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Incidence and antimicrobial susceptibility of *Salmonella*, *Listeria*, and *Campylobacter* spp. in raw “souvlaki” marketed in Thessaloniki (Northern Greece)

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Συχνότητα της παρουσίας και ευαισθησία στα αντιβιοτικά των *Salmonella*, *Listeria*, and *Campylobacter* spp. σε ωμά σουβλάκια από την αγορά της Θεσσαλονίκης (Β. Ελλάδα)

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ABSTRACT. Souvlaki is a popular Greek meat product consisting of small chunks or cubes of pork threaded on a small wooden or metal skewer. In the present study, 105 samples of raw pork souvlaki obtained from retail shops in Thessaloniki (Northern Greece) were screened for the incidence of *Salmonella*, *Listeria*, and *Campylobacter* and their susceptibility to various antimicrobial agents; serotyping of the isolates was also performed. Of the samples tested, 1.9% were positive for *Salmonella* and yielded 3 serovars (*S. SaintPaul*, *S. Fyris* and *S. Typhimurium*); 31.4% proved positive for *Listeria* spp. with 6.7% yielding *L. monocytogenes* isolates belonged to molecular serogroups 2 (serotypes 1/2c and 3c) and 4 (serotypes 4b, 4d, and 4e). *Campylobacter* spp. were not detected in any of the samples tested. The antimicrobial susceptibility to various antimicrobial agents of 11 *Salmonella* strains and 7 *L. monocytogenes* strains was also determined by disc diffusion method. *Salmonella* spp. were susceptible to a panel of 12 antibiotics but displayed intermediate resistance to tetracycline. *L. monocytogenes* isolates were resistant to nalidixic acid and ceftriaxone, partly resistant to clindamycin and cefotaxime, but sensitive to all antibiotics commonly used in veterinary and human listeriosis. Our findings indicate that souvlaki could be a potential vehicle of food borne infections due to strains of *L. monocytogenes* and *Salmonella* spp. in the case of inadequate thermal processing. In addition, good hygienic practices must be applied to avoid cross-contamination during preparation or handling of the product.

Keywords: *Salmonella*, *Listeria*, *Campylobacter*, souvlaki, incidence, antimicrobial susceptibility

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ΠΕΡΙΛΗΨΗ. Στην εργασία αυτή διερευνήθηκε η συχνότητα της παρουσίας καθώς και η ευαισθησία στα αντιβιοτικά των *Salmonella*, *Listeria*, και *Campylobacter* spp. σε ωμά σουβλάκια από χοίρειο κρέας. Συνολικά εξετάστηκαν 105 δείγματα, που προέρχονταν από την αγορά της Θεσσαλονίκης. *Salmonella* spp. απομονώθηκαν από ποσοστό 1,9% των δειγμάτων. Τα 11 απομονωθέντα στελέχη των σαλμονελών ανήκουν σε 3 ορότυπους (*S. SaintPaul*, *S. Fyris* και *S. Typhimurium*) και βρέθηκε ότι ήταν ευαίσθητα σε ένα σύνολο 12 αντιβιοτικών και μετρίως ανθεκτικά στην τετρακυκλίνη. *Listeria* spp. και *L. monocytogenes* απομονώθηκαν από ποσοστό 31,4% και 6,7%, αντίστοιχα, των δειγμάτων που εξετάστηκαν. Τα 7 απομονωθέντα στελέχη της *L. monocytogenes* ανήκουν στις ορολογικές ομάδες 2 (ορότυποι 1/2c και 3c) and 4 (ορότυποι 4b, 4d, και 4e) και βρέθηκε ότι ήταν ανθεκτικά στο ναλιδιξικό οξύ και την κεφτριαζόνη, μετρίως ανθεκτικά στην κλινδαμυκίνη και την κεφτριαζόνη, αλλά ευαίσθητα σε όλα τα αντιβιοτικά που χρησιμοποιούνται για τη θεραπεία της λιστερίωσης στον άνθρωπο και τα ζώα. *Campylobacter* spp. δεν ανιχνεύτηκαν σε κανένα από τα δείγματα. Τα αποτελέσματα της εργασίας έδειξαν ότι τα σουβλάκια είναι δυνατόν να καταστούν επικίνδυνα για τη Δημόσια Υγεία λόγω της δυνατότητας παρουσίας των παθογόνων *Salmonella* spp. και *L. monocytogenes* εάν δεν υποστούν επαρκή θερμική επεξεργασία. Επίσης, επιβάλλεται η εφαρμογή της καλής υγιεινής πρακτικής που θα αποτρέψει τη διασταυρούμενη μόλυνση κατά τη διάρκεια της προετοιμασίας του προϊόντος.

Λέξεις ευρητηρίας: *Salmonella*, *Listeria*, *Campylobacter*, σουβλάκι, συχνότητα παρουσίας, αντιμικροβιακή ευαισθησία

INTRODUCTION

Souvlaki is a popular Greek meat product consisting of small chunks or cubes of pork threaded on a small wooden or metal skewer. Traditionally, it is broiled over charcoal and generously salted and peppered; it may be served on the skewer for eating out of hand, in a pita sandwich with garnishes and sauces, or on a dinner plate, often with fried potatoes or pilaf. In modern years, it may be electric/gas grilled and made with other meats such as beef, lamb, chicken and sometimes fish (especially swordfish).

The word *souvlaki* is a diminutive of *souvla* (skewer) and is dating back several centuries.

Salmonella, *Listeria*, and *Campylobacter* are three major pathogens which continue to be of major concern for food industry, public health authorities and consumers. According to European Food Safety Authority (EFSA, 2014) campylobacteriosis was again the leading cause of zoonotic infections in the European Union, with 214,268 confirmed cases notified in 2012. Regarding *Salmonella*, the second most reported zoonotic infection in humans, it is worth noting that the number of cases showed a decrease for a fifth successive year in the European Union, with 91,034 confirmed human cases in 2012. Despite the observed reduction in salmonellosis cases, the largest number of reported food-borne outbreaks was

caused by *Salmonella* (28.6% of all outbreaks) in the European Union (EFSA, 2014). Although tackling salmonellosis and campylobacteriosis remains a top priority, listeriosis should be of particular concern because of the high mortality rate, especially among vulnerable groups. The number of infections from *Listeria* showed an increasing trend over the past five years in the European Union, with 1,642 confirmed human cases and 198 deaths in 2012 (EFSA, 2014).

Human salmonellosis, listeriosis, and campylobacteriosis can be acquired through the consumption of contaminated meats, the handling of contaminated raw meats and cross-contamination to other ready-to-eat products. Studies worldwide have shown that *Salmonella*, *Listeria*, and *Campylobacter* are often present in pork carcasses and pork meat (Zhao et al, 2001; Whyte et al, 2004; Busani et al, 2005; Fosse et al, 2008). The prevalence of *Salmonella* spp., *L. monocytogenes* and *Campylobacter* spp. in raw pork products at retail in Europe have been calculated to be 8.5%, 9.9% and 2.6%, respectively (Mataragas, 2008). The presence of these pathogens in raw pork meat could be attributed either to faecal contamination during evisceration and environmental contamination, or to food handlers.

In general, there is a paucity of data concerning the prevalence of food-borne pathogens in pork

products exposed to more extensive handling and processing e.g. souvlaki. The scarcity of relevant data in our country has led us to carry out this work determining the prevalence of *Salmonella*, *Listeria*, and *Campylobacter* in raw pork souvlaki in Northern Greece and the sensitivity of the isolates to various antimicrobial agents; serotyping of the isolates was also performed.

MATERIALS AND METHODS

Sample collection

A total of 105 samples of raw pork souvlaki were purchased at retail from 5 supermarket chains in Thessaloniki, Northern Greece. The samples were unpacked and chosen randomly from the point of sale during a 6-month period from January to June 2010, with a sampling frequency of 17 items per month. Immediately following purchase, samples of approximately 100-120 g were placed to sterile plastic sampling bags and transported on ice to the laboratory. All samples were examined within 1 h of arrival.

Isolation and identification procedures

Each sample comprised at least 100 g, was cut into small pieces using an aseptic procedure. From that, three test portions of 25 g were taken and analyzed for the presence of *Salmonella* spp., *Listeria* spp. and *Campylobacter* spp. All culture media and chemicals used were obtained from Merck KGaA (Darmstadt, Germany) unless otherwise stated.

Salmonella spp. were isolated using the procedures detailed in EN/ISO 6579:2002. Briefly, a sub-sample (25 g) was added to 225 ml of the pre-enrichment medium buffered peptone water, blended and incubated overnight (18-20h, 37°C). Afterwards, a selective enrichment was prepared by inoculating 0.1 ml and 1 ml of the pre-enrichment culture into 10 ml Rappaport-Vassiliadis broth (24h, 41.5°C) and 10 ml Muller-Kauffmann tetrathionate broth (24h, 37°C), respectively. Both broths were streaked onto each of two different selective solid media: Xylose Lysine Decarboxylase agar and Bismuth Sulfite agar (24h, 37°C). Up to five suspect colonies were then streaked to purity on Nutrient agar plates (24h, 37°C)

and confirmed biochemically (Triple sugar iron agar, L-lysine decarboxylation medium, Urea agar and indole reaction). Adequate quantity of each pure culture was stored at -80 °C in Nutrient Broth No. 2 (Oxoid CM67B) supplemented with 20% glycerol (BDH Laboratory Suppliers, Poole, UK) until further analysis. Serotyping of isolates was performed at the Centre of Report for Salmonella (Halkida, Greece).

Listeria spp. were isolated according to procedures detailed in ISO, 11290-1:1996/FDAM 1:2004 (E). Briefly, a sub-sample (25 g) was added to 225 ml of the primary enriched medium half Fraser broth, blended and incubated (24h, 30°C). Following this, a secondary enrichment was prepared by inoculating an aliquot (0.1 ml) into 10 ml Fraser Broth (48h, 30°C). Afterwards, a loopful (10 µl) of the primary and secondary enriched cultures were streaked onto Agar *Listeria* Otaviani Agosti-ALOA (Biolife, Milan-Italy) and Oxford agar and examined after 24 and 48 h (37°C). Five suspect *Listeria* spp. colonies from each plate were streaked for purity on Tryptone Soya agar with yeast extract (24h, 37°C). Adequate quantity of each pure culture was stored at -80 °C in Tryptone Soya broth supplemented with 20% glycerol (BDH Laboratory Suppliers, Poole, UK) until further analysis

Identification of *Listeria* spp. strains was conducted by using the multiplex PCR method, as described by Lawrence and Gilmour (1994). This assay uses genus-and-species specific primers and gives three results: a band indicative of bacterial DNA, *Listeria* spp. and *L. monocytogenes*.

Serotyping of strains identified as *L. monocytogenes* was performed using a second multiplex-PCR procedure according to Doumith et al. (2004), using four primer pairs specific for *L. monocytogenes* in addition to one primer pair specific for *Listeria* spp. This method clusters *L. monocytogenes* strains into four molecular serogroups (group 1: serotypes 1/2a, 3a; group 2: 1/2c, 3c; group 3: 1/2b, 3b, 7; and group 4: 4b, 4d, 4e). Appropriate positive and negative controls were included in all assays.

Campylobacter spp. were isolated and identified according to procedures detailed in ISO 10272-1:2006(E). In brief, a sub-sample (25 g) was added to 225 ml of the enrichment medium Bolton broth

(Oxoid CM0983), plus 5% laked horse blood (Oxoid SR0048) and supplements (Oxoid SR0183), and incubated under microaerophilic conditions in a jar (Genbox jar, Genbox Microaer Generator, Biomérieux) at 37°C for 4 h and then at 41,5°C for 44 ± 4 h. Afterwards, a loopful from the enriched culture was inoculated onto the surface of a selective medium mCCDA, (Oxoid CM739B; SR155E) as the first, and Karmali Agar (Oxoid CM935B; SR167E) as the second medium. Cultures were incubated under microaerophilic conditions at 41,5°C and colonies were examined after 44 ± 4 h. Five suspected *Campylobacter* spp. colonies from each plate were subcultured onto a Columbia blood agar plate and incubated under the above conditions for 44 ± 4 h. The isolates were then used for the examination of morphology, motility, and for the detection of catalase and oxidase activity, sensitivity to nalidixic acid and cephalothin, hippurate and indoxyl acetate hydrolysis. Adequate quantity of each pure culture was stored at -80 °C in Nutrient Broth No. 2 (Oxoid CM67B) supplemented with 5% lysed horse blood (Oxoid SR0048) and 20% glycerol (BDH Laboratory Suppliers, Poole, UK) until further analysis.

Antimicrobial susceptibility testing

The disk diffusion method according to Bauer et al. (1966) was used to determine the antimicrobial susceptibility of *Salmonella* and *L. monocytogenes* isolates to ampicillin (10 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cephalothin (30 µg), ciprofloxacin (5 µg), chloramphenicol (30µg), gentamicin (10 µg), kanamycin (30µg), nalidixic acid (30 µg), neomycin (30 µg), streptomycin (10 µg), sulfamethoxazole-trimethoprim (23.75/1.25 mg), and tetracycline (30 µg), (for all isolates), clindamycin (2 µg), erythromycin (15 µg), penicillin (10 U) and vancomycin (30 µg) (for *Listeria* spp). Isolates were classified as sensitive, intermediate or resistant according to the criteria recommended by Clinical and Laboratory Standards Institute (CLSI, 2008). Since no resistance criteria exist for *Listeria* spp. susceptibility testing in CLSI guidelines for the tested antimicrobials other than ampicillin and penicillin, criteria for staphylococci were applied in this study. *E. coli* ATCC 25922

and *Staphylococcus aureus* ATCC 29213 were used as reference strains.

RESULTS AND DISCUSSION

In our study, we analyzed 105 raw pork souvlaki samples for the occurrence of the three major food-borne pathogens and their antimicrobial profile. These bacteria have frequently been associated with pork and meat products and linked to a number of cases of human illness.

Several studies have indicated that *L. monocytogenes* and *Salmonella* spp. are present in retail raw pork. The reported prevalence of these pathogens in retail meats varies widely (Mayrhofer et al., 2004; Busani et al., 2005; Thénevot et al., 2006a). Variations in pathogens prevalence may be attributed to country of origin, type and size of meat analyzed, slaughterhouse sanitation, possible cross-contamination at retail level, sampling season and methodology used.

Salmonella spp. were isolated from two samples (1.9%). In this study although souvlaki is a meat preparation exposed to more extensive handling and processing however the observed contamination is relatively similar or lower than the most published data of retail fresh pork meat in other countries. The prevalence of *Salmonella* spp. found in this study is similar to that reported for retail pork meats from United Kingdom (Little et al., 2008). Relatively similar prevalence (1.8% and 2.0%) have also been reported in retail pork throughout Austria (Mayrhofer et al., 2004) and Canada (Aslam et al., 2012), respectively.

However, the contamination rate of the organism in other studies was much higher. Two studies conducted in Italy by Giovannini et al. (2004) and Busani et al. (2005) showed that 5.0% and 4.9%, respectively, of the fresh pork meat samples they examined were positive for *Salmonella*. In a study performed in pork cuts collected from retail stores in six continental United States cities (Duffy et al., 2001) *Salmonella* spp. were recovered from 9.6% of tested samples. A relatively large proportion of pork meat samples collected in swine abattoirs were found to be *Salmonella* positive (14.0%) in a Portuguese

investigation (Gomes-Neves et al., 2012). Studies in other countries have reported on the prevalence of *Salmonella* in retail pork meats, with contamination ranging from 0.4% in Germany (Schwaiger et al., 2012) to 26.7% in China (Yan et al., 2010) and 39.6% in North Vietnam (Thai et al., 2012).

In the current study serotyping results were available for 11 strains isolated from the two positive samples. In the former of the positive samples three different serovars (5 strains as *S. SaintPaul*, 2 strains as *S. Fyris* and 1 strain as *S. Typhimurium*) could be identified and in the latter sample all 3 isolates could be serotyped as *S. Typhimurium*. *S. Typhimurium* is the most frequently isolated serotype in humans in Europe and pigs are an important reservoir of this particular serotype (Boyen et al., 2008; EFSA, 2014). Three studies conducted on Irish raw pork samples showed that *S. Typhimurium* accounted for almost all of the isolated serotypes (Jordan et al., 2006; Prendergast et al., 2008; Prendergast et al., 2009). Other studies conducted in Italy (Busani et al., 2005), United Kingdom (Little et al., 2008) and Portugal (Gomes-Noves et al., 2012), also showed that it was the most frequent serotype obtained from pork meat samples and it was also predominant in processed meats which frequently involved pork.

Listeria spp. were detected in a greater proportion of raw pork souvlaki samples than that of *Salmonella* spp. Of the samples tested, 33 (31.4%) proved positive for *Listeria* spp. with 7 (6.7%) yielding *L. monocytogenes*. The higher incidence of *Listeria* spp. than that of *Salmonella* spp. in the present study was not unexpected because of the ubiquitous and environmentally tolerant nature of the pathogen. In addition, the organism is able to adhere and persist for long periods on the surface of equipment forming biofilms (Kornacki and Gurtler, 2007). *L. monocytogenes* can be found at all stages of pork meat industry with increasing prevalence from the slaughterhouse to the cutting room (Thénevot et al., 2006a; López et al., 2008; Wesley et al., 2008). Our results could confirm our previous study carried out on raw minced pork which showed that 35.0% and 8.0% of the samples examined proved positive for *Listeria* spp. and *L. monocytogenes*, respectively (Tzikas et al., 2011). Relatively data are reported by

Karakolev (2009) who isolated *L. monocytogenes* from fresh pork meat and minced pork in 5.0% and 9.2% of studied samples, respectively, in Bulgaria.

According to some previous reports, much higher incidences of *L. monocytogenes* were found in raw pork meat in other countries. Surveys at retail have shown contamination levels of 19.8% in the United States (Duffy et al., 2001), 22.0% in Austria (Mayrhofer et al., 2004), 24.0% in Canada (Bohaychuk et al., 2006), and 20% in China (Wang et al., 2013). In addition, two recent studies carried out in Italy by Pesavento et al. (2010) and Valero et al. (2014) showed that 21.4% and 14.4%, respectively, of the fresh pork cuts they examined were contaminated with *L. monocytogenes*.

Serotyping of *L. monocytogenes* is important from the epidemiological point of view and may have value as a virulence screening test. There are 13 serotypes of *L. monocytogenes* showing varied virulence potential, but only 4 serotypes (1/2a, 1/2b, 1/2c and specifically 4b) are associated with the majority of foodborne listeriosis outbreaks (Kathariou, 2002; Liu, 2006; Swaminathan et al., 2007). A Multiplex – PCR assay, developed by Doumith et al. (2004) can be utilized to detect the presence of virulence associated genes of *L. monocytogenes*. In this study, only 4 out of the 7 *L. monocytogenes* isolates could be classified in PCR groups of serotypes, using Doumith's method. Three isolates belonged to molecular serogroup 4, containing serotypes 4b, 4d, and 4e, while the other one isolate was identified as molecular serogroup 2, which includes serotypes 1/2c and 3c. As it was reported by Doumith et al. (2004), a disadvantage of Multiplex – PCR assay used for serotyping is that this method cannot differentiate serotype 4b from 4d and 4e and serotype 1/2c from 3c. However, this drawback would not decrease the efficiency of the multiplex PCR assay because as serotypes 3c, 4d and 4e are relatively rare in foods and rarely reported as implicated in human listeriosis (Doumith et al., 2004; Chen and Knabel, 2007), the isolated strains from souvlaki samples must be presumably serotypes 1/2c and 4b. There are only few data available on the serotyping of this pathogenic bacterium in pork meat. According to previous studies conducted in other countries on

pork products serotypes 1/2c and 4b are among the most common, as well as serotypes 1/2a and 1/2b (Thénevot et al., 2006a; Thénevot et al., 2006b). It is worth noticing that the 3 *L. monocytogenes* isolates from souvlaki samples were potentially identified as 4b serotype, which is the serotype most commonly causing human listeriosis (Kathariou, 2002; Gray et al., 2004; Liu, 2006). As for as the remaining 3 untypable isolates, they may belong to some clones of atypical strains (1/2a, 3a, 1/2c) which are not recognized in the expected PCR-types, using Doumith's assay (Kérouanton et al., 2010).

Campylobacter spp. were not detected in any of the samples tested in this study. This is in agreement with surveys conducted on retail pork products in other countries, such as Hong Kong (O'Toole, 1995), Northern Ireland (Madden et al., 1998), Ireland (Cloak et al., 2001) and Canada (Bohaychuk et al., 2006). Other studies at retail level have shown a low prevalence in pork meat. An Italian study revealed a 2.4% prevalence in fresh pork sausages (Zanetti et al., 1996), in the United States 1.6% of samples of pork ground meat and sausages were found to be contaminated with *Campylobacter* (Duffy et al., 2001) and a study in Belgium showed 2.5% positive pork mince meat (Ghafir et al., 2007). Although other studies conducted in United Kingdom (Little et al., 2008) and Italy (Pezzotti et al., 2003) have shown a higher incidence of the pathogen (5.0% and 10.3% in fresh pork meat, respectively), it is generally accepted that *Campylobacter* is not commonly isolated from pork at retail level (Duffy et al., 2001). The lower isolation rates of *Campylobacter* in retail pork meat may be due to the sensitivity of the bacterium to atmospheric oxygen and other environmental stresses during transport and storage of the products (Stern et al. 1984; Stern et al., 1985; Zhao et al., 2001).

No sample was contaminated with more than one food-borne pathogen.

The *Salmonella* strains isolated from raw pork souvlaki samples were subjected to antimicrobial resistance testing. Surprisingly, all 11 strains displayed intermediate resistance to tetracycline, an antibiotic commonly used in veterinary practice but were found susceptible against ampicillin, cefotaxime, cef-

triaxone, cephalothin, ciprofloxacin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, neomycin, streptomycin and sulfamethoxazole-trimethoprim. From a public health perspective, this finding is of particular importance, as these drugs are categorized by World Health Organisation (WHO, 2009) either as Critically Important (ampicillin, cefotaxime, ceftriaxone, ciprofloxacin, gentamicin, streptomycin) or as Highly Important Antimicrobials (cephalothin, chloramphenicol, kanamycin, nalidixic acid, neomycin, sulfamethoxazole-trimethoprim) in Human Medicine. As regards *L. monocytogenes*, the isolates were found resistant to nalidixic acid and ceftriaxone, and partly resistant to clindamycin and cefotaxime. Resistance to "modern" cephalosporins and to nalidixic acid was expected because it is considered an innate resistance (Troxler et al., 2000). In regard to clindamycin, according to Chen et al. (2010), its effect to *Listeria* species is a controversial issue as some studies suggested its sensitivity whereas another study reported high incidence of resistance. All seven isolates were found susceptible to antibiotics recommended for the treatment of listeriosis in humans, such as ampicillin or penicillin often in combination with gentamicin, or trimethoprim-sulfamethoxazole, as an alternative therapeutic option. Additionally, ampicillin or tetracycline is recommended for treatment of infections in animals (Charpentier et al., 1995).

Our findings indicate that souvlaki could be a potential vehicle of food borne infections due to *L. monocytogenes* and *Salmonella* spp. It is worth noting that during grilling of souvlaki, temperature can reach up to 250°C which quickly destroys these pathogens and especially *L. monocytogenes* (Sergelidis and Abraham, 2009). However, appropriate safeguards, such as avoiding the consumption of undercooked souvlaki and cross-contamination and the use of adequate hygienic practices must be taken for the protection of public health.

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