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Revised detection methods of *Salmonella* and *Listeria monocytogenes* in raw milk and dairy products from sheep/goat milk.

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1. Introduction

The aim of this study was to review the existing laws and regulations concerning the detection of *Salmonella* and *Listeria* bacterial species in sheep and goat raw milk and dairy products. The products under examination were raw sheep/goat milk, halloumi and anari cheese, the latter two being types of white cheese produced in Cyprus. The procedures in question were ISO 11290-1:1996 for *Listeria monocytogenes* and ISO 6579:2002 for *Salmonella species*. Additionally supplementary examinations were conducted using another method which is soon going to be used for the detection of these two bacterial species. The new method was introduced so that the detection of *Salmonella species* from raw and faecal materials can be done using just a single method than two different methods. In the case of *Listeria monocytogenes* we also used a different detection method which was proven to be more accurate and reliable than the method used in ISO: 11290-1:1996. All the procedures and experiments were conducted in accordance with the EU rules and regulations concerning the detection of *Salmonella species* and *Listeria monocytogenes*. Although all the results from the raw milk and the dairy products tested for *Salmonella* and *Listeria* were negative, the aim was to review the methods and materials

2. Literature review

2.1. Salmonella spp

The *Salmonellae* belong to the family of *Enterobacteriaceae* [1]. They are facultative anaerobes, gram negative, non-spore forming, and rod shaped bacteria. *Salmonella* is one of the most frequent causes of food borne gastroenteritis throughout the world and is also an important pathogen of animals. Salmonellosis; the clinical name of the disease caused by *Salmonella species*, is a zoonotic infection meaning it can be transmitted to humans from infected animal products such as milk, milk products and meat.

Salmonella nomenclature has been revised over the years and at the present it is based on biochemical and serological characteristics. Currently only two species of *Salmonella* are recognized. These are: (i) *Salmonella enterica* with 6 other subspecies and (ii) *Salmonella bongori*. The most important in foodborne infection is the one caused by *Salmonella enterica* of the subspecies enterica.

Furthermore the *Salmonella* genus is further divided into different serotypes. More than 2500 serotypes which are also called serovars belong to the species of *Salmonella enterica* and about 20 belong to *Salmonella bongori*. *Salmonella enterica subspecies enterica* contains nearly another 1500 serotypes where most of them are known to cause foodborne diseases. In addition each *Salmonella* serotype can be further divided by phage typing. A particular phage type can be denoted using the term PT. For example, *Salmonella enteritidis PT4* is an organism commonly associated with eggs and human illness. The most common serotypes concerning human illnesses are *Salmonella Typhimurium* and *Salmonella Virchow*. [2]

Animals can become infected with *Salmonella* [2] from contaminated feed and also from the environment around them. Also many foods and products originating from animals such as meat, milk, poultry, eggs and raw milk can be contaminated with *Salmonella* and therefore transmitting the pathogen to humans. Some studies in Europe showed that the highest occurrence of *Salmonella* occur in Portugal, Poland and the Czech Republic whereas the lowest occur in Sweden and Luxembourg. [3]

Cooked, ready-to-eat products may be contaminated as a result of cross contamination from raw food through direct contact. Contamination can also occur via food preparation surfaces and

equipment used which was not appropriately cleaned before use. Milk powder, ice cream, cheese and chocolate are all examples of products tested and found positive with *Salmonella*.

The most frequent infection from *Salmonella species* is non-typhoid. These species invade the cells lining the small intestine causing diarrhea, abdominal pain, and nausea, vomiting which might lead to dehydration in humans and most of the animal species. Immunosuppressed organisms such as elderly people might show septicemia or even reactive arthritis. Many *Salmonella* infections in animals are asymptomatic and are passed unnoticed by the farmers.

In humans the incubation time of *Salmonella* is 6-48 hours [(3)]. The infective dose is thought to vary widely and depends on the type of food consumed. Generally between 100-110 *Salmonella* cells are needed to cause an infection. Individuals recovering from *Salmonella* infection sometimes shed *Salmonella* bacteria in their stools for some time.

Most *Salmonella* serotypes can grow between 7-48^{o}C although growth is reduced at temperatures below 10^{o}C but some bacteria might survive. Some studies showed that some serotypes can even grow at temperatures as low as 4^{o}C. *Salmonella species* are also able to survive the chilling temperatures of a refrigerator.

The optimum pH for *Salmonella* multiplication is 6.5-7.5 but some serotypes can grow at a range of 3.7-9.5 of the pH scale. The survival of *Salmonella* in that pH range is also directly affected by the temperature and the type of acids present. Chilled temperatures up to 4oC favor *Salmonella* survival rather than freezing temperatures which are below 4oC. *Salmonella species* are also able to grow in water and are resistant to dried materials meaning that they are able to survive in dried products such as milk powder, chocolate, milk replacers and animal feed although the numbers are greatly reduced.

The majorities of *Salmonella* serotypes are not particularly heat resistant and are usually inactivated by pasteurization procedures or equivalent heat procedures. D-values are typically between 1 and 10 minutes at 60^{o}C and less than a minute at 70^{o}C. The typical z/values are 4-5^{o}C [4]. Products with high fat content or with low water content reduce the effectiveness of heat or pasteurization procedures.

According to the EU laws about *Salmonella* a HACCP approach is essential for the control of *Salmonella* in food production and food products either raw or cooked. Using the HACCP

approach the control should firstly start from the farm and then move to the raw animal products such as eggs, meat and milk. *Salmonella* can be effectively controlled by relative mild heat processing such as during pasteurization but it's essential that adequate measures are applied to avoid any cross contamination between raw and cooked products during processing. [4] HACCP plans should be used to identify and implement adequate controls for *Salmonella* so that the absence of the bacteria is ensured in all foods. In the case that *Salmonella* detection is positive various areas should be reviewed and reformulated to remove the bacteria and minimize the risk of contamination depending at which stage the infection occurred, either from the raw, the cooked products or if there is a fault in the processing and manufacturing procedure. General hygienic principles and procedures should be applied as well as effective temperature controls are of at most importance to minimize contamination and risks of transition of the pathogens either to animals or humans.

European Union Legislation concerning *Salmonella*

Legal frame work

Commission Regulation [5] (EC) No 2073/2005 on microbiological criteria for foodstuffs

Amended by Commission Regulation (EC) No 1441/2007

Reg. (EC) No178/2002– General food law [6]

Important changes of Commission Regulation No 2073/2005 [5]

MAIN OBJECTIVES:

- To ensure a high level of human health protection
- Reduction of human Salmonellosis and Listeriosis
- To harmonize microbiological criteria (MC)
- Uniform rules for food business operators

Community laws (contain microbial criteria) were repealed in 2006.

Directive 92/46/EC — Milk, milk products

The detection of *Salmonella* on the dairy products was in accordance with the ISO 6579:2002 of the European Union concerning the microbiology of food and animal feeding stuffs- Horizontal method for the detection of *Salmonella species*.

Additional ISO used:

1. ISO 6887-1: Microbiology of food and animal feeding stuffs- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination- Part 1: General rules for the preparation of the initial suspension and decimal dilutions.
2. ISO 7218:1996: Microbiology of food and animal feeding stuffs- General rules for microbiological examinations
3. ISO 8261: Milk and milk products- General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination.

2.2. Listeria spp

Listeria is a group of bacteria that are responsible for the disease called Listeriosis [7]. The *Listeria* genus contains ten other species of *Listeria* occurring worldwide. All of them are Gram positive, rod shaped coccobacillus [7]. The most pathogenic of the genus is *Listeria monocytogenes* which typically causes severe complications in pregnant women and furthermore disturbances in the central nervous system in humans. *Listeria monocytogenes* is a facultative anaerobic bacterium able to grow at low temperatures and a wide pH range 4.3 - 9.6. Listeriosis can be caused by ingesting contaminated food such as milk and undercooked or raw meat products. *Listeria* monocytogenes can be further divided into another eleven serovars from which serovars 4b and 1/2a are the most pathogenic [8].

Listeria monocytogenes is responsible for almost all infections in humans although rare cases of infection due to *Listeria ivanovii* and *Listeria seellgeri* have been reported. In animals *Listeria monocytogenes* is responsible for the majority of infections but *Listeria ivanovii* and *Listeria innocua* infections have also been reported. *Listeria ivanovii* has been associated with abortions and has been reported to very occasionally cause meningoencephalitis in sheep. [7]

Listeria monocytogenes has a very wide host range including humans, ruminants, pets, fish, crustaceans and even insects. The importance of Listeriosis in humans is that *Listeria* can cause disturbances in pregnant women. During the third trimester of pregnancy infection with *Listeria*

may lead to spontaneous abortion, fetal death and premature delivery of the fetus. In the pregnant mother, the infection gives rise to flu-like symptoms including septicemia, diarrhea, back pains and it is generally passed unnoticed since most of the disturbances are caused on the fetus.

Listeriosis in adult humans may affect the central nervous system causing meningitis. Other symptoms of human Listeriosis include high fever, tremors, ataxia and seizures. Another form of Listeriosis is called febrile gastroenteritis which is caused by a non-invasive form of Listeriosis. This form of infection shows the typical signs of gastroenteritis such as fever, diarrhea and vomiting.

The main way of transmission of *Listeria monocytogenes* is through the ingestion of contaminated food, raw milk and other meat and milk products. It can also be transmitted from the mother to the fetus transplacentaly [7] during pregnancy or via the birth canal during delivery of the baby. *Listeria* can be also transmitted directly to veterinarians and farmers handling infected animals during parturition or other obstetrical examinations.

The clinical manifestation of Listeriosis in animals includes encephalitis, septicemia and abortion, especially in sheep, goats and cattle. The septicemia form is relatively uncommon and generally occurs in neonates. It is marked by depression, in appetence, fever and eventually death. The encephalic form is sometimes referred to as circling disease because of the tendency of the infected animal to circle in one direction and it is the most common manifestation of the disease in ruminants. These signs include depression, anorexia, head pressing or turning of the head to one side and unilateral facial paralysis. Abortion is usually at late term of pregnancy. Rarely mastitis of ruminants has been associated with *Listeria monocytogenes* infection. Gastrointestinal Listeriosis can occasionally occur in sheep in some cases. The least susceptible animal to Listeriosis is the pig which is the most resistant of the animals. In cases where Listeriosis was found in pigs the clinical signs were septicemia, encephalitis and rarely abortions. Although birds are usually subclinical carriers' sporadic cases of Listeriosis have been reported. Most frequently, septicemia and far less commonly, meningoencephalitis were observed in infected birds. Avian Listeriosis may be the result of a secondary infection in viral disease conditions and Salmonellosis [8]

Listeria is able to survive in the soil, manure from farms, decaying vegetable matter, silage, and water [7]. Important routes of transition are the water supply, fresh and frozen poultry, fresh and

processed meats, raw milk, cheese and slaughterhouse wastes. Nowadays regular checks for *Listeria* are conducted in animal feedstuffs to prevent the infection of animals since Listeriosis is zoonotic. Also as with the case of *Salmonella* specific HACCP principles are applied in the case of *Listeria* to prevent and minimize the spread of the bacteria in the farms and in the manufacturing procedures.

The approximate infective dose of *Listeria monocytogenes* is estimated to be 10-100 million colony forming units in healthy hosts [7]. This number is much less in immunosuppressed humans such as AIDS patients. The incubation period can vary depending on the mode of transmission and dose received. The typical incubation period is 1-4 weeks but sometimes it might even extend to several months. Febrile gastroenteritis has the shortest incubation period of typically 18-20 hours.

Regulations for *Listeria monocytogenes*:

Regulation (EC) No. 178/2002 that provides the framework for food and feed law within the EU [6].

Regulation (EC) 2073.2005 concerning the microbiological criteria for feedstuffs [5].

Listeria monocytogenes must be absent in ready to eat foods intended for consumption by infants. [8]

ISO used for the detection of *Listeria*:

1. ISO 11290-1: 1996, microbiology of food and animal feeding stuffs- horizontal method for the detection and enumeration of *Listeria monocytogenes* – part 1: Detection method Amendment 1: 15-10-2004
2. ISO 6687: 1983, microbiology – general guidance for the preparation of dilutions for microbiological examinations.
3. ISO 7218: 1996, microbiology of food and animal feeding stuffs – general rules for microbiological examinations.

2.3 Nutritional values of sheep and goat milk

The values in the table below correspond to the nutritional values of goat and sheep milk per 100 grams milk.[9]

Table 1.

Constituents	**Units**	**Sheep**	**Goat**
Water	Gr	83.0	88.9
Protein	Gr	5.4	3.1
Fat	Gr	6.0	3.5
Carbohydrate	Gr	5.1	4.4
Energy	Kcal	95	60
	kJ	396	253
Sugar (Lactose)	Gr	4.9	4.4
Fatty Acids			
Saturated	Gr	3.8	2.3
Mono-unsaturated	Gr	1.5	0.8
Polyunsaturated	Gr	0.3	0.1
Cholesterol	Mg	11	10
Calcium	IU	170	100

Table 1: Nutritional values of goat and sheep milk 100/gr milk

3. Materials and methods

All the data used in these experiments came from sheep and goats grown in Cyprus. In Cyprus East Friesian milk sheep are commonly used for milk production. This sheep breed originates from north East Germany and are able to produce a milk yield of 500-700kg milk per lactation period with 6-7% milk fat. This breed is also used for wool production yielding about 4.5kg wool per ewe. [10] Also some farmers use Lakon [11] sheep for milk production which is a France originating breed. This breed produces an average of 260 liters in the lactation period. In the case of goats the milk used in the experiments came from Damascus (shami) goats which originated from the Middle East. The average milk production of shami goats is between 350-650kg milk per goat per lactation period containing 3.8-4.5% milk fat and 4-4.8% protein content. [12]

Halloumi [13] which is a type of hard rubber like cheese with a milky salty taste traditionally made in Cyprus. Halloumi is traditionally made from sheep and goat milk but nowadays commercial halloumi is made from cow milk as well. Traditional halloumi is made from "Machera" goat milk and Fat-tailed sheep milk and different manufacturers produce halloumi with different consistency and milk proportions in their recipes. Machera goats is a type of goat breed endemic in Cyprus and are used for halloumi production because of the higher fat content of milk fat which makes the cheese easier to become hard. Anari is another type of white cheese but it is sold in two different types. One type is in fresh soft form and the other one is in dried up form and hardened either salted or non-salted. Apart from the difference in milk fat content between halloumi and anari, halloumi also contains some aromatic plants in the recipe such as mint giving it a different taste than anari. Since halloumi is not yet registered as a purely Cypriot product the official ingredient consistency does not yet exist but in recent agreements with the European Union halloumi must have more than 45% sheep or goat milk content. For the past couple of years the Cyprus government is trying to patentee Halloumi as a Cyprus product and active talks with the European Union are in process.

All the experiments and analysis of the milk, halloumi and anari for *Salmonella species* and *Listeria monocytogenes* were conducted at the State Laboratory for the Control of Food of Animal Origin (LCFAO) located at the Athalassa area in Nicosia, Cyprus and is responsible for the clinical examinations of foods from animal origin as well as animal feeds. This laboratory is under the control of the Cyprus State Veterinary Services also located at Athalassa area.

During the experiments, I was working with Mrs. Afroulla Scotti who is a microbiologist working at the State Laboratory for the Control of Food of Animal Origin. Mrs. Afroulla is responsible for all the samples arriving at the laboratory for the detection of *Salmonella species* and *Listeria monocytogenes*. The whole detection methods took 10 days to complete and the results to be finalized and published. The collection of the raw milk samples took place in 3 different farms around the Nicosia area.

Table 2.

Department of agricultural research – Athalassa	
Number of raw milk samples=5 (2*20ml)	
	Ear tag ID number
Sheep	209040936
	235304534
Goat	209063887
	209071199
	208130871

Table 2: Ear tag ID from sample farm 1

Table 3.

Lykourgos Ntortzis Farm – Tseri	
Number of raw milk samples=5 (2*20ml)	
	Ear tag ID number
Sheep	24676283
Goat	245090063
	240707352
	243469772
	24346972

Table 3: Ear tag ID from sample farm 2

Table 4

Demetris Demetriou Farm – Nicosia Airport Area	
Number of raw milk samples=5 (2*20ml)	
	Ear tag ID number
Sheep	245298169
	239263413
	239270875
	246607382
	233810651

Table 4: Ear tag ID from sample farm 3

The dairy products samples that were tested for the presence of *Salmonella species* and *Listeria monocytogenes* bacteria came directly by samples that the different produces have to regularly submit to the State Veterinary services for the testing of *Salmonella* and *Listeria* in order to have the official clearing permit that is needed for them in order to sell their products.

Table 5.

Giannakis Theofanous	Paphos	CYS 6301002	4kg of Halloumi samples	Samples taken at the 26/08/13
Petros Ioannides	Paphos	CYS 6331027	2kg Goat Halloumi samples	Samples taken at the 26/08/13
Giannakis & Zoiro Stephani LTD	Paxna	0014	5 pieces of Halloumi	Samples taken at the 26/08/13
Giannakis & Zoiro Stephani LTD	Paxna	0014	5 pieces of fresh anari	Samples taken at the 26/08/13

Table 5: Dairy samples to be tested

Before starting the experiment for the detection of *Salmonella* and *Listeria* I had to get various authorizations from the Cypriot government. To begin with, I had to submit an official written statement concerning the aim of the study and the type of experiment. This official document had to be forwarded to the Senior General Manager of the State Veterinary Service of Cyprus for his

approval. Dr. Andreas Papaeustathiou gave his approval for starting the experiments and subsequently I had to go to Dr. Arsenoglou who is the head of the Department of State Laboratory for the Control of Food from Animal Origin which is under the control of the State Veterinary Service for his approval as well. Once all of the paperwork was completed we could start with the procedures as instructed by Dr. Papaeustathiou and follow all the protocols that are related with the procedures and all the needed safety rules of the State Veterinary service. In addition I had to get a special insurance from the government that allowed me to follow the government officials at their farm visits and get the raw milk samples,

All milk and dairy products from sheep and goat came from animals that do not show any clinical signs of infectious diseases that might be zoonotic or infectious. All the animals were in good general state of health and the milk sampling was done in early morning during milking to avoid overstressing the animals. The milk samples were taken from two different teats from the same animal. We collected a total of 40 ml milk from each animal. We used 2x20 ml sterile test tubes and took each sample from different teat canals. Each animal was carefully examined so that none of them had any visible udder wounds present. Prior to milking we carefully cleaned the udder using disinfectant solutions based on betadine to avoid contamination of the samples with any bacteria and other microorganisms that might be present in the environment or on the surface of the udder. All animals were fit for human consumption, meaning that none of the animals were treated with any antibiotics and did not show any clinical signs of illness thus there was no withdrawal period. To avoid spoiling the milk in the intense heat that exists in Cyprus we took the samples early in the morning and immediately placed the samples in a water cooler with some ice packs to cool it to around 8^{o}C for transport back to the lab. All of the procedures mentioned before comply with the regulation No. 853/2004 of the European Parliament and of the Council of 29 April 2004 – “Laying down specific hygiene rules for food of animal origin”. All the animals were randomly selected from the herd and were not in a dry period. When all of the samples arrived at the State Laboratory for the Control of Food of Animal Origin (LCFAO) the two samples from each animal were mixed into one 40 ml sample.

The sample collecting took place on the 26/8/13 at around 7.00 in the morning. By 10.00 in the morning all the samples collected from all 3 farms were transported using coolers to the State Veterinary institute and placed in a refrigerator to keep them fresh. Once all of them were cooled to about 8^{o}C the samples from each farm were taken out of the refrigerator and the milk from the

2 test tubes from each animal mixed thoroughly to achieve homogeneity in the sample. In total 20 ml from each sample are needed for each examination of the raw milk from each animal. 10 ml will be used for the detection of *Salmonella species* and the other 10ml for the detection of *Listeria monocytogenes*.

3.1. Salmonella detection procedure

Using a pipette we transferred 10ml from each of the raw milk sample into plastic bags called stomacher bags. Each sample was then labeled with a sequential number so we could identify each sample. In total we had 17 raw milk samples for *Salmonella* including a positive control sample and a negative control to test if all of the experiments components were correctly used. In each stomacher bag we then added 90ml of Buffered Pentone Water so that the total volume of the sample is 100ml. In order to completely homogenize the samples we placed the bags in a machine called a stomacher. This machine is a rapidly vibrating and moving machine that mixes the contents of the bag. Each sample needed about 2 minutes to be completely homogenized. All of the samples were then collected and placed in a collecting cylinder and placed in the incubator. The incubation time for the first enrichment for *Salmonella* is 18+/- 2 hrs at 37ºC (ISO 6579:2002.Annex A). Buffered Pentone Water is composed from:

Table 6.

Enzymatic digest of casein	10.0gr
Sodium chloride	5.0gr
Disodium hydrogen phosphate dodecahytrate	9.0gr
Potassium dihydrogen phosphate	1.5gr
Water	1000ml

Table 6: Contents of Buffered Pentone Water

The Buffered Pentone Water used was prepared the same morning the samples were collected. This buffer solution was used in order for the *Salmonella* bacteria to grow and form colonies while in the incubator. For the preparation of the buffer solution all the components were dissolved in water using slight heating to assist the process. No further pH adjustment was needed because the pH of the solution was close to 7.0 at 25ºC temperature. The whole solution was sterilized for 15 minutes in an autoclave at 121ºC and thereafter was ready for use. (ISO 6579:2002, Annex B.1.1-1.2. Composition and preparation of culture media and reagents)

After 18 hours in the incubator we removed the samples from the incubator and we firstly extracted 0.1 ml of the culture and transferred them into 10ml of RVS broth (Rappaport-Vassiliades medium with soya). After the transfer we placed the samples back into the incubator

for another 24 +/- 3 hrs at 41.5ºC. Rappaport-Vassiliades medium with soya is composed of three different solutions and was prepared the day of use. Solution A of the RVS broth contains:

Table 7

Enzymatic digest of soya	5.0gr
Sodium chloride	8.0gr
Potassium dihydrogen phosphate	1.4gr
Dipotassium hydrogen phosphate	0.2gr
Water	1000ml

Table 7: Solution A of Rappaport – Vassiliades

All of the above mentioned components were dissolved in the water while heating it at around 70ºC. This first solution will be then added to the other two solutions to complete the RVS solution later on used for the second cultural enrichment. (ISO 6579:2002, Annex B.2.1.1-2.1.2. Composition and preparation of culture media and reagents).

Solution B of the Rappaport-Vassiliades medium with soya consists of the following components:

Table 8.

Magnesium chloride hexahydrate	400.0gr
Water	1000ml

Table 8: Solution B of Rappaport – Vassiliades

The magnesium chloride hexahydrate will be dissolved in the water at room temperature but since the magnesium chloride hexahydrate is very hygroscopic it is advisable to use salt from a newly opened container. The made solution can be kept for up to 2 years in a dark glass bottle with a tight stopper. This solution B will then mixed with solutions A and C to make the final Rappaport-Vassiliades solution used for the second part of the cultural enrichment of *Salmonella*.(ISO 6579:2002, Annex B.2.2.1-2.2.2. Composition and preparation of culture media and reagents).

The third and final solution making up the Rappaport-Vassiliades broth is made up of:

Table 9.

Malachite green oxalate	0.4gr
Water	100ml

Table 9: Solution C of Rappaport – Vassiliades

The malachite green oxalate is dissolved in the water at room temperature and the resulting solution is kept in a dark brown glass bottle at room temperature for at least 8 months. (ISO 6579:2002, Annex B.2.3.1-2.3.2. Composition and preparation of culture media and reagents).

Then the complete medium is made using different portions of the 3 solutions mixed together.

Table 10.

Solution A	1000ml
Solution B	100ml
Solution C	10ml

Table 10: Mixture of all solutions

After the addition of the mentioned ratios of the 3 solutions adjustment of the pH to 5.2 is needed. Sterilization at 115ºC for 15 minutes in an autoclave is also needed before storage of the solution until it is used. After the mixing of the three solutions the final ratios of all the ingredients are as follows:

Table 11.

Sodium chloride	7.2g/l
Potassium dihydrogen phosphate	13.4g/l
Magnesium chloride hexahydrate	28.6g/l
Enzymatic digest of soya	4.5g/l
Malachite green oxalate	0.036g/l

Table 11: Contents of final Rappaport - Vassiliades solution

We then divided the resulting RVS culture medium solution (green colored) into 10ml culture test tubes and transferred 0.1ml of the sample culture into it and placed them into the incubator at

41.5ºC for 24 +/- 3hrs.(ISO 6579:2002, Annex B.2.4.1-2.4.2. Composition and preparation of culture media and reagents)

From the incubated culture with Buffered Pentone Water we extracted another 1ml using a pipette and transferred it to 10ml of MKTTn solution (Muller – Kauffmann tetrathionate – novobiocin broth). This culture will be incubated for 24 +/- 3 hrs in an incubator at 37.1ºC. (ISO 6579:2002.Annex A).

The Muller – Kauffmann tetrathionate – novobiotin broth contains three solutions in it which were all made and used on the same day.

The first solution needed for the MKTTn base medium needed has the following components:

Table 12.

Meat extract	4.3gr
Enzymatic digest of casein	8.6gr
Sodium chloride	2.6gr
Calcium carbonate	38.7gr
Sodium thiosulfate pentahydrate	47.8gr
Ox bile for bacteriological use	4.78gr
Brilliant green	9.6mg
Water	1000ml

Table 12: First MKTTn solution contents

For the preparation of the medium we dissolved the dehydrated basic components in the water by boiling and adjusting the pH if necessary with appropriate buffer solution at 8.2+/- 0.2 at 25oC. This medium can be stored up to 4 weeks at low temperature of 2-3oC. (ISO 6579:2002, Annex B.3.1.1-3.1.2. Composition and preparation of culture media and reagents).

The second solution needed for the MKTTn broth medium we use iodine as the main component. The rest of the components include:

Table 13.

Iodine	20.0gr
Potassium iodide	25.0gr
Water	100ml

Table 13: Second MKTTn solution contents

We completely dissolved the potassium iodide in 10ml of the water ratio and then we added the iodine and thereafter we added the rest of the water. The resulting solution can be stored in a dark cupboard with a tightly closed container at room temperature. (ISO 6579:2002, Annex B.3.2.1-3.2.2. Composition and preparation of culture media and reagents).

The third and final solution needed for the preparation of the Muller – Kauffmann tetrathionate – novobiocin broth is the novobiocin solution. The components of this solution are as follows:

Table 14

Novobiocin sodium salt	0.04g
Water	5ml

Table 14: Third MKTTn solution contents

For the preparation of the novobiocin solution we had to dissolve the novobiocin sodium salt in the 5ml of water and sterilize the solution by filtration. The resulting solution could be stored for up to 4 weeks at 3^{o}C. (ISO 6579:2002, Annex B.3.3.1-3.3.2. Composition and preparation of culture media and reagents).

To prepare the complete Muller – Kauffmann tetrathionate – novobiocin broth we had to add appropriate ratios of the 3 solutions.

Table 15.

Base medium	1000ml
Iodine- iodide solution	20ml
Novobiocin solution	5ml

Table 15: Final solution contents of MKTTn

We then divided the resulting MKTTn solution (blue coloured) into glass test tubes. Each test tube contained about 20ml of the MKTTn medium and then placed in the incubator at 37.1^{o}C for another 24 +/- 3hrs.

For the preparation of the complete MKTTn broth we add 5ml of the novobiocin solution to the 1000ml of base medium and mix them thoroughly. Then we add to this solution the iodine-iodide solution and again mix them well to homogenize the solution. (ISO 6579:2002, Annex B. 4.3.2. Composition and preparation of culture media and reagents).

On Wednesday, 21st of August we removed the RVS and MKTTn culture mediums from the incubators. We firstly observed if we could see any color changes on the culture medium that might indicate the presence of *Salmonella* in any of the samples. The only samples were some discoloration occurred was the sample with the positive control colonies. The next step in the *Salmonella* detection method is the transfer of any resulting colonies from the RVS and MKTTn broths to petri dishes. Two types of growth media were used for the petri dish detection. The first one was the XLD agar (Xylose lysine deoxycholate agar, red colored) and the second culture media was the BGA agar (Brilliant green agar, red colored). For this procedure we used a steel wired loop to transfer a drop from each broth media onto each agar plate and use the loop to widely spread the solution in order to obtain as widespread as possible any resulting *Salmonella* colonies. In case of positive detection of *Salmonella* the XLD agar changes color from red to black. All the *Salmonella* colonies that might be present produce black colored characteristic rounded colonies. In the case of the BGA agar any resulting positive result, produce pinkish colonies surrounded by a reddish ring. (ISO 6579:2002.Annex A). Using the wire loop we transferred a drop from each sample from the MKTTn broth onto two separate petri dishes and the same thing was also done for the RVS culture medium. One petri dish contained XLD agar and the other one contained BGA agar.

Therefore, in total we had 92 samples. In order to avoid any confusion below is an explanation and a table illustrating the number of samples along with their additives; meaning the drop of suspected *Salmonella* cultures.

Table 16.

	MKTTn	RVS	
	19 petri dishes containing BGA with a drop of MKTTn culture	19 petri dishes containing BGA with a drop of RVS culture	
	19 petri dishes containing XLD with a drop of MKTTn culture	19 petri dishes containing XLD with a drop of RVS culture	
	2 positive controls for BGA with a drop of MKTTn culture	2 positive controls for BGA with a drop of MKTTn culture	
	2 negative controls for BGA with a drop of MKTTn culture	2 negative controls for BGA with a drop of RVS culture	
	2 positive controls for XLD with a drop of MKTTn culture	2 positive controls for BGA with a drop of RVS culture	
	2 negative controls for XLD with a drop of MKTTn culture	2 negative controls for BGA with a drop of RVS culture	
Total number of samples	46	46	92

Table 16: Samples for MKTTn and RVS in details

For the MKTTn culture:

One drop from each of the 19 samples from the MKTTn original sample cultures were added to 19 petri dishes containing BGA giving us 19 samples, one drop from each of the MKTTn original sample cultures were added to 19 petri dishes containing XLD giving us another 19 samples, 2 samples of positive control transferred to BGA containing petri dishes, and 2 samples of negative control transferred to BGA containing petri dishes,2 samples of positive control transferred to XLD containing petri dishes, and 2 samples of negative control transferred to XLD containing petri dishes, altogether constituting 46 samples.

For the RVS culture:

One drop from each of the 19 samples from the RVS original sample cultures were added to 19 petri dishes containing BGA giving us 19 samples, one drop from each of the RVS original

sample cultures were added to 19 petri dishes containing XLD giving us another 19 samples, 2 samples of positive control transferred to BGA containing petri dishes, and 2 samples of negative control transferred to BGA containing petri dishes,2 samples of positive control transferred to XLD containing petri dishes, and 2 samples of negative control transferred to XLD containing petri dishes. Altogether 46 samples.

In the end we had 92 samples, which were then placed in the incubator for 24 +/-3hrs at 37ºC. (ISO 6579:2002.Annex A. 9.5.2. Microbiology of food and animal stuffs- Horizontal method for the detection of *Salmonella* spp.)

For the conformation of the presence of *Salmonella* we use 2 different agar media. The first on as I mentioned is the XLD culture agar. Xylose Lysine Deoxycholate agar contains the following ingredients for its preparation

Table 17.

Yeast extract powder	3.0gr
Sodium chloride	5.0gr
Xylose	3.75gr
Lactose	7.5gr
Sucrose	7.5gr
L-Lysine hydrochloride	5.0gr
Sodium thiosulfate	6.9gr
Iron(III) ammonium citrate	0.8gr
Phenol red	0.08gr
Sodium deoxycholate	1.0gr
Agar	9-18gr
Water	1000ml

Table 17: Contents for XLD

In order to prepare the XLD agar we had to dissolve the dehydrated base components such as the yeast in the water. To assist the procedure slight heating was required as well as frequent stirring of the mixture. We allowed the water to boil but keeping in mind the temperature so that the water solution did not overheat. Appropriate buffers might be used to adjust the required pH to

7.4 +/-0.2 at 25ºC. We continue the heating process until the whole medium boils and the agar dissolves completely. Then we immediately pure the XLD agar gel into the petri dishes while in a water bath at 44-47ºC and then we allow the agar gel to solidify. The newly prepared agar plates can be stored for up to 5 days after preparation when kept at 3ºC. (ISO 6579:2002, Annex B. 4.1.1- B.4.2 Composition and preparation of culture media and reagents).

For the preparation of the Brilliant Green Agar we used the following ingredients.[14]

Table 18.

Enzymatic Digest of Animal Tissue	5.0gr
Peptone	10.0gr
Yeast Extract	3.0gr
Lactose	10.0gr
Sucrose	10.0gr
Sodium phosphate, dibasic	1.0gr
Sodium phosphate, monobasic	0.6gr
Brilliant green	0.0047gr
Phenol red	0.09gr
Agar	12.0gr
Water	1000ml

Table 18: Contents of BGA

In order to prepare the BGA agar we dissolved the enzymatic digest of the animal tissue into the water using heat and frequent agitation of the mixture. We allowed the mixture to boil avoiding overheating until all of the ingredients were completely dissolved. Then we poured the medium into petri dished and allowed it to solidify at 25ºC. Special precautions were taken such as protective glasses, and the use of a fume cabinet to prevent eye irritation as the mixture in question is known to be an eye irritant as well as skin and respiratory tract irritant. (Anon. 1981, International Organisation for Standardization, Microbiology- General guidance on methods for the detection of *Salmonella*. Ref. method, ISO 6579-1981)

After the 24hrs incubation period, the petri dishes containing the BGA and the XLD agar mediums are removed from the incubator and are observed for any *Salmonella* colonies. In the

case of negative results the other four marked colonies should be examined for a definite negative confirmation. In the case of positive results from the agar mediums the colonies using a sterile wire loop should be transferred onto another nutrient agar medium and incubated for another 24 +/-3hrs at 37ºC. For the confirmation we take each of the dishes of each selective medium and take at least one colony which is considered to be the typical *Salmonella* colony and transfer the colonies onto the surface of pre dried nutrient agar plates. The colonies should be spread in such a way that they will be well isolated and allow space for the colonies to develop. The nutrient agar plates will then be incubated for 24 +/-3hrs at 37ºC. The colonies that are well developed on the nutrient agar will be then used for biochemical and serological confirmation for the presence of *Salmonella*.(ISO 6579:2002.Annex A. 9.5.2. Microbiology of food and animal stuffs- Horizontal method for the detection of *Salmonella spp*)

At this point as our results came out to be negative, we reached the end of our experimentation and testing phase. However, the standard procedure for the detection and identification of *Salmonella* is to be discussed in the following pages.

The resulting isolated colonies of *Salmonella* are then transferred to nutrient agar petri dishes and incubated. Thereafter the resulting colonies will be used for biochemical and serological identification methods in order to correctly identify the species of the *Salmonella*. For the preparation of the nutrient agar we use the following ingredients and procedures.

Table 19.

Meat extract	3.0gr
Peptone	5.0gr
Agar	9-18.0gr
Water	1000ml

Table 19: Composition of nutrient agar

During the preparation of the nutrient agar we dissolve all of the components of the medium into the water using heat if necessary to assist the procedure. After the complete dissolvement of the components pH adjustment with appropriate buffers might be needed to reach the necessary pH of 7.0 +/- 0.2 at 25ºC. Then we transfer the culture medium into test tubes or small bottles and sterilize the medium using an autoclave for 15 minutes at 121ºC. Each petri dish contains

approximately 15ml of the sterile melted nutrient agar gel. The nutrient agar with the resulting colonies is incubated for 24 +/-3hrs at 37 +/- 1ºC. (ISO 6579:2002, Annex B. 5.1.- B.5.3 Composition and preparation of culture media and reagents).

3.1.1 For the biochemical confirmation of Salmonella:

For the biochemical confirmation the recommended tests are the:

1) TSI agar test,
2) Urea agar test,
3) L-Lysine decarboxylation medium test,
4) Detection of β- galactosidase, Medium for Voges – Proskauer (VP) reaction and
5) Medium for indole reaction.

The first test that can be used is the TSI agar test. For this test we transfer the colonies from the nutrient agar gel onto the TSI (Triple Sugar Iron Medium) agar using a steel transfer loop. Using the wire loop we swipe the colonies onto the agar slant surface and then press the wire loop deep into the gel to reach the bottom. The TSI agar gel is then incubated in an incubator for 24 +/-3hrs at 37ºC. The Triple sugar iron agar medium contains the following components:

Table 20.

TSI components	Meat extract	3.0gr
	Yeast extract	3.0gr
	Peptone	20.0gr
	Sodium chloride	5.0gr
	Lactose	10.0gr
	Sucrose	10.0gr
	Glucose	1.0gr
	Iron (III) citrate	0.3gr
	Sodium thiosulfate	0.3gr
	Phenol red	0.024gr
	Agar	9-18gr
	Water	1000ml

Table 20: TSI components

For the preparation if the TSI agar (originally red in color) we have to dissolve all of the components into the water and heat if necessary. When all of the ingredients have dissolved into the water a slight adjustment to the pH might be needed using an appropriate buffer to reach the desired pH of 7.4 +/- 0.2 at 25ºC. Then we pour the mixture into petri dishes or test tubes containing approximately 15ml of the agar gel. Using an autoclave we sterilize the petri dishes for 15 minutes at 121ºC. After sterilization we allow the gel to set in a sloping position to give a butt of depth of 2.5cm to about 5cm. (ISO 6579:2002, Annex B. 6.1- B.6.2 Composition and preparation of culture media and reagents).

The interpretation of the changes in the TSI agar gel test is the following:

- *Butt:*
    1. If the color observed turns yellow then the glucose in the gel were used therefore glucose positive.
    2. If the color observed remains red then the glucose in the gel was not used therefore glucose negative.
    3. If the color observed turns to black therefore formation of hydrogen sulfide.
    4. If bubbles or cracks are observed in the TSI agar gel is seen then gas bubbles were produced from the glucose in the gel.
- *Slant surface:*
    1. If the color observed turned yellow then lactose and/or sucrose was used therefore lactose and/or sucrose positive.
    2. If the color remained red then the test is lactose and/or sucrose negative thus meaning that lactose and/or sucrose were not used.

In the TSI butt test *Salmonella* show alkaline (red) slants and acid (yellow) butts with gas formation in most of the cases. Also *Salmonella* produces hydrogen sulfide which is responsible for the black color of the TSI agar in case of positive results. In the TSI slant test *Salmonella* turns the color to yellow giving a positive result for lactose and/or sucrose used. (ISO 6579:2002.Annex A. 9.5.3.1. Microbiology of food and animal stuffs- Horizontal method for the detection of *Salmonella spp*)

The second test that can be used for the biochemical verification of the presence of *Salmonella* is the urea test. For this test two different solution mixtures are used and then mixed together into a complete medium. The first solution is the base medium and the second solution is the actual urea solution. The first base medium contains the following:

Table 21.

Peptone	1.0gr
Glucose	1.0gr
Sodium chloride	5.0gr
Potassium dihydrogen phosphate	2.0gr
Phenol red	0.012gr
Agar	9-18gr
Water	1000ml

Table 21: Base medium for the urea solution

In order to prepare the base medium we dissolve all of the different components into the water using slight heat if necessary. When all the ingredients have dissolved a pH adjustment might be needed to reach the desired pH of 6.8 +/- 0.2 at 25^{o}C. Then we sterilize the medium using an autoclave at 121^{o}C for 15 minutes. (ISO 6579:2002, Annex B.7.1.1- B.7.1.2. Composition and preparation of culture media and reagents).

The second solution called the urea solution contains the following:

Table 22.

Urea	400gr
Water to a final volume of	1000ml

Table 22: Urea solution

To make the solution we dissolve the urea into the water and filter the solution to remove any dissolved particles and sterilize. Then the two solutions are mixed together to form the urea agar solution which is red in color initially. For the final medium we add 50ml of the urea solution into 950ml of the base medium under aseptic conditions. Then we divide the medium into sterile

tubes each containing approximately 10ml. (ISO 6579:2002, Annex B. 7.3.1- B.7.3.2 Composition and preparation of culture media and reagents).

For the urea test we use a steel wire loop and transfer some of the colonies formed on the nutrient agar gel onto the surface of the urea gel. Using the wire loop and swiping motion we transfer the colonies on the slant surface of the urea agar and then incubate the samples at 37^{o}C for 24 +/- 3 hrs in an incubator and periodically check the samples to observe any changes in the appearance. In the presence of *Salmonella* the test is positive and *Salmonella* spits the urea liberating ammonia which causes a change in color from red, which is the initial color of the test, to rose red and later on to deep cherry red color. In the case of urea positive test the results may start to show quite early meaning from 2 hrs and onwards.

The third test for the biochemical verification of *Salmonella* is the L-Lysine decarboxylation medium test. The initial color of the L-Lysine decarboxylate is purplish red. In case of positive reaction with *Salmonella* species the color changes to purple and the medium turns turbid. In case of negative results for *Salmonella* the color changes to yellow. The components of the L-Lysine decarboxylate are the following. (ISO 6579:2002.Annex A. 9.5.3.4. Microbiology of food and animal stuffs- Horizontal method for the detection of *Salmonella spp*)

Table 23.

L-Lysine monohydrochloride	5.0gr
Yeast extract	3.0gr
Glucose	1.0gr
Bromocresol purple	0.015gr
Water	1000ml

Table 23: L-Lysine decarboxylate components

For the preparation of the L-Lysine decarboxylase medium we add the components to the water until they are dissolved. Heating may be necessary to completely dissolve the components. After the complete dissolvement of the components some adjustments to the pH might be needed to reach the desired pH of 6.8 +/- 0.2 at 25^{o}C. Then the medium is transferred to test tubes by pouring 2-5 ml in each test tube which is then sealed with screw caps. Then sterilize the tubes in

an autoclave for 15 minutes at 121ºC. (ISO 6579:2002, Annex B.8.1- B.8.2 Composition and preparation of culture media and reagents).

The fourth test that can be used for the biochemical verification of *Salmonella* is the β-galactosidase detection test. The actual reagent is composed of two different solutions which are then mixed together to form the complete reagent. The original color of the reagent is colorless and in the case of positive result the color changes to yellow. The first solution used is the buffer solution and is consisting of the following ingredients.

Table 24.

Sodium dihydrogen phosphate	6.9gr
Sodium hydroxide, 10mol/l solution	3ml
Water, to a final volume of	50ml

Table 24: Buffer solution for the β- galactosidase reagent

For the preparation of this buffer solution we have to dissolve the sodium dihydrogen phosphate into 45ml of the water and then we adjust the pH using an appropriate buffer to 7.0 +/- 0.2 at 25ºC using the sodium hydroxide solution. Then the rest of the water is added to reach 50ml of volume. (ISO 6579:2002, Annex B.9.1.1- B.9.1.2 Composition and preparation of culture media and reagents). The second solution used in the β- galactosidase detection test is the actual ONPG solution. The ONPG solution consists of:

Table 25.

o-nitrophenyl β-D-galactopyranoside (ONPG)	0.08gr
Water	15ml

Table 25: ONPG solution components

For the preparation of the ONPG solution we simply dissolve the ONPG in the water at approximately 50ºC. Then we allow the solution to cool down and then we mix the two solutions to form the complete reagent. For the complete reagent we use 5ml of the buffer solution and 15ml of the ONPG solution and mix them together. (ISO 6579:2002, Annex B.9.2- B.9.3.2 Composition and preparation of culture media and reagents).

For the test we use transfer the *Salmonella* colony into a tube containing 0.25ml of saline solution and then add one drop of toluene and shake the tube. Then place the test tube in a water bath at 37ºC for a few minutes and then add 0.25ml of the β-galactosidase reagent and mix it. After thorough mixing of the solution plate the test tube back into the water bath for 24 +/- 3 hrs and examining the test tube periodically because the change in color might occur from the first 20 minutes of the reaction. For a positive result for the detection of *Salmonella* the color changes from colorless to yellow proving the presence of *Salmonella*. (ISO 6579:2002.Annex A. 9.5.3.5. Microbiology of food and animal stuffs- Horizontal method for the detection of *Salmonella spp*)

The fifth test that can be used for the detection of *Salmonella* using biochemical reactions is the use of the Voges- Proskauer (VP) reaction. The Voges- Proskauer reaction is composed of four solutions mixed together to form the complete reagent. The first solution is called the VP medium, the second is the creatine solution (N-amidinosarcosine), the third solution is the 1-Naphthol, ethanolic solution and finally the fifth solution is called the potassium hydroxide solution. The first component of the Voges- Proskauer reagent is the VP medium as I mentioned before and is composed of the following ingredients.

Table 26.

Peptone	7.0gr
Glucose	5.0gr
Dipotassium hydrogen phosphate	5.0gr
Water	1000ml

Table 26: VP medium components

For the preparation of the VP medium we have to dissolve all of the components in the water where slight heating might be necessary to assist the dissolvment. After the complete dissolve of all of the ingredients in the water a slight adjustment to the pH might be needed to reach the desired pH of 6.9 +/- 0.2 at 25ºC. After all of the adjustments approximately 3ml of the medium is transferred to test tubes and sterilized in an autoclave at 121ºC for 15 minutes. (ISO 6579:2002, Annex B.10.1.2 Composition and preparation of culture media and reagents).

The second solution making the Voges – Proskauer reagent is the Creatine solution which is only made up from 0.5gr of Creatine monohydrate and 100ml of water. For the preparation of the creatine solution we completely dissolve the creatine monohydrate into the water. (ISO 6579:2002, Annex B.10.1-B.10.2.2 Composition and preparation of culture media and reagents).

The third solution making up the Voges – Proskauer reagent is the 1- Naphthol, ethanolic solution. This solution is only made up from 6gr of 1- Naphthol which is dissolved into 100ml of 96% Ethanol. (ISO 6579:2002, Annex B.10.3.1- B.10.3.2 Composition and preparation of culture media and reagents).

The fourth and final solution of the Voges – Proskauer reagent is the Potassium hydroxide solution which contains only 40gr of Potassium hydroxide completely dissolved into 100ml of water. (ISO 6579:2002, Annex B.10.4.1- B.10.4.2 Composition and preparation of culture media and reagents).

After the preparation of all the components of the Voges – Proskauer reagent medium we transfer with a wire loop some of the suspected *Salmonella* colonies into a sterile test tube which contains the 3ml of the VP medium and incubate it for 24 +/- 3hrs at 37^{o}C. After the incubation period we add two drops of the creatine solution into the tube and followed by three drops of the 1- Naphthol, ethanolic solution. After that we add two drops of the final solution which is the Potassium hydroxide solution. After each addition of each of the solutions we have to shake the test tube to completely mix the reagents together. A positive reaction is indicated by a change in color from yellowish to reddish color within 15 minutes from the start of the reaction. (ISO 6579:2002.Annex A. 9.5.3.6. Microbiology of food and animal stuffs- Horizontal method for the detection of *Salmonella spp*)

The sixth and final biochemical verification test for *Salmonella* is the Indole reaction. The Indole reaction is composed of two separate reactions. The first is the Tryptone/tryptophan medium and the second one is the Kovacs reagent. The Tryptone/tryptophane medium is composed of the following ingredients.

Table 27.

Tryptone	10.0gr
Sodium chloride	5.0gr
DL-Tryptophan	1.0gr
Water	1000ml

Table 27: Tryptone/ Trypophane medium components

For the preparation of the medium we have to dissolve the components into boiling water and adjust the pH if it is necessary by using an appropriate buffer to reach the desired pH of 7.5 +/- 0.2 at 25ºC and then disperse approximately 5ml of the medium into separate test tubes and sterilize the tube in an autoclave for 15 minutes at 121ºC. (ISO 6579:2002, Annex B.11.1.1- B.11.1.2 Composition and preparation of culture media and reagents).

The second component of the Indole reagent is the Kovacs reagent and is composed of the following ingredients.

Table 28.

4- Dimethylaminobenzaldehyde	5.0gr
Hydrochloric acid	25.0ml
2-Methylbutan-2-oi	75.0ml

Table 28: Kovacs reagent components

For the complete preparation of the Kovacs reagent we have to mix all of the ingredients into the 2-Methylbutan-2-oi. After the complete dissolvement of all of the components the reagent is ready to be used. (ISO 6579:2002, Annex B.11.2.1- B.11.2.2 Composition and preparation of culture media and reagents).

For the Indole reaction we transfer some of the suspected colonies of *Salmonella* from the nutrient agar culture into the test tubes that contain approximately 5ml of the Tryptone/tryptophane medium and incubate them for 24 +/- 3hrs in an incubator at 37ºC. After the end of the incubation period we add one drop of the Kovacs reagent to the test tube. A positive result proving the presence of *Salmonella* is seen as a red ring on the top of the medium.

A negative result is represented as a yellowish brown ring formation. (ISO 6579:2002.Annex A. 9.5.3.7. Microbiology of food and animal stuffs- Horizontal method for the detection of *Salmonella spp*)

Furthermore some serological test can be performed to verify the presence of *Salmonella* and differentiate between the different antigens of *Salmonella*. *Salmonella* has three types of antigens. O-, Vi- and H- antigens and these can be tested by slide agglutination tests with the appropriate sera from positive pure colonies and after auto agglutinable strains that have been eliminated. . (ISO 6579:2002.Annex A. 9.5.4.1. Microbiology of food and animal stuffs- Horizontal method for the detection of *Salmonella spp*)

For the elimination of auto agglutinable strains we can use one drop of saline solution dropped onto a clean glass slide and using a wire loop spread the drop on the slide to homogenously cover the slide. Then we take a part of the suspected colony and mix it well with the saline drop. Then by means of shaking the slide for one minute to mix the saline with the colonies we observe the slide under a dark ground microscope. During observation under the microscope we focus on the colonies and see if the bacteria have clumped together into more or less distinct units, if they did then the strain is considered as auto agglutinable thus no further testing is needed because the antigen detection is not possible. (ISO 6579:2002.Annex A. 9.5.4.2. Microbiology of food and animal stuffs- Horizontal method for the detection of *Salmonella spp*)

All the above biochemical tests are performed in order to accurately determine the *Salmonella species* present in the samples. For the definite diagnosis of the specific *Salmonella species* all the test have to be combined to reach the precise identification of the species. The following table illustrates how we can determine the *Salmonella* species accurately.

Table 29

Salmonella Strain										
Test	*Salmonella Typhi*		*Salmonella Paratyphi A.*		*Salmonella Paratyphi B.*		*Salmonella Paratyphi C.*		Other Strains	
	Reaction	%	Reaction	%	Reaction	%	Reaction	%	Reaction	%
TSI acid from glucose	+	100	+	100	+		+		+	100
TSI gas from glucose	-	0	+	100	+		+		+	92
TSI acid from lactose	-	2	-	100	-		-		-	1
TSI acid from sucrose	-	0	-	0	-		-		-	1
TSI hydrogen sulfide produced	+	97	-	10	+		+		+	92
Urea hydrolysis	-	0	-	0	-		-		-	1
Lysine decarboxylation	+	98	-	0	+		+		+	95
β–Galactosidase reaction	-	0	-	0	-		-		-	2
Voges–Proskauer reaction	-	0	-	0	-		-		-	0
Production of indole	-	0	-	0	-		-		-	1

Table 29: Salmonella strain identification

Furthermore in order to detect the specific antigens from each of the *Salmonella species* we have to perform the antigen tests. In the *Salmonella species* we have three different types of antigens that can be detected using serological examinations. The three antigen types are the O- antigen, the Vi- antigen and finally the H- antigen. For the detection of the antigens the procedure is the same more or less. For the detection of the O- antigen we use one non-autoagglutinating pure colony and by using one drop of the anti-O serum and a drop of saline solution we observe agglutination. If the saline droplet and the anti-O serum agglutinate then the reaction is positive and the colony has the O- antigen. The same procedure is done for the Vi- antigen with the only difference that the anti-Vi serum is used instead of the anti-O serum. Again if the saline drop

containing the anti-Vi serum agglutinates then the reaction is positive proving the existence of the Vi- antigen in the specific *Salmonella species* tested. For the detection of the H-antigen the procedure is a bit different. For this detection we have to inoculate the semi-solid nutrient agar gel with a pure non-autoagglutinable colony and incubate the medium in an incubator for 24+/- 3hrs at 37ºC. After the incubation period we use this culture for the examination of the H-antigen. Using the same technique as above meaning using a non-autoagglutinating colony we add only one drop of anti-H serum to the colony instead of using an additional drop of saline solution as before. If the reaction agglutinates then the reaction is considered to be positive for the presence of H-antigen in the suspected *Salmonella* colony. (ISO 6579:2002.Annex A. 9.5.4.3.- 9.5.4.5 Microbiology of food and animal stuffs- Horizontal method for the detection of *Salmonella spp*).

All of the above methods for the detection of *Salmonella spp* are performed in accordance with the ISO 6579:2002 directive from the European Union. Apart from this method we also used the MSRV (Rappaport Vassiliades Medium Semisolid Modified) technique. [15] This detection method is mainly used for the detection of *Salmonella* from feces samples [16] at the moment. In search of a universal method for detection of *Salmonella* from fecal and raw samples the European Union is trying to use this method as a universal detection method. At the moment this detection method is only a draft and is scheduled to be an official method by the year 2015 in order to make the detection easier and less time consuming. This method is easier to use and less time consuming for the microbiologist performing the tests because there is no need to use two different detection methods for the samples coming from feces and from raw milk. The initial color of the MRSV medium is blue. In the case of a test being positive, [17] for the presence of *Salmonella* the color of the medium changes to light blue with a turbid whitish zone [18] around the suspected culture droplets.

The MSRV medium also uses Novobiotin for the rapid detection of motile *Salmonella* species as it is found in the RVS medium used in ISO 6579:2002. For this method the first steps of enrichment of the samples with BPW is the same as described above. The incubation period with the Buffered Peptone Water is 18 +/- 2 hours at 37ºC. After the incubation period we use a pipette and draw 1ml from the sample containing the BPW. Using the pipette we transfer the 1ml of the sample onto the petri dish containing the MSRV medium dividing the sample into 3

droplets on the surface of the plate. Then we incubate the cultures at 41°C using an incubator for 24 +/- 3hrs. The next day we remove the cultures from the incubator and observe the cultures for any color changes. Using a sterile wire loop we transfer a drop from the edge of the colonies to a petri dish containing XLD medium. From there on, the procedure is the same for both detection methods. The advantage of this method apart from harmonizing both detection methods is that any color change on the MSRV culture medium can be show suspicion of the presence of *Salmonella* in the sample tested. For the sample to be suspected for the presence of *Salmonella* a grayish white turbid zone around the sample droplet has to be formed. [19] If the color of the MSRV medium remains bluish then the sample can be seen as negative for the presence of *Salmonella* but further test have to be performed to reach a definite result. That's why the rest of the detection method is the same as the one used before in order to determine the *Salmonella species* and the *Salmonella* antigen in the sample. This test is especially sensitive in the detection of motile [20] *Salmonella species* and less sensitive to immotile *Salmonellas*.

The MSRV culture medium used contains the following contents. For the preparation of the culture medium special precautions must be taken into consideration because the preparation procedure might be potentially irritating to the eyes, the respiratory system and the skin. For this reason the preparation procedure is done under a fume cupboard and the use of protective glasses and gloves is strongly recommended.

Table 30

Enzymatic Digest of Casein	4.59gr
Casein Acid Hydrolysate	4.59gr
Sodium Chloride	7.34gr
Potassium Dihydrogen Phosphate	1.47gr
Magnesium Chloride, anhydrous	10.93gr
Malachite Green Oxalate	0.037gr
Agar	2.7gr
Novobiocin	20mg
Water	1000ml

Table 30: MSRV Ingredients

For the preparation we dissolve the ingredients except the Novobiotin into the water and heat slightly to assist the complete dissolve of the materials used. Stirring of the solution might be needed to assist the procedure. When all of the components are dissolved into the water we allow the solution to cool to approximately 40ºC and then we add the Novobiotin into the mixture and mix well. Slight adjustment to the pH might be needed to reach the preferred pH of 5.6 +/- 0.2 at 25ºC. Then we can divide the medium into petri dishes and allow them to cool down and solidify. The color of the medium is bluish due to the presence of the Malachite Green Oxalate which acts as a color indicator.[16]

3.2. Listeria detection procedure

For the detection of *Listeria monocytogenes* we used the same raw milk and dairy products that we used for the detection of *Salmonella*. The detection of *Listeria monocytogenes* was done parallel with the detection procedure for *Salmonella* and also took one week for the results to be published. For the detection of *Listeria monocytogenes* we used the ISO 11290-1:1996. (Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes*) given by the European Union.

Using 10ml from the original raw sample and 10gr from the dairy products samples we add 90ml of Buffer Peptone Water and pour them into a stomacher bag as we did for the *Salmonella* detection and place the bags for each of the samples into the stomacher machine. Using this machine which vibrated and mixes the samples completely we transfer 10ml of the contents to the primary enrichment medium called Half Fraser broth. In this we add 90ml of the Half Fraser broth plus Acriflavine and Nalidixic acid. The following ingredients and procedures were used to prepare the Half Fraser broth with the Acriflavine and the Nalidixic acid to form the complete medium used. The whole procedure is divided into six different stages for the preparation of the complete medium used for the primary enrichment. We have to incubate the Half Fraser broth medium containing the sample in an incubator for 24 +/- 3hrs at 30ºC.

The six solutions that make up the complete Half Fraser broth medium used are:

1) Base medium
2) Lithium chloride solution
3) Sodium salt of nalidixic acid solution
4) Acriflavine hydrochloride solution
5) Ammonium iron (III) citrate solution
6) Final complete medium solution

The first thing to prepare for the Half Fraser broth is the base medium. For this base medium the following ingredients and procedure is used as indicated in the ISO 11290-1:1996.

Table 31

Meat peptone (peptic digest of animal tissue)	5.0gr
Tryptone (peptic digest of casein)	5.0gr
Beef extract	5.0gr
Yeast extract	5.0gr
Sodium chloride	20.0gr
Disodium hydrogen phosphate dehydrate	12.0gr
Potassium dihydrogen phosphate	1.35gr
Aesculin	1.0gr
Water	1000ml

Table 31: Ingredients of Base medium used in the Half Fraser broth

For the preparation we have to dissolve the base components in the water. If needed a slight heating might be used to assist the procedure. When all of the ingredients are completely dissolved a slight adjustment in the pH might be needed using an appropriate buffer to reach the desired pH of 7.2 +/- 0.2 at 25ºC. Then we can separate the base medium into smaller test tubes containing approximately 20ml of the medium where the lithium and the nalidixic acid solution will later be added into it and sterilize the tubes using an autoclave at 121ºC. (ISO:11290-1:1996, Annex B: B.1.1.1-B.1.1.2: Composition and preparation of culture media and reagents).

The second solution to be made for the complete Half Fraser broth media is the Lithium chloride solution which after its completion it is going to be added to the base medium. Special precautions must be used for the preparation of this solution because lithium chloride when added to the water produces a lot of heat since the reaction with water is very exothermic. Also the whole procedure has to be done under a fume cupboard because the solution might be irritating to the mucus membranes of the nose, mouth, eyes and skin surface. The lithium chloride solution is a simple solution made up just from 3grams of Lithium chloride which is dissolved into 10milliliters of distilled water. (ISO:11290-1:1996, Annex B: B.1.2.1-B.1.2.2: Composition and preparation of culture media and reagents).

The third solution in line to be prepared is the Sodium salt of nalidixic acid solution. Again this is a very simple solution to be prepared since it is only composed of 0.1 grams of Sodium salt of nalidixic acid dissolved into 10 milliliters of Sodium hydroxide containing 0.05 mol/ solution. When we dissolve the two components we have to use a filtration flask to remove any undissolved particles. (ISO:11290-1:1996, Annex B: B.1.3.1-B.1.3.2: Composition and preparation of culture media and reagents).

The fourth solution to be prepared for the Half Fraser broth medium for the detection of *Listeria monocytogenes* is the Acriflavine hydrochloride solution. For this solution we add 0.25 grams of Acriflavine hydrochloride powder into 100 milliliters of distilled water and mix them thoroughly until the acriflavine is completely dissolved into the water and then filter the solution in order to remove any undissolved acriflavine particles. (ISO:11290-1:1996, Annex B: B.1.4.1-B.1.4.2: Composition and preparation of culture media and reagents).

The fifth and final component of the Half Fraser broth medium is the Ammonium iron (III) citrate solution. This last solution is composed of just 5.0 gram of Ammonium iron (III) citrate powder dissolved into 100 milliliters of distilled water. After we have dissolved the Ammonium iron (III) citrate into the water we filter the solution to remove any resulting undissolved Ammonium particles. (ISO:11290-1:1996, Annex B: B.1.5.1-B.1.5.2: Composition and preparation of culture media and reagents).

After we have prepared all of the solutions that make up the Half Fraser broth complete medium we have to mix all of the ingredients together to form the complete medium. The final medium solution is composed of the following proportions of the previously mentioned ingredients and the ingredients are mixed right before use of the medium as previously mentioned into test tubes. (ISO:11290-1:1996, Annex B: B.1.6.1-B.1.6.2: Composition and preparation of culture media and reagents).

Table 32

Base medium	100ml
Lithium chloride solution	1.0ml
Sodium salt of nalidixic acid	0.1ml
Acriflavine hydrochloride	0.5ml
Ammonium iron (III) citrate	1.0ml

Table 32: Ingredients of complete Half Fraser broth

The next day when the incubation period for the primary enrichment with Half Fraser broth has finished we remove the samples from the incubator and using a pipette we transfer 0.1ml from the sample even if we see a color change into the primary enrichment tube into test tubes containing half Fraser medium which is going to be the secondary enrichment for the growth of the *Listeria* in order to produce bacterial colonies if indeed the sample is infected with *Listeria* bacteria. At the same time using a sterile wire loop we transfer a drop of the sample onto two different nutrient agar petri dishes. One of the petri dishes contains ALOA (Agar *Listeria* according to Ottaviani and Agosti) growth medium and the other petri dish contains PALCAM growth medium. Using the steel wire in both cases we apply slight pressure on the sample drop and we spread the drop evenly throughout the plate in order to obtain as widespread sample as possible and obtain as clear colonies as possible. (ISO: 11290-1:1996. Annex A: A.9.3-9.4 : Microbiology of food and animal feeding stuffs- Horizontal method for the detection and enumeration of *Listeria monocytogenes*).

The use of ALOA (Agar for *Listeria* demonstration according to Ottaviani and Agosti) is preferred than the use of the recommended Oxford agar plating medium because ALOA agar medium is considered to be more accurate and give more constant accurate results for the detection. [21]

For the production of the first selective growth medium which is the ALOA the following procedures and ingredients were used. The complete ALOA growth medium is composed of three solutions mixed together to make up the complete growth medium. The solutions needed are:

1) ALOA
2) ALOA Selective Supplement
3) ALOA Enrichment Supplement

The first solution to be made is the actual ALOA agar growth medium. The following ingredients were needed for the preparation of the ALOA agar.

Table 33.

Meat peptone	18.0gr
Tryptone	6.0gr
Yeast extract	10.0gr
Sodium pyruvate	2.0gr
Glucose	2.0gr
Magnesium glycerophosphate	1.0gr
Magnesium sulphate	0.5gr
Sodium chloride	5.0gr
Lithium chloride	10.0gr
Disodium hydrogen phosphate anhydrous	2.5gr
5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside	0.05gr
Agar	13.5gr

Table 33: Ingredients for ALOA agar

For the second solution which is the ALOA Selective Supplement that we have to make, the following ingredients were needed.

Table 34.

Nalidixic acid	10mg
Ceftazidime	10mg
Cycloheximide	25mg
Polymyxin B	38350 IU

Table 34: Ingredients for ALOA Selective Supplement

The third and final component of the ALOA agar plating medium is an already made compound containing 1.0 gram of L-α-phosphatidylinositol which later on will be added to the rest of the compounds to make up the complete ALOA agar plating medium.

To complete the production of the ALOA agar plating medium we had to add 10 milliliters of the ALOA Selective Supplement and 40 milliliters of the ALOA Enrichment Supplement into 1000 milliliters of the ALOA agar and mix them until they completely mix. Heating up the components to 45-50^{o}C might be needed to assist the mixing of the materials used. When all of the components have mixed we can pour the complete ALOA plating agar into petri dishes until they are needed. The whole procedure has to be done using a fume cupboard since the Cycloheximide found in the ALOA Selective Supplement is potentially toxic causing irritation to the skin and the mucus membranes of the nose and mouth. The final complete ALOA agar plating medium should have a pH of 7.2 +/- 0.2. After pouring the complete ALOA on the petri dishes we allow the agar to solidify and cool to room temperature. [22]

In case of positive detection of *Listeria monocytogenes* the color of the agar plate changes to green blue surrounded by an opaque halo from the yellowish of the original color. The ALOA agar plating medium with the suspected sample is then incubated in an incubator for 24 +/- 3 hrs at 37^{o}C +/- 1^{o}C.

The second petri dish in which we transferred a drop of the culture from the half Fraser broth medium contains PALCAM agar medium. PALCAM agar base medium is usually used as a selective medium for the detection of *Listeria* monocytogenes. PALCAM is made up of four different compounds. The compounds making up the PALCAM agar medium are:

1) Agar base
2) Polymyxin B sulfate solution
3) Acrifalvine hydrochloride solution
4) Sodium ceftazidine pentahydrate

The first compound to be made is the agar base medium and it’s composed of the following ingredients:

Table 35.

Peptones	23.0gr
Starch	1.0gr
Sodium chloride	5.0gr
Yeast extract	3.0gr
Agar	9.0-18.0gr
D-glucose	0.5gr
D-mannitol	10.0gr
Aesculin	0.8gr
Ammonium iron (III) citrate	0.5gr
Phenol red	0.08gr
Lithium chloride	15.0gr
Water	960ml

Table 35: Components of the Agar base medium for PALCAM

For the preparation of the PALCAM agar base we had to dissolve all of the components into the 960ml of distilled water. The procedure was enhanced by boiling the water at the same time. After we completely dissolved the ingredients the pH should be 7.2 +/- 0.2 at 25ºC. If the pH is not the desired then the use of an appropriate buffer could be used to achieve the desired pH of 7.2. we then sterilize the agar base using an autoclave at 121ºC for 25 minutes. (ISO:11290-1:1996, Annex B: B.4.1-B.4.1.2: Composition and preparation of culture media and reagents).

The second solution that we had to prepare as part of the PALCAM agar plating medium was the Polymyxin B sulfate solution. This solution is made up from the following two ingredients. The first ingredient is the Polymyxin B sulfate from which we use 0.1 grams of it and dissolve it into the second ingredient which is 100 milliliters of distilled water. After we add the polymyxin b sulfate to the distilled water we stir the solution and pass the solution through a filter in order to remove any resulting undissolved powder particles. (ISO:11290-1:1996, Annex B: B.4.2.1-B.4.2.2: Composition and preparation of culture media and reagents).

The third solution is the Acriflavine hydrochloride solution. This solution is made from 0.05 grams of Acriflavine hydrochloride dissolved into 100 milliliters of distilled water and then the

solution is passed through a filter paper to remove any powder particles that might not dissolved in the water. (ISO:11290-1:1996, Annex B: B.4.3.1 -B.4.3.2: Composition and preparation of culture media and reagents).

The fourth and final solution that makes up the PALCAM agar plating medium is the Sodium ceftazidine pentahydrate solution. The solution is made by mixing 0.116 grams of Sodium ceftazidine pentahydrate into 100 milliliters of distilled water and passing the solution through a filter again to remove any particles that remained undissolved. (ISO:11290-1:1996, Annex B: B.4.4.1-B.4.4.2: Composition and preparation of culture media and reagents).

After we prepared all of the components of the PALCAM agar medium we had to mix all of the compounds and solutions together to make the complete medium. For this we used:

Table 36.

Agar base	960ml
Polymyxin B sulfate solution	10ml
Acriflavine hydrochloride solution	10ml
Sodium caftazidine pentahydrare solution	20ml

Table 36: Contents of the complete PALCAM medium

For the preparation of the complete medium we had to heat the agar base to 47oC so that it melts and then add the Polymyxin, Acriflavine and the Sodium ceftazidine to it and mix them well until the medium is homogenized. Then we pour approximately 15ml of the complete medium onto petri dishes and then allow the medium to cool down and solidify to room temperature. The original color of the PALCAM agar plating medium is pinkish purple. In the first 24 hours of incubation period for the PALCAM medium *Listeria* colonies appear as small greenish colonies with black centers and a black halo around them. If the PALCAM agar medium is incubated for 48 hours then the *Listeria* colonies appear as green colonies surrounded by a black halo and the colonies appears to be depressed into the agar gel. The PALCAM agar plating mediums are incubated for 48 +/- 2 hrs at 37oC +/- 1oC in an incubator. (ISO:11290-1:1996, Annex B: B.4.5-

B.4.6: Composition and preparation of culture media and reagents). (ISO: 11290-1:1996, Annex A: 9.4.4.2 : Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes*).

The second enrichment with full Fraser broth will be incubated using an incubator at 37ºC for 48 +/- 3hrs. From there on we will transfer any resulting colonies that might form onto ALOA and PALCAM agar gel for further colony isolation. For the preparation of the full Fraser broth we use the following ingredients and preparation techniques. The full Fraser broth medium is made up of four parts:

1) Base medium
2) Acriflavine hydrochloride solution
3) Ammonium iron (III) citrate solution
4) Complete Fraser broth medium

For the base medium used in the full Fraser broth the following ingredients are used:

Table 37.

Meat peptone (peptic digest of animal tissue)	5.0gr
Tryptone (peptic digest of casein)	5.0gr
Meat extract	5.0gr
Yeast extract	5.0gr
Sodium chloride	20.0gr
Disodium hydrogen phosphate dehydrate	12.0gr
Potassium dihydrogen phosphate	1.35gr
Aesculin	1.0gr
Lithium chloride	3.0gr
Sodium salt of nalidixic acid	0.02gr
Water	1000ml

Table 37: Components of base medium used in the Full Fraser broth

For the preparation of this initial base for the full Fraser broth we dissolve all the ingredients into the water. Slight heating the water might be needed to completely dissolve the ingredients and slight adjustment to the pH using an appropriate buffer to reach the needed pH of 7.2 +/- 0.2 at 25ºC. After preparing and mixing all of the ingredients we pour the base medium into test tubes and sterilize the test tubes using an autoclave at 121oC for 15 minutes. (ISO:11290-1:1996, Annex B: B.2.2.1-B.2.2.2: Composition and preparation of culture media and reagents).

The second solution is the Acriflavine hydrochloride solution which is the same as the one used in the Half Fraser broth medium described earlier and is only made by 0.25 grams of Acriflavine hydrochloride powder mixed into 100 milliliters of distilled water. After mixing the water and the Acriflavine together we pass the solution through a filter to remove any undissolved Acriflavine particles that might be left. (ISO:11290-1:1996, Annex B: B.2.2: Composition and preparation of culture media and reagents).

The third solution to be made is the Ammonium iron (III) citrate solution which is again the same solution as the one used in the preparation of the Half Fraser broth. The solution contains 5.0 grams of Ammonium iron (III) citrate powder dissolved into 100 milliliters of water. Then we mix the ammonium iron citrate powder into the water and pass the solution through a filter to remove any undissolved powder particles that might remained. (ISO:11290-1:1996, Annex B: B.2.3: Composition and preparation of culture media and reagents).

The fourth and final step in the preparation of the full Fraser broth is the mixing of all of the solutions and the base medium together to form the complete medium. For the final complete medium we add 0.1ml from each of the solutions into 10 ml of the base medium and pour them into appropriate test tubes until we transfer the 0.1ml of the sample from the half Fraser broth onto them. The second enrichment with full Fraser broth will be incubated in an incubator for 48 +/- 3hrs at 37 +/- 1ºC. (ISO:11290-1:1996, Annex B: B.2.3: Composition and preparation of culture media and reagents).

After the 48 hours incubation period of the second enrichment with the full Fraser broth the cultures are removed from the incubator and any color changes from the original yellow color is noted. Usually the presence of *Listeria monocytogenes* in the full Fraser causes a change in color from the original yellowish color into dark black color. Then by using a sterile wire loop we

transfer one drop from the full Fraser broth onto an agar plate containing ALOA agar plating medium and another drop onto a petri dish containing PALCAM agar plating medium. Using the wire loop we try to swipe and spread the droplet that might potentially contain *Listeria* bacteria as much as possible in order to get as pure single *Listeria* colonies as possible. The PALCAM and ALOA agar plating mediums used in this stage of the investigation are the same as the ones used before so the preparation technique and all of the ingredients used are the same as described at p.42 – p.44. The petri dishes containing PALCAM agar plating medium will be incubated at 37ºC for 24 +/- 3hrs in an incubator whereas the petri dishes containing the ALOA agar plating medium will be incubated in an incubator for 48 +/- 2hrs at 37ºC.

Table 38.

Table of the number of samples	Original samples	Primary enrichment	Secondary enrichment	Total number of PALCAM samples	Total number of ALOA samples
	15 raw milk	15 raw milk	15 raw milk	45 raw milk	45 raw milk
	4 dairy products	4 dairy products	4 dairy products	12 dairy products	12 dairy products
	1 positive control	1 positive control	1 positive control	3 positive controls	3 positive controls
	1 negative control	1 negative control	1 negative control	3 negative controls	3 negative controls
Total number	21 samples	21 samples	21 samples	63 samples	63 samples

Table 38: Total number of samples in details

At this point as our results came out to be negative, we reached the end of our experimentation and testing phase. However, the standard procedure for the detection and identification of *Listeria species* and *Listeria monocytogenes* is to be discussed in the following pages.

From there on the confirmation stage starts for the confirmation of *Listeria spp.* After we remove the PALCAM and the ALOA agar plating mediums and we observe any colonies formed we

precede with the confirmation techniques. For the confirmation of just any *Listeria species* we can use four different tests. These tests are:

1) Selection of colonies using TSYEA agar
2) Catalase reaction
3) Gram staining
4) Motility test

For the first test for the confirmation of the presence of *Listeria species* we have to transfer any resulting colonies from the PALCAM and the ALOA agar plating medium into TSYEA (Tryptone Soya Yeast Extract Agar) agar medium. The TSYEA agar will be used to enhance the formation of *Listeria* colonies for further investigation and correct identification of each of the *Listeria* species using other techniques that will be described later on. Using a wire steel sterile loop we scrape the colonies formed on either the PALCAM or the ALOA agar medium and transfer them onto petri dishes that contain the TSYEA agar. We then place the TSYEA plates in an incubator for 24 hrs at 37ºC. Positive *Listeria* colonies produce convex colorless or opaque in color. The ingredients and the production procedure for TSYEA agar are the following. (ISO: 11290-1:1996, Annex A: 9.5.1.1 : Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes*).

Table 39.

Tryptone	17.0gr
Soya peptone	3.0gr
Sodium chloride	5.0gr
Dipotassium phosphate	2.5gr
Glucose	2.5gr
Yeast extract	6.0gr
Agar	9-18gr
Water	1000ml

Table 39: Contents of TSYEA agar

For the production of the TSYEA agar we have to dissolve all of the above components into the distilled water. To assist the faster dissolvement of the ingredients we can boil the water before adding the components. When all of them are dissolved completely we measure the pH which

should be 7.3 +/- 0.2 at 25ºC and if we have a difference in pH an appropriate buffer could be used to reach the desired pH and then disperse the agar into petri dishes and allow the mixture to cool down and partly solidify at room temperature. We can sterilize the mixture using an autoclave at 121ºC for 15 minutes. (ISO:11290-1:1996, Annex B: B.5.1 - B.5.2: Composition and preparation of culture media and reagents).

The resulting colonies formed on the TSYEA agar plate will be used for the other three following methods that are used for the confirmation of *Listeria species*. The next test that can be performed is the Catalase test. For the catalase test we transfer one of the colonies formed on the TSYEA agar onto a microscope slide and add a drop of hydrogen peroxide solution onto it. If we observe gas bubble coming out of the hydrogen peroxide drop then the test is positive for the presence of *Listeria spp.* (ISO: 11290-1:1996, Annex A: 9.5.2 : Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes*).

The third test for the conformation of *Listeria* species is the Gram staining test. In this test we use the standard Gram staining technique where *Listeria* species are represented as gram positive slim and short rods. (ISO: 11290-1:1996, Annex A: 9.5.3 : Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes*).

The final test for the confirmation of the presence of *Listeria species* in our samples is the Motility test. For the motility test we take one of the colonies formed on the TSYEA agar using a sterile wire loop and transfer it onto a plate containing TSYEB agar. We then incubate the sample in an incubator set at 25ºC for 16 hours or until the medium turns cloudy. (ISO: 11290-1:1996, Annex A: 9.5.4: Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes*).

The TSYEB (Tryptone Soy Yeast Extract Broth) culture medium contains the following components:

Table 40.

Tryptone	17.0gr
Soya peptone	3.0gr
Sodium chloride	5.0gr
Dipotassium phosphate	2.5gr
Glucose	2.5gr
Yeast extract	6.0gr
Water	1000ml

Table 40: Components of TSYEB

For the preparation of the liquid culture broth we have to dissolve all of the above ingredients into the distilled water. Slight heating of the water might be needed to speed up the reaction. After that we adjust the pH using an appropriate buffer if needed to reach a pH of 7.3 +/- 0.2 at 25ºC and then we divide the medium into test tubes and sterilize the medium using an autoclave at 121ºC for 15 minutes. (ISO:11290-1:1996, Annex B: B.6.1 - B.6.2: Composition and preparation of culture media and reagents).

After the 16 hours incubation period in the incubator have passed we remove the TSYEB broth medium from the incubator and using a sterile wire loop we transfer a drop from the medium onto a clean microscope slide and place a cover slip over it. We observe the slide under the microscope where the *Listeria* bacteria are seen as short rods, slim in appearance with observed motility. These are the test that can be performed for the confirmation of any *Listeria* species in the samples that are tested. (ISO: 11290-1:1996, Annex A: 9.5.4 : Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes*).

After the confirmation of the presence of *Listeria* in the samples we can perform separate tests to see which *Listeria* species is present in our samples. For the presence of *Listeria monocytogenes* the following tests can be performed:

1) Haemolysis test
2) Carbohydrate utilization test

3) CAMP test

The first test for the confirmation of the presence of *Listeria monocytogenes* is the haemolysis test. For this test we will use sheep blood agar plates in order to see any haemolytic reaction on the plates proving the presence of *Listeria monocytogenes*. The sheep blood agar is made up of:

Table 41.

Meat peptone	15.0gr
Liver digest	2.5gr
Yeast extract	5.0gr
Sodium chloride	5.0gr
Agar	9.0-18.0gr
Water	1000ml

Table 41: Contents of sheep blood agar

For the preparation of the sheep blood agar we have to dissolve all the previous mentioned components into boiling water and adjust the pH if needed to reach the required pH of 7.2 +/- 0.2 at 25^{o}C. After that we have to add 5.0-7.0 milliliters of defibrinated sheep blood and we add the blood to the base medium produced before. We then allow the blood medium to cool down to 47^{o}C and mix the blood medium well. We then sterilize the medium using an autoclave at 121^{o}C and then divide the medium into Petri dishes. We then dry the agar surface and take a colony using a wire sterile loop and transfer the colony from the TSYEB onto the sheep blood agar. Then using the same wire loop we stab the medium. Onto the same plate we use a positive *Listeria monocytogenes* sample and a negative *Listeria innocua* control culture. We then incubate the plates in an incubator at 37^{o}C for 24 +/- 2hrs and then examine all of the cultures produced. In case of positive cultures produced by *Listeria monocytogenes* the cultures show narrow light zones with β- haemolysis. On the other hand *Listeria innocua* does not show clear zones around the stab produced by the wire loop. *Listeria seeligeri* only shows a weak zone of hemolysis on the sheep blood agar. The most striking appearance is seen with *Listeria ivanovii* which shows wide, clearly delineated zones of β-haemolysis on the sheep blood agar. (ISO: 11290-1:1996, Annex A: 9.6.1: Microbiology of food and animal feeding stuffs – Horizontal

method for the detection and enumeration of *Listeria monocytogenes*). (ISO:11290-1:1996, Annex B: B.7.1 - B.7.3.1: Composition and preparation of culture media and reagents).

The second test to be performed for the identification of each of each of the *Listeria* species is the Carbohydrate utilization test. For this test we use a wire loop and transfer a colony from the LSYEB and transfer it into each of the carbohydrate utilization broths. The carbohydrate utilization broths are composed of rhamnose and xylose. The carbohydrate broth is composed of two parts: the base medium and the carbohydrate solutions. The base of the carbohydrate utilization broth is made up of the following:

Table 42.

Proteose peptone	10.0gr
Meat extract	1.0gr
Sodium chloride	5.0gr
Bromocresol purple	0.02gr
Water	1000ml

Table 42: Contents of Carbohydrate utilization broth

For the preparation of the carbohydrate utilization broth we have to dissolve the components into the water and use slight heating if necessary. We might need to adjust slightly the pH using an appropriate buffer to reach the necessary pH of 6.8 +/- 0.2 at 25ºC and separate the medium into test tubes. We then sterilize the medium at 121ºC in an autoclave for 15 minutes. (ISO:11290-1:1996, Annex B: B.8.1 - B.8.1.2: Composition and preparation of culture media and reagents).

The next part is the complete composition of the carbohydrate solutions. We simply add 5.0 grams of L- Rhamnose or D- xylose into 100 milliliters of water. We mix them thoroughly and sterilize the medium using a filter to remove any powder residues that remain undissolved. For the complete media we add 1 milliliter of the carbohydrate solution into 9 milliliters of the base. Positive reactions which are proven by acid formation are indicated as a yellow change in color and it starts to appear after 24 hours. The reaction of carbohydrate utilization might take as long as 48 hours for the change in color to appear. (ISO: 11290-1:1996, Annex A: 9.6.2 : Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes*).

The final test to be performed of the identification of each of the *Listeria* species is the CAMP test. For this test we have to use *Staphylococcus aureus* and *Rhodococcus equi* cultures as well to assist the identification of the *Listeria* species. For this test we use sheep blood agar plates and we transfer using a sterile wire loop *Staphylococcus aureus* and *Rhodococcus equi* cultures onto the plate. The two cultures are arranged parallerly onto the sheep blood agar but making sure that the two colonies do not touch and interfere with the identification. On the same plate we use a different sterile wire loop and transfer control cultures of *Listeria monocytogenes*, *Listeria innocua* and *Listeria ivanovii*. We also transfer colonies produced from the TSYEB agar plate onto the sheep blood agar for identification. We then incubate the blood agar plates in an incubator at 37ºC for 24 hours. A positive reaction to the test is when a thick zone of β-haemolysis is seen at the intersection of the test strains with each of the cultures of the *Staphylococcus aureus* and the *Rhodococcus equi* cultures. A positive reaction with *Rhodococcus equi* is seen as an arrow head shape heamolysis. The test is considered to be negative if a weak zone of haemolysis is produced. A positive reaction with *Staphylococcus aureus* is seen when a small zone of haemolysis between the *Staphylococcus aureus* and the *Listeria monocytogenes* cultures. (ISO: 11290-1:1996, Annex A: 9.6.3: Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes*).

Table 43.

Species	Haemolysis	Production of acid		CAMP test	
		Rhamnose	Xylose	*Staphylococcus aureus*	*Rhodococcus equi*
Listeria monocytogenes	+	+	-	+	-
Listeria innocua	-	Variable	-	-	-
Listeria ivanovii	+	-	+	-	+
Listeria seeligeri	(+)*	-	+	(+)	-
Listeria welshimeri	-	Variable	+	-	-
Listeria grayi subspecies grayi	-	-	-	-	-
Listeria grayi subspecies murrayi	-	Variable	-	-	-

Table 43: Reactions for the identification of *Listeria* species

*(+) might produce a positive result or an inconclusive result

These were all the materials and methods that we used in the States Veterinary Services Control of Food of Animal Origin for the detection of both *Salmonella* and *Listeria monocytogenes* in raw sheep/goat milk and sheep/goat dairy products. After the completion of all of the previously described experiments an official certificate from the Control of Food of Animal Origin was issued proving that all of our samples did not contain neither *Salmonella* nor *Listeria monocytogenes*. With this certificate the products tested can be used and sold in the market. The certificate issued by the Laboratory of Control of Food of Animal Origin is also certified by the State Veterinary Services.

4. Results

Table 44

Table of results from the Departments of Agricultural research in Athalassa area in Nicosia			
Number of samples 5 (2*20ml)			
Sheep	Ear Tag Identification Number	Result of *Salmonella* detection	Result of *Listeria* detection
	209040936	Negative	Negative
	23534534	Negative	Negative
Goat	209063887	Negative	Negative
	209071199	Negative	Negative
	208130871	Negative	Negative

Table 44: Results of *Salmonella* and *Listeria* detection in samples from farm 1

Table 45

Table of results from Lykourgos Ntortzis Farm – Tseri area			
Number of samples 5 (2*20ml)			
Sheep	Ear Tag Identification Number	Result of *Salmonella* detection	Result of *Listeria* detection
	24676283	Negative	Negative
Goat	245090063	Negative	Negative
	240707352	Negative	Negative
	243469772	Negative	Negative
	24346972	Negative	Negative

Table 45: Results of *Salmonella* and *Listeria* in samples from farm 2.

Table 46

Table of results from Demetris Demetriou Farm – Nicosia Airport Area			
Number of samples 5 (2*20ml)			
Sheep	Ear Tag Identification Number	Result of *Salmonella* detection	Result of *Listeria* detection
	245298169	Negative	Negative
	239263413	Negative	Negative
	239270875	Negative	Negative
	246607382	Negative	Negative
	233810651	Negative	Negative

Table 46: Results of *Salmonella* and *Listeria* detection in samples from farm 3.

Table 47

Giannakis Theofanous	Paphos	CYS 6301002	4kg of Halloumi samples	Results of *Salmonella* detection: Negative	Results of *Listeria* detection: Negative
Petros Ioannides	Paphos	CYS 6331027	2kg goat Halloumi samples	Results of *Salmonella* detection: Negative	Results of *Listeria* detection: Negative
Giannakis & Zoiro Stephani LTD	Paxna	0014	5 pieces of Halloumi	Results of *Salmonella* detection: Negative	Results of *Listeria* detection: Negative
Giannakis & Zoiro Stephani LTD	Paxna	0014	5 pieces of fresh anari	Results of *Salmonella* detection: Negative	Results of *Listeria* detection: Negative

Table 47: Results from the detection of *Salmonella* and *Listeria* from dairy product samples

All of our samples listed above in tables 44- 47 that we collected and tested with the different methods described above were proven to be negative for both *Salmonella* and *Listeria monocytogenes* bacteria. All the samples were free from both bacteria therefore the raw milk and the dairy products could be used for trading and consumption. After the completion of the tests the Department of Control of Food of Animal Origin issued a certificate certifying that all the samples that were tested were negative for the presence of *Salmonella* and *Listeria monocytogenes* bacteria. The same certificate was also reviewed by the State Veterinary Services and the raw milk and dairy products were certified for human consumption or trade.

5. Discussion

In this research we sought out to review the existing ISO: 6579:2002, Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella species* and ISO: 11290 – 1:1996, Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes* and investigate the possibility of new techniques and improvements for the detection of *Salmonella* and *Listeria monocytogenes* in raw milk and dairy products derived from sheep/ goat milk.

All the raw milk samples that we collected from three different sheep and goat farms around Nicosia area were from two different mammary glands and then mixed to homogenize the milk. The samples for the dairy products were obtained from samples that were sent to the State Veterinary Services of Cyprus directly from the producers and were sent to the Department of Control of Food of Animal Origin for testing for the presence of *Salmonella* and *Listeria monocytogenes* before being released for human consumption.

The whole experiment included the use of ISO: 6579:2002 for the detection of *Salmonella* and ISO : 11290 – 1: 1996 for *Listeria monocytogenes* as well as some improvement techniques that might in the future help in the faster detection for the presence of the two bacteria.

For the detection of *Salmonella* in our samples we pipette 10ml of the samples into 90ml of Buffered Peptone water and using a stomacher machine homogenized the samples and then incubated them for 18 hours. Then 0.1 ml of the incubated samples were transferred to 10ml of RVS medium and incubated for 24 hours at 41.5^{o}C. Another 1ml from the incubated samples was transferred using a sterile pipette onto 10 ml of MKTTn broth and incubated for 24 hours at 37^{o}C. The next day the using a sterile wire loop a drop from the RVS sample was transferred onto an XLD agar plate and using the same loop the drop of the suspected culture was spread as wide as possible in order to achieve the purest possible colony in case of positive results thus those resulting colonies could be used for further identification techniques. The XLD plates were incubated for 24 hours at 37^{o}C. From the same RVS sample another drop was transferred using a sterile wire loop onto BGA growth medium. Using the same wire loop we also spread the drop of the suspected culture onto the BGA plate. The BGA plates were incubated for 24 hours at 37^{o}C as well. Again for the MKTTn samples that we incubated the previous day we used the same

procedure using a sterile wire loop to transfer a drop of the suspected culture onto two separate plates. The first plate contained XLD medium and the second one contained BGA medium and both plates were incubated for 24 hours in an incubator at 37ºC. The next day all the plates were removed from the incubators and examined for any colonies that might have formed overnight. Initially the original color of the XLD plates is bright red and in case that of a positive result, the color changes to yellow. Also any *Salmonella* colonies that might be present produce black colored colonies on the XLD agar. In the case of the BGA agar the original color of the medium is yellowish and for a positive result bluish green colonies are produced by *Salmonella* species. After the incubation of the XLD and the BGA agar plates all of our samples we proved to be negative but following the indications given by the ISO: 6579:2002 the procedures that would be used in case of positive results were also mentioned. These indications included different biochemical and serological methods for the detection of *Salmonella* until a definite result could be obtained.

During the experiment for the detection of *Salmonella* we also used another method. This method is normally used for the detection of *Salmonella* from feacal material. This method uses MRSV agar medium and is thought that in the near future it will be used as a universal method for the detection of *Salmonella* from both feacal and raw products. The initial enrichment of *Salmonella* with Buffered Peptone Water is the same as the one described in the ISO: 6579:2002 but then we transferred 1 ml of the culture onto a petri dish that contained MRSV medium and divided the droplet into 3 equal parts. The initial color of the MRSV medium is blue. In case of a positive test for the presence of *Salmonella* the color of the medium changes to light blue with a turbid whitish zone around the suspected culture droplets. For further investigation the same technique meaning the transfer of the colonies onto XLD and BGA agar plates could be used for the clear detection of *Salmonella* as in the ISO: 6579:2002. This method apart from the harmonization of the two different detection procedures that are used now from samples derived from fecal materials and from raw milk and dairy products it can be used as an easier and more rapid detection of *Salmonella*. Apart from being cheaper for the different laboratories it also takes less time for the microbiologists to complete the testing for the detection of *Salmonella.* This is because the positive *Salmonella* samples can even be detected just by using the MRSV growth medium and the changes on the growth medium are easily detected. Also the clear distinction between negative and positive tests allows the microbiologists to conduct

examinations from both fecal and raw samples simultaneously without having to change the detection technique and the materials used for the detection. For me it was easier and faster to detect the color differences on the MRSV agar than having to wait another day until the incubation period of the XLD and the RVS mediums ended. Since all of our results were negative the MRSV trials were also negative but the difference in the time taken for a positive result to be interpreted was realized when we used the positive *Salmonella* control tests. The results for the presence of *Salmonella* were obvious as soon as the MRSV mediums were removed from the incubator instead of having to transfer any resulting colonies to the XLD and the RVS agars.

The only limitation of the MRSV medium is that it is especially sensitive to the detection of motile *Salmonella* species thus the use of the MRSV medium for the detection of immotile or damaged *Salmonella* species is less sensitive thus false positive or false negative results might be obtained making the detection more difficult and the use of the supplementary XLD and BGA agars should be used to reach a definite and accurate result.

For the detection of Listeria monocytogenes we used 10 ml of the raw milk samples and 10 ml from the dairy products sample and mixed it with 90 ml of Buffered Peptone Water in individual stomacher bags. The stomacher bag samples were then one by one placed in the stomacher machine and mixed well for approximately two minutes to homogenize the sample with the Peptone buffered water. Then all of the samples were collected and using a sterile pipette we transfer 10ml of the sample into 90ml Half Fraser broth which contains Acriflavine and Nalidixic acid. The resulting solution is then placed in an incubator set at 30^{o}C for 24 +/- 3 hrs to be incubated. When the incubation period has ended the next day the samples are removed from the incubator and two procedures are carried out.

0.1 ml of the incubated sample that might be suspected for the presence of *Listeria monocytogenes* are used to be transferred using a sterile pipette into 10 ml of Full Fraser broth medium which is going to be incubated in an incubator for 48 +/- 3 hours at 37 +/- 1^{o}C. At the same time we use a sterile wire loop to transfer a droplet of the sample onto an agar plate containing ALOA growth medium and another droplet onto a petri dish containing PALCAM agar and placed them into an incubator. For the ALOA petri dish the samples are incubated for

24 +/- 3 hours at 37 +/- 1ºC. For the petri dishes that contain the PALCAM detection agar we placed the samples into the incubator for 48 +/- 2 hours at 37 +/- 1ºC.

The next day the samples containing the Full Fraser broth are removed from the incubator and using a sterile wire loop we transfer a drop of the culture on to two new Petri dishes. One petri dish contains ALOA detection agar and the other petri dish contains PALCAM detection medium. Using the wire loop we spread evenly the droplet and try to spread it so that we can obtain as pure colonies as possible. The original color of the ALOA detection medium is yellowish and the original color of the PALCAM is pinkish purple. In case of positive detection of *Listeria monocytogenes* using the ALOA detection medium the bacteria appear as greenish colonies with black centers and surrounded by an opaque halo around them. On the other hand on the PALCAM detection medium *Listeria monocytogenes* appear as depressed green colonies surrounded by a black halo. The ALOA containing petri dish will be incubated using an incubator for 24 +/- 3 hours set at 37 +/- 1ºC. The PALCAM containing petri dishes will be incubated in an incubator for 48 +/- 2 hours set at 37 +/- 1ºC.

At the same time we remove the ALOA petri dish that we incubated the previous day and observe the detection petri dish for the presence of *Listeria monocytogenes* which as mentioned before will form greenish colonies with black centers surrounded by an opaque halo.

The next day we removed the samples that were incubated in the incubator that contained the ALOA from the Full Fraser broth and we observed for the presence of *Listeria monocytogenes* as described above. Also we remove from the incubator the samples with PALCAM detection agar that we directly incubated containing Half Fraser broth and observe for the presence of *Listeria monocytogenes* that in case of positive result will appear as depressed greenish colonies surrounded by a black halo.

On the final day of the detection procedure we removed the samples of the PALCAM petri dishes from the incubator and we observed for the presence of the bacteria as described before.

After the incubation of the detection mediums containing ALOA and PALCAM mediums our results came out to be negative and our investigation stopped but using the indications in ISO: 11290-1:2002 the procedures for the identification of the different *Listeria* species that might be present in the sample were also described. The rest of the methods that are described in the

methods and materials part of the *Listeria monocytogenes* identification are used for the correct identification of each of the Listeria species that might be presence in our sample

In this experiment we also used a method that was not in the ISO: 11290-1:1996. In the ISO: 11290-1:1996 the detection agar mediums used are the PALCAM and the Oxford detection mediums. In our case we used the ALOA and the PALCAM detection mediums. We used the ALOA detection medium because it is able to produce more accurate and reliable results in the detection of *Listeria monocytogenes* instead of the Oxford detection medium. ALOA detection medium according to the study: Improvement of the detection of *Listeria monocytogenes* by the application of ALOA, a diagnostic, chromogenic isolation medium[21], is able to obtain 4.3% more positives and 25.0% less false negative results than the Oxford detection medium. The ALOA detection medium that is used is also able to be used for the identification between *Listeria monocytogenes* and *Listeria innocua* as well as other non- pathogenic *Listeria* species which usually appear as bluish colonies without the surrounding halo on the detection medium. The use of ALOA is proven over the years to be able to reduce the time needed for the detection of *Listeria monocytogenes* and also to reduce the costs needed for the detection compared to Oxford detection medium.

6. Summary

The detection of the *Salmonella species* and *Listeria monocytogenes* is a widely known and universally accepted procedure that is especially carried out every day in all of the Laboratories that have to do with animal products that are intended to be used either by humans or by other animals. Everyday thousands of samples from raw milk, milk products, ready-to-eat products, and meat and egg samples are sent to laboratories to be tested for *Salmonella* and *Listeria monocytogenes* because of the risk of zoonotic transmission. This means that these two bacteria can infect humans and cause them quite severe health issues. An example to show the severity of such an infection would be the case of infection of pregnant women with *Listeria monocytogenes* which could lead to abortions and severe diarrhea and dehydration in the case of infection with *Salmonella* species. That's why it is very important for us to use fast and accurate techniques in the detection of the two bacterial species.

This study was inspired by the increased attention these bacteria have gained over the last 20 years which has yielded new techniques and detection methods. In this study, we firstly used the existing ISO guidelines that arrive directly from the European Union for the detection of *Salmonella* and *Listeria monocytogenes.* We then explored the two new methods to investigate whether they were indeed less time consuming, more accurate and more cost effective.

For the *Salmonella* detection, the additional method of detection used was the MSRV detection medium. This method is currently used in the detection of *Salmonella* from fecal materials. With the use of MSRV for the detection of motile *Salmonella* bacteria from raw milk the two procedures could be done simultaneously, meaning that a single detection method can compensate for two in the detection of *Salmonella* from raw and fecal materials. This will save time in the preparation of the different samples from both raw and fecal materials, and cost for the laboratories.

In the case of *Listeria monocytogenes* we also used a different method than the one used in ISO 11290-1:1996. In our investigation we used ALOA and PALCAM as detection mediums instead of the proposed Oxford and PALCAM. The ALOA detection method proved to be more accurate and faster in detecting *Listeria monocytogenes* thus saving time and cost to the laboratories that use these technique.

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Appendix 2

Abbreviations

1. ^{o}C: Degrees Celcius
2. gr: Grams
3. hrs: Hours
4. ml: Milliliters
5. spp: Species
6. mg: Milligrams
7. IU: International Units
8. HAACCP: Hazard Analysis And Critical Control Points
9. Kcal: Kilo Calories
10. Kj: Kilo Joules
11. Kg: Kilograms
12. BPW: Buffered Pentone Water
13. RVS broth: Rappaport-Vassiliades medium with soya
14. MKTTn solution: Muller – Kauffmann tetrathionate – novobiocin broth
15. XLD agar: Xylose Lysine Deoxycholate agar
16. BGA agar: Brilliant Green Agar
17. TSI medium: Triple Sugar Iron Medium
18. ONPG: o-nitrophenyl β-D- galactopyranoside
19. VP reaction: Voges- Proskauer reaction
20. MSRV medium: Rappaport Vassiliades Medium Semisolid Modified
21. ALOA agar: Agar for Listeria demonstration according to Ottaviani and Agosti
22. TSYEA agar: Tryptone Soya Yeast Extract Agar

23. TSYEB broth: Tryptone Soy Yeast Extract Broth

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