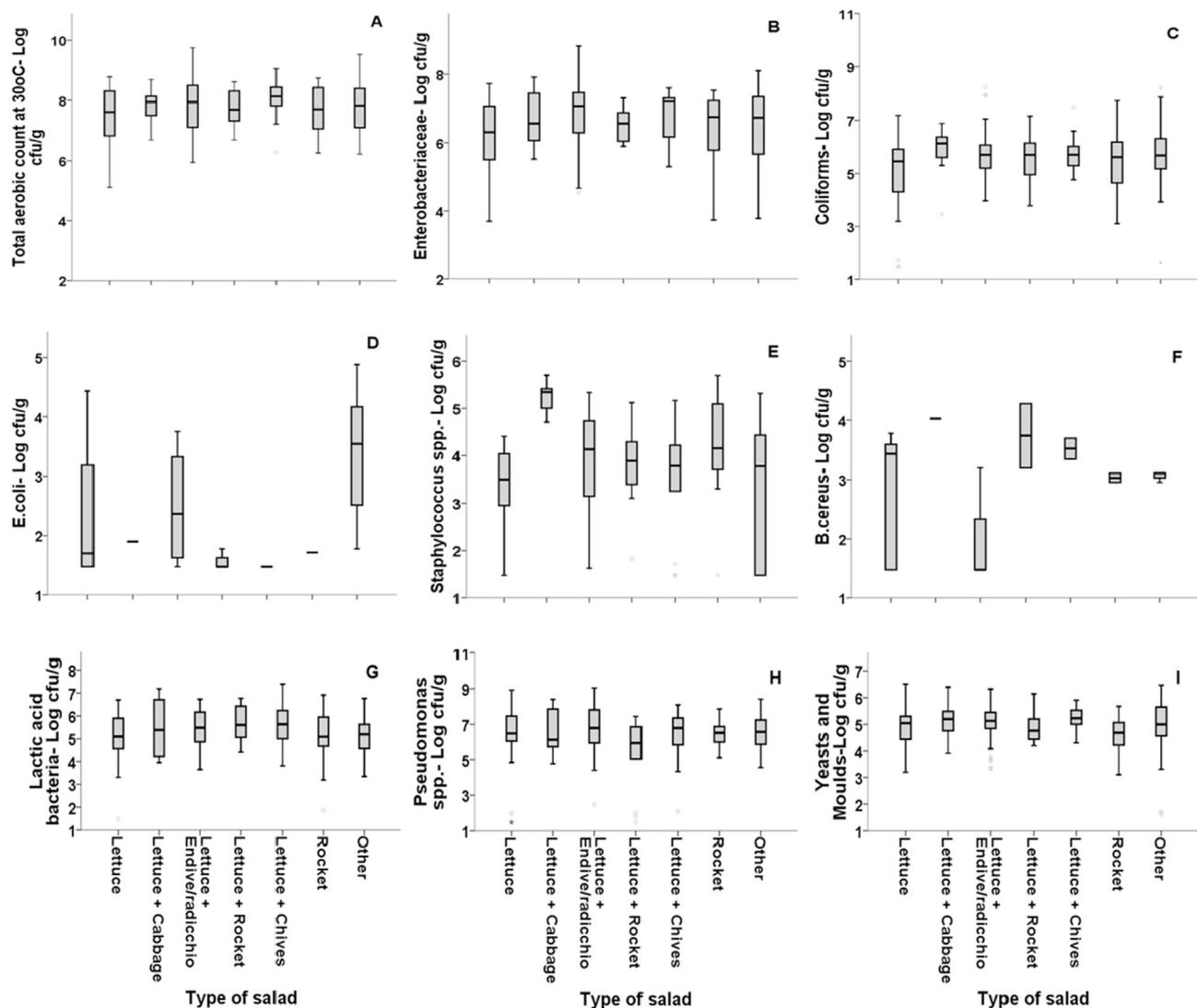
Fig. 3. Microbiological quality of different types of ready to eat salads collected during one year period from supermarkets in Cyprus. Results include only positive samples for each microorganism tested and are the mean value ± standard deviation. Other = Lettuce +2 or more ingredients. Each box contains 50 per cent of cases and whiskers represent the rest. The line across the inside of the box represents the median value.

of lettuce and cabbage revealed the lowest (0.73 mg GAE/g Fwt) (Table 1). Low antioxidant capacity was found (by FRAP and ABTS assays) in salads with lettuce and cabbage combination, whereas the combination of lettuce with two or more ingredients had the highest ($P < 0.05$) antioxidants. Salads with only rocket or lettuce showed the lowest %CO_2 values (4.10 and 4.73%, respectively), whereas the combination of lettuce with cabbage had the highest value (13.73%).

Three way ANOVA analysis shown in Table 1 revealed that sampling period affected the antioxidant activity (DPPH and ABTS) of salads ($P < 0.001$) and FRAP ($P < 0.01$), whereas phenols and %CO_2 production were not significantly affected ($P \geq 0.05$). Salad producer significantly affected FRAP, ABTS ($P < 0.001$), DPPH and phenols ($P < 0.01$); however %CO_2 was not significantly affected ($P \geq 0.05$). The %CO_2 production was significantly ($P < 0.001$) affected by the type of salad. Phenols, DPPH ($P < 0.01$), FRAP and ABTS ($P < 0.05$) were also affected. Total phenolic content and antioxidant activity (DPPH, FRAP and ABTS) were significantly ($P < 0.001$) impacted by the interaction of producer*salad. The interactions of period*producer and period*salad did not significantly (P ≥ 0.05) affect total phenols, antioxidant activity (DPPH, FRAP and ABTS) or %CO_2 production of salads. Finally, the interaction of period*producer*salad only influenced ABTS ($P < 0.05$).

4. Discussion

Safety and quality of fresh produce are of great concern as both are demanded by the food industry and the consumers. To our knowledge there is limited information regarding the microbiological safety of ready-to-eat salads with leafy vegetables in Cyprus. In a previous study by Eleftheriadou et al. (2002) it was observed that 1.70% and 27.21% of mixed salads and vegetables in Cyprus were found positive for the presence of *B. cereus* (> 4 log cfu/g), *E. coli* (> 2 log cfu/g) and *L. monocytogenes*. The survival of *L. monocytogenes* and *Salmonella enterica* in fresh-cut salads even in low numbers has been previously mentioned by Manios et al. (2013).

Our findings regarding TVC are in accordance with several studies that have investigated the aerobic microbial counts of leafy vegetables, which ranged between 3 and 8 log cfu/g (Ailes et al., 2008; Korir et al., 2016; Nousiainen et al., 2016). However, high TVC counts (> 6 log cfu/g) do not necessarily indicate low microbiological safety of food, as it may be due to harmless (e.g., spoilage) epiphytic (predominant) microorganisms (Faour-Klingbeil et al., 2016a). Furthermore, high Enterobacteriaceae and coliforms levels in ready-to-eat salads have been linked with the preharvest application of untreated manure and immoderate postharvest handling (Faour-Klingbeil et al., 2016b). For

Table 1
Effect of sampling period, salad producer and type on total phenolic content (mg GAE/g Fwt), antioxidants (mg trolox/g Fwt) and %CO_2 measured after samples transportation to the lab for the ready to eat salads collected during one year period from supermarkets in Cyprus.

	Phenols	DPPH	FRAP	ABTS	% CO_2
Sampling period					
Autumn	1.40 ± 0.74 a	1.56 ± 0.92 a	0.97 ± 0.74 a	1.31 ± 0.53 a	4.79 ± 1.98 b
Winter	1.14 ± 0.59 ab	1.56 ± 0.44 a	0.72 ± 0.59 ab	0.58 ± 0.22 b	5.10 ± 3.85 b
Spring	1.04 ± 0.52 b	1.45 ± 0.69 a	0.46 ± 0.37 bc	1.39 ± 0.38 a	8.22 ± 4.19 a
Summer	1.05 ± 0.65 b	0.42 ± 0.37 b	0.33 ± 0.39 c	0.54 ± 0.26 b	6.40 ± 4.39 ab
Salad producer					
A	1.34 ± 0.75 a	1.55 ± 0.83 a	0.80 ± 0.75 a	1.18 ± 0.56 ab	5.91 ± 3.91 bc
B	1.26 ± 0.54 ab	1.58 ± 0.97 a	0.74 ± 0.48 a	1.41 ± 0.60 a	9.83 ± 6.34 a
C	0.85 ± 0.27 b	0.88 ± 0.27 b	0.36 ± 0.27 b	0.84 ± 0.41 b	8.19 ± 4.33 ab
D	1.12 ± 0.64 ab	1.33 ± 0.74 ab	0.64 ± 0.53 ab	1.03 ± 0.50 b	4.91 ± 1.78 bc
E	1.33 ± 0.74 a	1.35 ± 0.86 ab	0.70 ± 0.57 ab	1.03 ± 0.53 b	3.46 ± 1.92 c
Type of salad					
Lettuce	1.40 ± 0.63 a	1.83 ± 0.75 a	0.80 ± 0.54 a	1.16 ± 0.60 ab	4.73 ± 3.21 c
Lettuce + Cabbage	0.73 ± 0.28 b	1.11 ± 0.35 a	0.28 ± 0.17 b	0.76 ± 0.43 b	13.73 ± 3.66 a
Lettuce + Endive/radicchio	0.89 ± 0.42 ab	1.22 ± 0.45 a	0.41 ± 0.38 ab	0.94 ± 0.44 ab	7.94 ± 3.62 b
Lettuce + Rocket	1.26 ± 1.14 ab	1.90 ± 1.63 a	0.83 ± 1.32 a	0.94 ± 0.59 ab	5.68 ± 2.73 bc
Lettuce + Chives	0.86 ± 0.25 ab	1.12 ± 0.25 a	0.41 ± 0.32 ab	0.99 ± 0.45 ab	7.06 ± 1.85 bc
Rocket	1.35 ± 0.39 a	1.16 ± 0.24 a	0.50 ± 0.25 ab	1.16 ± 0.42 ab	4.10 ± 3.78 c
Other	1.37 ± 0.74 a	1.73 ± 0.79 a	0.92 ± 0.65 a	1.29 ± 0.59 a	5.77 ± 3.35 bc
Three-way Anova	Phenols	DPPH	FRAP	ABTS	% CO_2
Period	ns	***	**	***	ns
Producer	**	**	***	***	ns
Salad	**	**	*	*	***
Period*Producer	ns	ns	ns	ns	ns
Period*Salad	ns	ns	ns	ns	ns
Producer*Salad	***	***	***	***	ns
Period*Producer*Salad	ns	ns	ns	*	ns

Results shown are the mean value ± standard deviation and values followed by the same letter in each column are not significantly different ($P \geq 0.05$). .ns, *, **, and *** indicate non-significant or significant differences at $P \leq 5\%$, 1% and 0.1%, respectively, following three-way ANOVA. Other = Lettuce +2 or more ingredients.

instance, Jeddi et al. (2014) have mentioned coliforms levels ranging between 1.9 and 6.0 log cfu/g with the majority of fresh-cut vegetables having > 5 log cfu/g. It is worth mentioning that increased numbers of coliforms and *E. coli* in leafy vegetables from field to the marked has been observed in another study by Faour-Klingbeil et al. (2016b). It has been previously mentioned the environmental contamination with the use of untreated manure, contaminated water or soil during preharvest, as well as the excessive handling during postharvest might result to high loads of coliforms in ready-to-eat vegetables (Aycicek et al., 2006).Therefore, this confirms the possibility of contamination with faecal matter throughout the food supply or microbial proliferation due to improper chilling or inappropriate packaging conditions (e.g., failure in flushing or maintenance of a modified atmosphere in the package). Nousiainen et al. (2016) reported a positive correlation (Pearson correlation; r = 0.661, P < 0.001) between TVC and coliforms counts and this is also evidence in our results with r = 0.583 and P < 0.001. Coliforms have been used as sanitary indicators in foods for their presence in high numbers (higher than most foodborne pathogens) and their relatively quick and easy identification (HPA, 2009). However, coliforms presence in ready-to-eat salads may vary along the different processing steps and the end product (Faour-Klingbeil et al., 2016a). This complicates the efforts for obtaining useful information about the contamination occurrence and the adequacy of processing fresh produce for ready-to-eat salads production.

It has been mentioned in previous studies that the prevalence of *E. coli* on leafy green vegetables can be below 5% (Sagoo et al., 2003; Ailes et al., 2008; Santos et al., 2012; Cardamone et al., 2015), whereas some others reported levels around 15% and higher (de Oliveira et al., 2011; Campos et al., 2013; Nousiainen et al., 2016). According to our findings *E. coli* was found in 11.57% of samples and these are in accordance with a study by Abadias et al. (2008) that mentioned the presence of *E. coli* on 11.4% of fresh-cut vegetables including rocket, carrot, corn salad, endive, lettuce, spinach and mixed salads. The highest *E. coli* levels in

our study were observed during autumn similarly to the results of another study in Italy where *E. coli* was detected in 27% of ready-to-eat salads and high levels were reported in autumn for both lettuce and arugula (Caponigro et al., 2010). In that study, Caponigro et al. (2010) reported that the short periods of concentrated rain often happen in autumn in Italy. This phenomenon is also observed in Cyprus as in other Mediterranean countries resulting to high moisture levels in field. Additionally, relatively high temperatures for the season are also common in Cyprus and thus the presence of *E. coli* on fresh produce might be favoured.

The assessment of *E. coli* isolates in our study showed that 2.62% of samples harboured ESBL resistant genes and this has also been reported by Chau et al. (2017) in a microbiological survey of ready-to-eat salads in Singapore, where the corresponding values were of 2.3%. Additionally, Campos et al. (2013) have isolated β-lactamase producing bacteria (other than *E. coli*) from ready-to-eat salads in Portugal including *Rahnella aquatilis*, *Citrobacter freundii*, *Raoultella terrigena*, *Hafnia alvei*, *Enterobacter cloacae*, *Enterobacter aerogenes* and *Cronobacter sakazakii*. It is worth mentioning that in a study in Switzerland, 5% of ready-to-eat salads were found to harbour β-lactamase producing *Enterobacteriaceae* and environmental bacteria (minor ESBLs) and this suggests the possible presence of these bacteria throughout the food supply chain (Nuesch-Inderbinen et al., 2015). These findings, suggest that food safety in Cyprus is within ranges found in developed countries as Portugal and Switzerland.

Survival and growth of *L. monocytogenes* and *Salmonella* spp. in RTE salads is influenced by storage and packaging conditions and one of the most important factors is storage temperature (Nousiainen et al., 2016). It has been shown that *L. monocytogenes* can survive and multiply at 7 and 15 °C on leafy vegetables (Sant'Ana et al., 2012a). Storage of fresh produce at abusive conditions i.e. temperatures above 4 °C have shown increased microbial growth and metabolic activities of plant tissue leading to pathogen growth and food spoilage (Caleb et al., 2013).

Furthermore Poimenidou et al. (2016) found that antimicrobial treatments might not be effective against *L. monocytogenes* cells once fresh produce (i.e cherry tomatoes and lettuce leaves) has been contaminated with cells incubated at low temperatures (5 °C) as the bacterium cells might be able to resist and survive the treatments applied after fresh produce contamination. In our study, 3.7% of samples stored at 7 °C were found to harbour *L. monocytogenes* and these results resemble those of other studies conducted in the UK and Brazil with prevalence of *L. monocytogenes* of 4.8% and 3.1%, respectively (Little et al., 2007; Sant'Ana et al., 2012b). On the other hand, other studies have reported lower (0.66%) or higher (6%) prevalence of *L. monocytogenes* in ready-to-eat salads (Santos et al., 2012; Ssemanda et al., 2017). However it should be noted that as it has been mentioned by Angelidis et al. (2015) not all *Listeria* spp. isolates might actually be *Listeria* spp. and thus further molecular identification is essential.

Caponigro et al. (2010) reported the presence of *Salmonella* spp. in postharvest washing areas (6.7%) suggesting possible contamination of fresh produce during processing, whereas similar (5%) and lower (0.3%) prevalence has also been mentioned by others (Jeddi et al., 2014; Losio et al., 2015). Our results showed the absence of *S. enterica* which is in accordance with HPA guidelines for ready-to-eat food (HPA, 2009). These findings imply that improved preventive measures have been taken by the food industry in Cyprus regarding this pathogen. This is further supported by a previous study of Eleftheriadou et al. (2002) who reported 0.68% prevalence of *Salmonella* spp. in mixed salads and vegetables. *L. monocytogenes* was found on some vegetable samples due to possible mishandling or contamination pre (irrigation water, soil, handling) and/or postharvest (handling, cutting, prior washing) and since it is able to survive at low temperatures (4 °C) during cold storage. As it has been previously mentioned, the disinfectants used in the food industry (mainly sodium hypochloride) are not able, in the currently used concentrations, to completely remove all pathogen and spoilage microorganisms from fresh produce (Losio et al., 2015). It is of great significance that the vegetable industry implement and control *Listeria* inside processing plants as RTE salads are widely consumed and vulnerable groups (children, pregnant women and elderly) might be exposed to great risk as mortality rate for these groups is quite high and ranges from 20 to 30% (Farber and Peterkin, 1991).

The correlation of microbial load of vegetables and the antioxidant activity of plant tissue is not commonly studied. However it should be considered and investigated further as it will possibly give insights on how the plant tissue reacts to the presence of foodborne pathogens as well as spoilage microorganisms and prevents or enhances bacterial growth. The correlation matrix of microbial and physiological responses is presented in Fig. 4. Phenols and DPPH are positive correlated with *Staphylococcus* spp.*, Pseudomonas* spp., *E. coli,* and *B. cereus*. This may suggest that excessive handling increases microbial load as well as plant stress. However, the above microbes are negatively correlated to the ABTS antioxidant activity. It should be mentioned that several *Pseudomonas* have been associated with spoilage. For example, the presence of *P. chlororaphis* on lettuce, onions, potatoes and carrots may lead to plant cell wall degradation and inevitably in spoilage of fresh produce (Lee et al., 2013). This might explain the higher antioxidant levels and phenolic content as a part of plant defence mechanisms against biotic stress. Furthermore, it can be assumed that the presence of *B. cereus* and *Pseudomonas* spp. (at high levels) may have contributed to the low prevalence of *L. monocytogenes* and *S. enterica* in our study as it has been previously reported by Santos et al. (2012) that those bacteria antagonize each other.

The relative expression in microbial and physiological responses in fresh cut salads was examined in relation to the season, the salad producer (supplier) and type of salads, as presented by heat map in Fig. 5. In spring, *Staphylococcus* spp., *Pseudomonas* spp., LAB, *Enterobactereaceae*, and TVC but also CO_2 and ABTS activity were relatively increased (Fig. 5A). In summer, *E. coli* and coliforms were relatively increased, while LAB, yeast and moulds as well as antioxidants
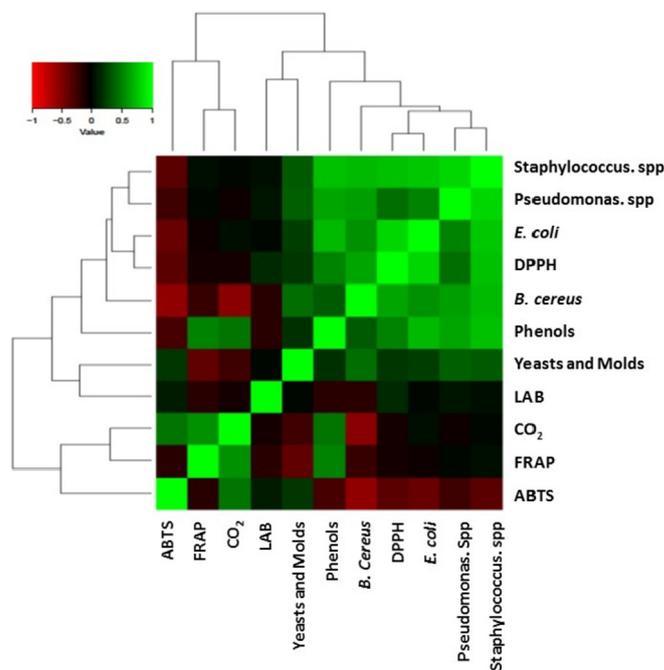

Fig. 4. Heat-map matrices of the correlation between microbial and physiological responses in fresh cut salads. Each square indicates r (Pearson's correlation coefficient of a pair of responses).

(DPPH, FRAP, ABTS) were decreased. Our findings are in accordance with Caponigro et al. (2010), who also reported LAB in ready-to-eat salads in Italy during spring and summer. Yeast and moulds were relatively increased during winter, whereas antioxidants and phenols were increased in autumn. A decrease of coliforms and *Pseudomonas* spp. and CO_2 was also observed during autumn. Our results are partly confirmed by the observations of Caponigro et al. (2010) where they reported higher average visual quality and lower microbial load in winter and spring compared to summer and autumn.

Differences among salad producers might be due to different processing procedures and possible excessive handling (Sant'Ana et al., 2012a). Salad producing factories in Cyprus consist of small and big companies (exports to more than 25 countries) and the current packaging practices include the use of modified packaging atmosphere (some of them), different packaging materials (single-layer OPP and double-layer PE), as well as the use of chlorine-based disinfectants. Samples from producer A were found to have increased antioxidant levels and phenolic content, while samples from producer B had relatively increased CO_2 and microbial load (*Enterobacteriaceae*, coliforms, *Staphylococcus* spp. and yeast and moulds) (Fig. 5B). Producer C samples had relatively increased microbial load (*B. cereus*, *Pseudomonas* spp. and TVC) and low antioxidant levels. *E. coli* was increased in producer's D samples, whereas samples from producer E had relatively low microbial load, meaning safer produce, and CO_2 production.

The combination of lettuce with rocket had increased microbial load, while yeast and moulds were decreased in plain rocket salads. Low TVC and yeast and moulds counts in rocket salads have also been reported by Spadafora et al. (2016). *E. coli* and antioxidants (DPPH, FRAP) were increased in the combination of lettuce with two or more ingredients, whereas TVC, *Enterobacteriaceae* and coliforms were low (Fig. 5C). Samples with plain lettuce had decreased microbial numbers (LAB, TVC, *Enterobacteriaceae*, coliforms, *Pseudomonas* spp. and *Staphylococcus* spp.). Increased microbial load was observed by the combination of lettuce with chives (LAB, TVC, *Pseudomonas* spp., coliforms, *Enterobacteriaceae* and yeast and moulds) and endive/radicchio (*Enterobacteriaceae*, coliforms, *Staphylococcus* spp. and *Pseudomonas* spp.). The combination of lettuce with cabbage had increased microbial load
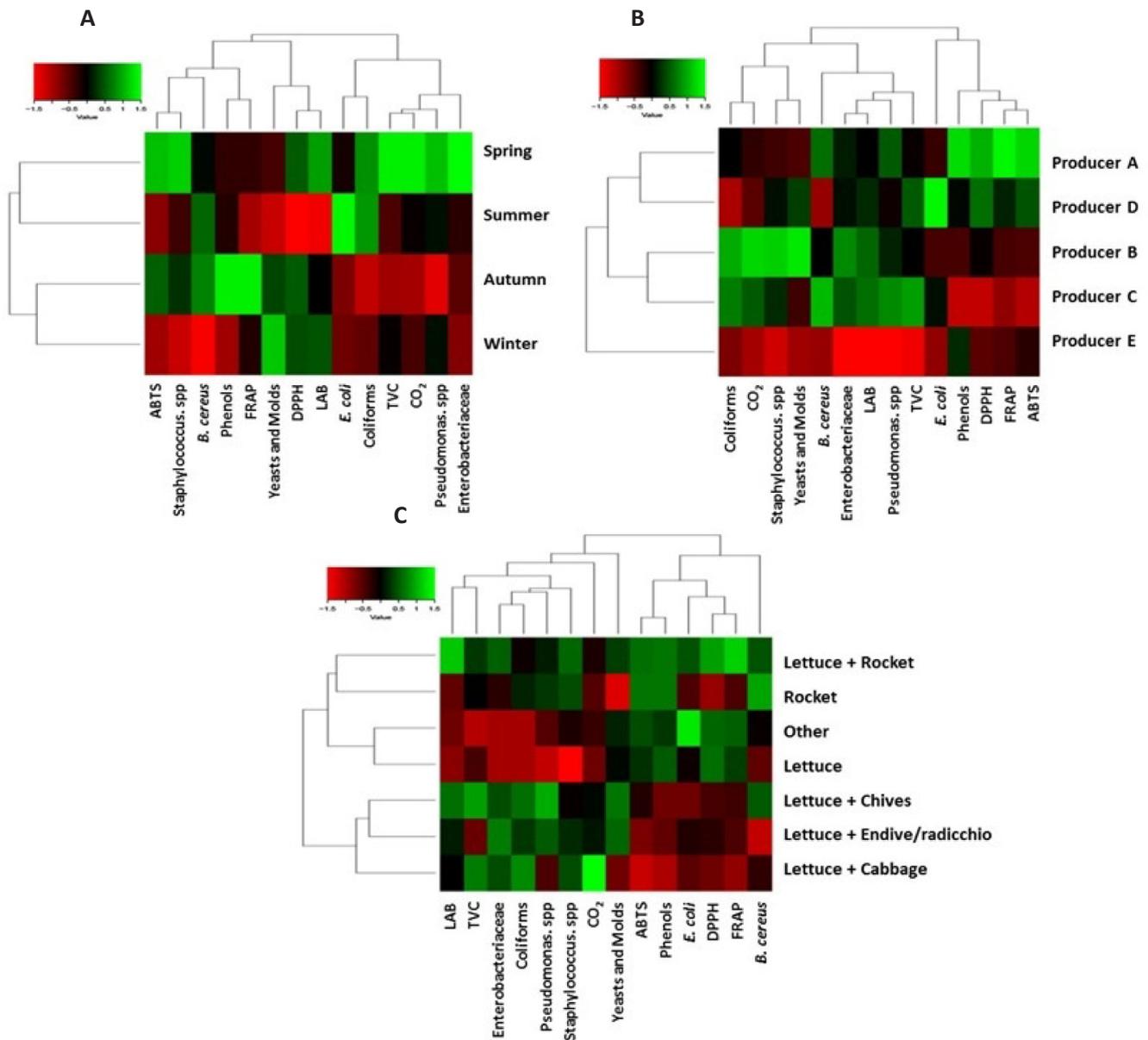
Fig. 5. Microbial and physiological changes in fresh cut salads. Heat map representing relative expression of responses in (A) season (B) producer/supplier and (C) salad type.

(TVC, *Enterobacteriaceae*, coliforms, *Staphylococcus* spp.) and CO_2. Nousiainen et al. (2016) reported CO_2 production ranging between 0.10 and 22.20% and this findings are similar to our results (0.20–20.49%) suggesting that microbial load and the type of vegetable affect respiration of salads and may contribute to spoilage.

The study of TVC and *Enterobacteriaceae* as part of the normal micro flora of vegetables and their correlation with the presence of pathogens may contribute to the establishment of preventive microbiological criteria and less time consuming analysis of ready-to-eat salads (i.e. 24 h instead of 3–4 days). Furthermore the combination of these analyses with physicochemical attributes of RTE leafy vegetables might provide useful insights for assessing and preventing microbiological contamination of these products, as well as for preserving their nutritional value.

Microbiological quality and safety and physicochemical attributes of fresh produce are of great importance. The microbiological quality of ready-to-eat salads has been previously studied in many countries e.g. Brazil (de Oliveira et al., 2011), Italy (Cardamone et al., 2015),

Portugal (Campos et al., 2013), and United States of America (Korir et al., 2016); whilst a number of them further assessed organoleptic attributes (Caponigro et al., 2010; Santos et al., 2012) or physiological parameters such as O_2/CO_2 packaging atmosphere composition (Nousiainen et al., 2016) of these products. The presence of foodborne pathogens in RTE salads arises consumer safety concerns and the on-going need for understanding the prevalence and persistence of pathogens in fresh produce, towards better management practices and safety policies development. Future studies are required to fully understand the factors affecting the entrance, presence and survival of foodborne spoilage and pathogenic organisms in ready-to-eat salads, as well as the physiological attributes of vegetables in order to identify the gaps of the food supply chain that allow their establishment in the food industry.

5. Conclusions

The present study was examining a number of metabolic variables

(antioxidant activity, CO_2 production) besides the presence of spoilage and pathogen bacteria; in order to link the microbiological load of ready-to-eat salads and the response of plant tissue towards biotic (pathogens) and abiotic (storage) stress. Spring is the season that ready-to-eat salads are more pronounced on microbial load. *Salmonella enterica* was not found in any of the samples tested, whereas *Listeria monocytogenes* and ESBL *E. coli* were present in 3.70% and 2.62% of samples, respectively. The content of phenolics and the activity of antioxidants were positively correlated with the presence of *Staphylococcus* spp., *Pseudomonas* spp., *E. coli* and *Bacillus cereus*, whereas fresh produce processing accelerates microbial load and antioxidative mechanisms due to the plant stress. Various salad types are respiring differently through the respiration metabolic process. Furthermore, the interaction of salad producer*type of salad affected greatly the total phenolic content and the antioxidant activity of salads, as different processing practices may cause more or less plant stress and subsequently affecting the quality of fresh produce.

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