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Investigation of the Presence of Different Animal Species within Processed Meat and Meat Products using PCR Procedures and Development of Risk Models based on Consumer Health

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Abstract

Fraudulent imitation and adultery of meat and meat products are fooling the consumers, jeopardizing their health, economical situation and potentially causing harm to religious beliefs. The aim of this project was to search for the existence of such fraudulent imitations and adulteries within processed meat products across different sale points (them being markets) found within 11 municipalities of the Marmara Regionusing PCR procedures. According to the findings gathered during the study, 25 of the collected samples (4.54

Index terms— meat, meat products, adultery, fraud, pathogens, DNA typing, PCR.

1 I. Introduction

aving access to sufficient quantities of food which is produced in a high quality and trustworthy environment while guaranteeing its safety is a fundamental right for the well physical, mental and psychological development of every human being. Even though the application of food safety is one of the most prioritized policies of the European Union (EU), when it comes to the management of the quality of meat and meat products throughout the whole process starting at the barn, ending on the table, solely the labeled information cannot actually guarantee the food safety (1). That's why, it's vital for meat and meat products to be checked in order to determine from which animals they are produced from, to validate the labeled information found on their packages, to detect substances that can harm the consumer health(carcass products high in BSE, undesirable fats, illegal addition of animal species into meat products, insect and rodent contamination of the same products because of the lack of proper hygiene, etc.). In the Notification entitled "Instructions for the application of the Notification on meat and meat products" issued in our country on February 2013 (2), the following statement can be found: "Species, as mentioned in its corresponding article in the Notification, can only be mixed with themselves. For example, chicken-turkey mixture or a calf-sheep mixture." which has thus rendered illegal to mix different animal species in meat and meat products.

Listeria monocytogenes is an important grampositive, facultative anaerobic microorganism that is being frequently isolated from nearly all food products and that can cause sporadic and epidemic infections. As it can live and thrive in active soil, it can survive in vegetables, dairy and dairy products, potable or waste water, as well as poultry meat and poultry products. In turn, this infectious agent can be transmitted to humans or other animals via fecal-oral route (3). Main causes of human listeriosis are pasteurized/non-pasteurized dairy and dairy products, meat and meat products, poultry meat and poultry products, poultry fodders, vegetables and contaminated waters (4). Patients with suppressed immune system because of diseases such as HIV, hepatitis or cancer, as well as pediatric and geriatric cases along with pregnant women form the primary risk group for the human listeriosis.

Escherichia coli are aerobic/facultative aerobic microorganisms that can be found within the normal flora of the intestinal system of humans and warmblooded animals. Even though some coliform groups as well as some E.coli strains are harmless, these aforementioned agents can also possess pathogenic strains. Total coliform

bacteria quantity and the presence of E.coli is being reported as an indicator of poor hygienic conditions and fecal contaminations (5). Among the main sources of the contamination of aforementioned microorganism groups are; willingly or unwillingly introducing foreign animal tissues that weren't subjected to the obligatory food safety inspection system, tissues that come from the same species but shouldn't be put in meat products (such as renal or lung tissues), toilets with poor hygienic conditions and the end consumer or the food production personnel who don't follow the hygienic necessities after using the toilet.

When meat and meat products, all having an important role in human consumption, are acquired from healthy animals and processed within appropriate conditions, they are regarded as microbiologically safe. Unless necessary precautions are taken during elevation and slaughtering, meat and meat products might end up causing serious health problems among the consumers. Also, fraudulent imitation and adulteration done in order to decrease cost and thus increase profit margin may lead to the introduction of undesirable animal species (horse, donkey, pig, etc.) in meat and meat products. Furthermore, in establishments processing than one meat product (mainly establishments processing cattle and poultry meats under the same roof), tissues belonging to foreign animals might unwillingly get introduced into these processed meats. Moreover, in some cases of adultery of meat and meat products, unwanted tissues not coming from a foreign animal (nail, kidney, brain, lung, etc.) might be added willingly or somehow end up unwillingly contaminating these said products.

The aim of this project is to search for the existence of fraudulent imitation and adultery within processed meat products across different sale points (them being markets) found within 11 municipalities of the Marmara Region (Edirne, Tekirda?, K?rklareli, ?stanbul, Kocaeli, Yalova, Sakarya, Bursa, Bilecik, Bal?kesir and Çanakkale) using PCR procedures.

2 II. Materials and Methods

3 a) Sample collection

Over the course of this study, across 11 different municipalities of the Marmara Region (Edirne, Tekirda?, K?rklareli, ?stanbul, Kocaeli, Yalova, Sakarya, Bursa, Bilecik, Bal?kesir and Çanakkale), from a total of 5 different meat product types (Beef Salami, Beef Garlic Flavoured Sausage, Chicken Sausage, Bresaola, Braised Meat), a grand sum of 550 samples were gathered (50 from each municipality, in each municipality 10 samples for each meat product type). The gathered samples, which were put in transportation boxes that were rendered sterile according to the rules of asepsis and antisepsis, were brought to our university inside transportation containers with 4°C inner temperature. Samples were kept at -20°C until the analyses. Detailed information on collected samples is shown on Table **??**.

4 Table 1: Detailed information on the sample collection program.

Tablo 1: Örnek toplama program? hakk?nda detayl? bilgi b) Microbiological Analyses -E. coli: From swabsticks containing the growth medium which comes from where the sampling was made, passages have been made, in accordance with asepsis conditions, into TBX agar growth medium that was previously prepared and poured into petri dishes. The petri dishes were then incubated for 24 hours in 44°C. Following this incubation period, typical colonies that formed were counted. About 98% of E. coli serotypes contain the enzyme ?-D glucuronidase. This aureus in order to detect whether the isolates possess the CAMP factor (6).

5 c) DNA Extraction

The DNAs of all the isolates were extracted via the commercial DNA extraction kit, in accordance with the kit protocol. The extracts were stored in -20°C to be used later on as target DNA during PCR procedures.

6 d) PCR

On Table **??** is shown species specific primer sets used during the PCR procedure.

Table **??**: Species specific primer sets used during the PCR procedure (7)(8)(9)(10)(11). Besides species specific primers, PCR procedures have been made on colonies that were microbiologically isolated and evaluated as suspicious in order to identify (i) E. coli, one of the most important food pathogen which jeopardizes consumer health, (ii) L. monocytogenes, which can be isolated and identified in 7 to 10 days and also can be hard to identify due to all the different chemical tests made during its identification process. These two aforementioned food pathogens and the primer sets we have used for them can be found on Table **??**. **??**: Primer sets designed according to the different serotypes used in our study and their properties (10,(12)(13)(14)(15)(16).

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Tablo 3: Çal??mam?zda kullan?lan farkl? serotipler için haz?rlanm?? primer setleri ve onlar?n özellikleri (10,(12)(13)(14)(15)(16)

8 Primer No

Sequence (5' -3') Target Gene / Amp (bp) Target microorganism The real-time PCR procedure is as follows:

-50-100 mg of tissue from samples were sliced or crushed to bits and then were put in microcentrifuge tubes.

-400 µL of SH solution was added into the samples in microcentrifuge tubes and mixed via vortex. -To the homogeneous-looking mixture were added 8 µL of proteinase K and 40 µL of SLS solution. After mixing well enough, the mixture was kept under 60°C for 2 hours for the cells to open up. -Following the 60°C incubation, 300 µL of SP solution was added to the mixture which was then stirred via vortex for 30 seconds. -The mixture was centrifuged at 12000 rpm for 30 minutes. The supernatant was then moved into an empty tube. -500 µL of isopropanol was added to the supernatant, stirred via vortex and then incubated under -20°C for 1 hour. -Following the incubation, the mixture was centrifuged at 12000 rpm for 20 minutes. The supernatant was thrown away.

-After adding 0.5 mL of ethanol to the pellet remaining at the bottom of the tube, the pellet was dissolved by gently vortexing and then centrifuging at 12000 rpm for 5 mins. -The ethanol was thrown away and the sedimenting DNA was left to dry. -With the ethanol completely evaporated, on the remaining pellet was added 150 µL of SE solution and then it was kept overnight for the DNA to dissolve under room temperature.

-The dissolved DNA was measured via UV spectrophotometer and was diluted to reach a concentration of 50 ng/µL. Afterwards, the following heat cycle protocol was executed, 1. 95°C for 10 minutes 2. 95°C for 10 seconds 3. 60°C for 15 seconds 2 nd and 3 rd steps were repeated 35 times in a cycle.

9 III. Results

10 a) Foreign species identification and detection of fraud and adultery

In this study, a total of 550 samples of processed meat was collected from different sale points (supermarkets, markets, local bazaars etc. / being local brands, if present), found within 11 municipalities of Marmara Region (Edirne, Tekirda?, K?rklareli, ?stanbul, Kocaeli, Yalova, Sakarya, Bursa, Bilecik, Bal?kesir and Çanakkale) and from these collected samples, existence of voluntary and involuntary (in establishments processing meats of different species, improper equipment use/surfaces/personnel borne improper procedure applications?) fraud and adultery was researched using PCR procedures. These aforementioned fraud and adultery applications were analyzed by taking into account 8 different animal species (pork, poultry, cattle, sheep, horse, donkey, cockroach and house fly). Details concerning the collected samples and findings are shown on Table 4. ? 2 products (0.36%) had horse DNA as foreign species. ? 4 products (0.72%) had cattle DNA as foreign species.

11 b) Microbiological analyses

All of our samples were analyzed according to 2 food pathogens (Escherichia coli and Listeria monocytogenes) which can seriously harm consumer health. Table 5 shows analysis details of the collected samples during the study, in relation with the chosen food pathogens. ? Even though no pork DNA was found within any of the samples of our study, pork nano-drop measures were also included since it is important in our country for religious reasons. **??**———? The microbiological load on adulterated samples is statistically significantly higher than it is on unadulterated samples. For every group (them being adulterated and unadulterated samples), group differences were made according to the samples that are positive on microbiological parameter. For these microbiological parameters, samples which didn't show any growth were omitted. ? As L. monocytogenes wasn't found in any of the samples, it wasn't evaluated in this table. Table 9: Evaluation of group differences between adulterated and unadulterated products in relation with their negative effects on consumer health, using microbiological parameters (According to Pearson Chi Square method).

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The results obtained on this table shows within the products not suitable for human consumption the group differences between adulterated and unadulterated meat products. ? For every group (them being adulterated and unadulterated samples), group differences were made according to the samples that are positive on microbiological parameter. For these microbiological parameters, samples that didn't show any growth were omitted.

? As L. monocytogenes wasn't found in any of the samples, it wasn't evaluated in this table.

13 IV. Discussion

Even though the application of food safety is one of the most prioritized policies of the European Union (EU), when it comes to the management of the quality of meat and meat products throughout the whole process starting at the barn, ending on the table, solely the labeled information cannot guarantee the food safety (17,18). Fraudulent imitation and adultery of meat and meat products are fooling the consumers, jeopardizing their health, economical situation and potentially causing harm to religious beliefs.

According to the findings gathered during the study, 25 of the collected samples (4.54%) contained poultry DNA, 5 of them (0.90%) contained house fly DNA, 6 of them (1.09%) contained sheep DNA, 2 of them (0.36%)

contained cockroach DNA, 2 of them (0.36%) contained horse DNA and 4 of them (in chicken sausages / 0.72%) contained bovine DNA as foreign species. No pork DNA was found in the collected samples. 100% of the adulterated or fraudulent samples are made up from openly sold brandless or local brand products. Adultery and fraudulent imitation was not found in samples collected from brands producing and marketing nationwide or internationally. According to the results, it could be seen as a high probability that firms producing meat products either without any brand or under a local brand license are processing more than one species of animals and end up accidentally mixing up tissues belonging to different animal species. Another possible cause would be the staff working at the aforementioned firms lacking anytraining on proper hygiene which leads to the mechanical contamination of meat products due to the lack of training or attention. Another possibility is the thought that these aforementioned firms are willfully executing adultery and fraudulent imitation in order to make profits.

In one study conducted in the United States, Hsieh et al. (19) reported that in 90% of the minced meat samples contained poultry meat introduced willingly or unwillingly and therefore adulterated meat was being marketed. Türky?lmaz et al. (20) found that within 121 meat and meat products analyzed using AGID method, 3 of them (2.5%) contained equidae meat, 2 of them (1.7%) contained pork meat. As a result of the study of 223 samples, Türk et al. (21) has found that 16 of the samples (7.1%) contained pork meat, 12 of them (5.3%) contained equidae meat and 6 of them (2.6%) contained a mixture of pork-equidae meat. Within 410 samples of meat and meat products acquired in Bursa and Istanbul, Gün?en et al. (22) has found, using ELISA method, that 14 of these samples (3.41%) contained horse meat. Results in our study are lower in relation to the detected species when compared to the aforementioned studies. In addition to the results obtained by these previously mentioned researchers, in our study, in 2 samples (0.36%) cockroach DNA and in 5 samples (0.90%) house fly DNA was detected. The presence of cockroach and house fly DNA in results makes us think that in their corresponding manufacturers, poor hygiene conditions are present, food safety regulations are not applied and these manufacturers are inefficient when it comes to the general cleaning, disinfection, staff hygiene and self-care.

Throughout literatures in our county and around the world, the causes for the acquisition of different results on this subject would be the different physical conditions of the sales points along with presence or lack of the application of food safety protocols, deficiencies in processing and/or usage of the same equipment for establishments processing more than one species of animal meat, intentional or unintentional application of adultery and fraudulent imitation and staff's lack of knowledge on applied procedures. It's thought that, at the root of the results obtained in our study lies the deficiencies of the inspection of food safety systems as well as staff's lack of knowledge.

According to the results obtained in this study, 102 of the samples (18.5%) were found to be positive for E. coli and therefore not suitable for human consumption. One of the most remarkable findings in our study would be the fact that a significant number of E. coli positive samples come from those which were adulterated and fraudulent (Table 5). As explained above, in establishments having really poor hygienic conditions (most of them producing adulterated and fraudulent products), our results show that poor toilet hygiene can also be present. Another possible risk factor is that personnel infected with E. coli can easily transmit the bacteria to their surroundings (places such as homes, public transportations, public toilets, local bazaars with lots of people in it, malls, cinemas, schools, etc.).

For samples that weren't "suitable for human consumption" according to the E. coli parameter, highest value was 3.7 x 10 4 cob/gr, whereas lowest was 1.2 x 10 2 cob/gr. According to the results obtained, for samples not suitable for human consumption in relation to their E. coli parameter of total coliform bacteria quantity, highest value was found within beef salami (in which poultry DNA was found) and chicken sausage (in which house fly and cockroach DNA was found). There are studies which report that poultry meat does also contain E. coli. In a study conducted in Egypt, Abdul-Raouf et al. (**??**3) studied E. coli O157:H7 in various foods. In this study, from samples gathered from slaughterhouses, supermarkets and barns, 3 out of 50 samples (6.0%)of minced bovine meat and 2 out of 50 samples (4.0%) of poultry meat contained E. coli. In a study conducted by Doyle and Schoeni (24,25), from samples gathered from cattle, sheep, pork and chicken meat, E. coli O157:H7 was tried to be isolated. At the end of the study, E. coli O157:H7 was found in 3.7% of cattle meat, 2% of sheep meat, 1.5% of pork meat and 1.5% of chicken meat. The agent was detected in chicken wing samples and again in another study, within chicken nugget samples, E. coli O157:H7 serotype was found (24). One of the main reasons of this difference would be that water activity (a w) in poultry meat is higher when compared to other butchered meats. It is thought that high water activity levels directly influence the total coliform bacteria and E. coli parameters. The results also show us that samples containing cockroach and house fly DNA also contain high amounts of E. coli. As mentioned in above paragraphs, flies and cockroaches can transmit, as a primary or secondary contamination source, a high quantity of bacteria, parasite, protozoa and virus to its environment by physical contact.

These insects originating mainly from toilets are thought to transmit E. coli to meat products mechanically. Another reason for these aforementioned findings would be the deficiencies in application of hygiene protocols within establishments that produce and sell meat products. Even though during our study, neither establishment hygiene nor critical control points (CCP) within establishments were inspected, in establishments from which samples containing high quantity of E. coli and total coliform bacteria were gathered, by external inspection, we can conclude that they are lacking minimum hygiene applications. A different reason for this would be the

possibility that these previously mentioned high quantities of E. coli and total coliform bacteria were already present inpoultry meat.

In our study, L. monocytogenes was one of the investigated parameters. Nevertheless, none of the samples contained L. monocytogenes.

Another parameter investigated in our study was the difference in potential risks to the consumer between adulterated/fraudulent products and unadulterated/non-fraudulent products. For this reason, a two-way relationship analysis was done using the Pierson Chi Square method. One of the relationship analyses was made to evaluate the relationship analysis between adulterated/fraudulent products and unadulterated/non-fraudulent products. Another relationship analysis was made to investigate the statistical significance between adulterated/fraudulent products and unadulterated/non-fraudulent products both not suitable for human consumption. According to the results obtained in our study, for both of the relationship analyses, statistically significant differences were found on the basis of E. coli. For this microbiological parameter which is significant when it comes to the consumer, possible risks were found in favor of adulterated and fraudulent products (among all the products not suitable for human consumption, adulterated and fraudulent ones were found to contain statistically significantly higher quantities of risk factors on the basis of E. coli). Since in none of the samples L. monocytogenes was detected, relationship analyses were not done on this factor.

In our country and throughout the world, adultery and fraudulent imitation either occurs willfully and illegally in order to increase profits or accidentally, in establishments processing different species of animal meat, by keeping the production of different animal species on the same space or lack of staff training, poorly executed food safety applications or quality management. Especially, adultery and fraudulent imitation done to increase profits brings with itself G serious microbiological risks that can endanger consumer health. Since such willful adultery and fraudulent imitation is executed illegally, inspection and control procedures don't work on them which can create innumerous microbiologically critical control points during processing. Furthermore, no ante-mortem or post-mortem inspections are done on foreign animal borne meats as well as slaughtered animals. Additionally, control over the processes of extraction of internal organs, meat mincing, packaging and transportation remains impossible. Not identifying microbiological, parasitic, chemical risks throughout the whole process of the arrival of meats to customers can end up creating innumerable risk factors. In our study, L. monocytogenes was in none of the adulterated or fraudulent meats. When it comes to E. coli, it's found in significantly more adulterated/fraudulent meats than unadulterated/non-fraudulent meats. Our findings show significant differences between unadulterated/nonfraudulent products that are not suitable for human consumption and adulterated/fraudulent products, in terms of microbiological risks that can be brought upon the consumer. In the light of these findings, it can be said that adultery and fraudulent imitation can end upseriously jeopardizingthe consumer health. [1] [2] [3] [4]

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Tablo 4: Örnek toplama program? hakk?nda detayl? bilgiler.

REGION	MUNICIPALITY	SAMPLE NAME	SALE POINT	TOTAL SAMPLE COUNT	POSITIVE SAMPLE COUNT	FOREIGN ANIMAL SPECIES
		Beef Salami	Market	10	1 (10%)	Poultry
		Beef Garlic Flavoured Sausage	Market	10	2 (20%)	Poultry [×2]
Marmara	Edi	Chicken Sausage	Market	10	1 (10%)	House Fly
		Bresaola	Market	10	0	—
		Braised Beef	Market	10	1 (10%)	Sheep

Figure 1: Table 4 :

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Tablo 5:

Figure 2: Table 5 :

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Volume XVII Issue I Version I (D D D D) G	Microbiological parameter	Sample name
Medical Research	Escherichia coli	Beef salami Beef Garlic Flavoured Sausage Chicken Sausage Bresaola Braised Beef
Global Journal of	Listeria monocytogenes	Beef Garlic Flavoured Sausage Chicken Sausage Bresaola Braised Beef

Tablo 6: Çal??mam?zda elde edilen PCR sonuçlar?n?n ISO 16140 de?erlendirme parametrelerine gö

	Relative accuracy (%)	Relative specifity (%)
E. coli	88.90	97.34
L. monocytogenes	——	— –

[Note: ? As L. monocytogenes wasn't found in any of the samples, it wasn't evaluated.]

Figure 3: Table 6 :

7

Tablong/µl 7: DNA type	A260	A260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
1822.46	36.952	18. 691	1.76	1.82	50.00	230	19.002	3.499
2786.21	51.203	24.266	1.89	1.92	50.00	230	26.782	4.001
3893.03	72.089	37.668	2.07	1.99	50.00	230	36.988	3.600
3055.92	62.580	31.352	2.09	2.01	50.00	230	31.616	5.900
3211.87	66.873	34.002	2.13	1.87	50.00	230	32.043	4.999
3100.21	65.660	33.992	2.12	1.43	50.00	230	31.234	5.203
3343.455	71.650	37.231	1.72	1.89	50.00	230	36.902	5.453

Figure 4: Table 7 :

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	Microbiological parameter	Related variable	Value	Asymp. Sig
Pearson Chi Sq	Escherichia coli	Adultered samples / All of the unadultered samples	9.653	.000
Pearson Chi Sq	Listeria monocyotgenes	Adultered samples / All of the unadultered samples	-	

[Note: Tablo 8: Ta??i? yap?lan ve ta??i? yap?lmayan et ürünleri aras?ndaki grup farkl?l?klar?n?n tüketici sa?l???n? riske etmesi aç?s?ndan analiz edilen mikrobiyolojik parametreler için s?nanmas? (Pearson Chi Square yöntemine göre). Tablodaki sonuçlar ta??i? yap?lmad??? tespit edilmi? tüm örneklerin toplam? ve ta??i? yap?lm?? et ürünleri aras?ndaki grup farkl?l?klar?n? yans?tmaktad?r.]

Figure 5: Table 8 :

9

	Microbiological parameter	Related variable
Pearson Chi Sq	Escherichia coli	Adultered samples / Unadulte
Pearson Chi Sq	Listeria monocyotgenes	Adultered samples / Unadulte

? Values marked with red are statistically significant since they are lower than P<0.005.
? In values marked with red, the positive relationship correlation for adulterated products is positive.
Adulterated meat products, compared to unadulterated meat products, are significantly harmful to the consumer health when microbiological parameters are taken into account.

Figure 6: Tablo 9 :

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