UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II

Ph.D.

# Ambiente, Prevenzione e Medicina Pubblica

XXV cycle

Food chains Hygiene :

monitoring of retail products and characterization of isolated microbial species (virulence factors, ability to form biofilm, antibiotic resistance).

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**Academic year 2012-2013**

*to my father*

# Abstract

Hygienistic food monitoring can be performed at various supply chain levels; the thesis "Food chains Hygiene: monitoring of retail products and characterization of isolated microbial species (virulence factors, ability to form biofilm, antibiotic resistance)" focused attention on the final phase of this distribution chain. Different types of meat and vegetables samples were investigated on microbiological basis. Samples were collected in Naples city (Italy).

According to the the CE Regulation 2073/2005, the following microbiological protocols were assessed: total bacterial count, *Escherichia coli*, *Salmonella spp*. .

The microbiological monitoring was additionally performed to evaluate: Sulphite-reducing Clostridia*, Clostridium difficile, Listeria spp and L. monocytogenes, Staphylococcus spp. and S. aureus, Campylobacter spp., E. coli O157: H7, Clostridium perfringens, Enterococcus spp., Aeromonas spp* ..

In addition, virulence factors studies were realized. The statistical study involved the verification of positivities registered and the frequency distributions analysis. These evaluations provided useful input to the risk assessment associated with production chains.

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# Premises

Meat is primarily composed of nitrogen compounds (proteins, water-soluble substances, collagen, lipids, carbohydrates and minerals), whose percentages depend on the considered animal species, age, race, nutritional and husbandry conditions.

Meat has a high nutritional value due to its proteins content and high digestibility. It traditionally is a very expensive food, although its consumption is linked to several potential health care problems.

Generally, meat is divided by color assumed after slaughter in: "white ", "red" and "black" meat. White meat gathers poultry meat such as chickens, hens, capons, ducks, turkeys, guinea fowls, pigeons, geeses. Red meat includes cattles, buffaloes, sheeps, horses, goats, pigs. Wild animals' meat includes black meat, boars, deers, roe deers, pheasants, partridges, quails, wild ducks.

In Italy, pig represents 42,3% consumption, followed by cattle (27,2%) and poultry (18,2%); in developed countries, consumption is almost equally distributed. The livestock products demand depends on several factors including the number and type of consumer settlement (rural and urban), their income and the product prices, the quality of nutrition and hygiene. In addition, consumers preferences are influenced by taste, religion, traditions (e.g. Islamic countries dominant consumption is represented by beef but they do not eat pork). Individual meat consumption has grown over the last decade.

The production of beef in Italy reveals an important agro-industry trade. Over the past fifty years there has been a huge increase in production and consumption of meat: in fact, it increased from 18,8 kg / person in the 1950's to 83,8 kg / person in the 1990's and this trend keeps on growing. Despite the crisis linked to Creutzfeldt Jakob Syndrome (also known as mad cow disease) has led to a decline in beef consumption, this was quickly offset by the pork and by chicken meat. The outbreak of bird flu in early 2004 has put the entire poultry industry at risk, resulting in lower demand and increased consumption of other meat and, in particular, jobs. After the bird flu there have been no other turbulent effects: to confirm this, we noted that, in 2004, domestic purchases of meat have

increased by almost one percentage point against 2003. In particular, the growth of beef (2,9%) and swine (1,6%) was offset by the decrease in poultry (-2,6%), while the consumption of sheep and goat meat remains unchanged. The value of the production sector (base price 2006) stood at around 3,4 billion euros, representing 7,3% of the value of domestic agricultural production and a value close to 39% of the entire sector Livestock for meat.

Italy is one of the EU countries that shows one of the highest consumption of beef per capita, equal to 25,4 kg.

# 1. THE HEALTH RISKS

Food contamination is one of the main problems in the food industry. In order for the food to arrive to the consumer at its best, it is important to fulfill good hygiene practices within every stage of the production.

The health risk for beef is linked to a primary contamination, caused by the illness of the animal from possible secondary contamination, throughout the chain of processing, storage and distribution. Microbial contamination (in particular, bacteria), causes 91,5% diseases contractable from meat.

The etiology of bacterial diseases can be summarized through the following types:

- infection, when the microorganism enters the body and directly causes the disease. The severity of the damage is proportional to the ability of invasive bacteria, the microbial load and the receptivity of the host;
- poisoning due to toxins already preformed regardless of the presence or absence of germs that they produce;
- poisoning caused by the presence of living bacteria that are able to produce toxins. It occurs within hours after ingestion of the food and causes nausea, vomiting, abdominal pain, diarrhea, sometimes fever.

There are numerous sources of contamination of food but the main changes are related to microbial contamination.

The diseases linked to meat consumption may also be associated to metazoaria, protozoal, and viral animals.

Contamination throughout the supply food chain production is now an alarming reality though put through constant checks and application of risk reduction methodologies. There are also many health rules governing the types of control and the allowable limits for the protection of consumer health, but it is important to consider the detection of new emerging pathogens.

The European Union has issued a series of measures to simplify, consolidate and harmonize the legislation concerning veterinary checks, animal health and food hygiene by introducing the Hygiene Package which entered into force in 2006.

Thanks to this package, the European Union's attention focuses on companies that are the first leaders and organs of control that must be appropriately controlled and managed.

The Hygiene Package incorporates and extends the concepts already given by Lex 178/2002 which are the basis of the European Food Safety Authority (EFSA):

- The protection of human health as a main precaution.
- The use of hazard analysis and risk assessment in production units.
- The adoption of microbiological criteria and temperature control.
- Codes of good hygiene procedures.
- Control of food hygiene of the competent authorities.
- The responsibility of industry to marketing.

The aim of the new hygiene rules, general and specific, is to ensure a high level protection of consumers with regard to food safety from the place of primary production to point of sale or export with an integrated strategy. Every food business operator, along the whole food chain, should ensure that safety is not compromised.

# 2. ZOONOSES AND FOOD-BORNE OUTBREAKS

In order to begin the study of food contamination, it is useful to picture an epidemiological scheme of food-borne diseases in the world, in Europe and in Italy. Shown below there are the data for the outbreaks related to more representative germs.

## 2.1. Food and beverages surveillance and monitoring in Italy: year 2010

To prevent the public health risk, the official control of food and beverages is vital in order to verify and ensure the conformity of products, protecting the interests of consumers. Monitoring regards Italian products, or other sources, in order to be marketed throughout the country and those destined for another EU Member State or exported to another country. Official controls should be carried out at any stage of production, processing, distribution, storage, transport, marketing and administration. The controls include inspections at various stages of production and are divided into several stages: inspection, sampling, laboratory analysis of samples, personnel hygiene control, examination of written material and documents of various kinds, check of verification systems operated by the company and its results.
The official control has to consider:

- The status, hygiene and related uses of the plants, equipment, tools, facilities, structures and means of transport;
- raw materials, ingredients, processing aids and all other products used;
- production or preparation for consumption;
- semi-finished products;
- finished goods;
- materials and articles intended to come into contact with food;
- the procedures for disinfection, cleaning and maintenance;
- the technological processes of production and food processing;

- labeling and presentation of foodstuffs;
- the means of preservation.

The Health Ministry, under official supervision, is responsible for planning, directing and coordinating the inspection moments. Within each Region, this task is entrusted to the Councils, while the functions of control over the production, trade and service of food and beverage, is mainly responsibility of the municipalities that exercise through the Local Health Authorities.

The Ministry holds a key role, availing of Food Safety and Nutrition Directorate General (with its Regional Offices), the Office of Maritime Health, Air Frontiers (USMAF) and Peripheral Veterinary Offices, including the Border Inspection Posts (BIPs) and Veterinary Offices for Communitarian Obligations (UVAC).

Through whole country jurisdiction and articulated structures at the peripheral level, there are Carabinieri for the Health Protection (NAS).

The Regions and the Autonomous Provinces of Trento and Bolzano operate locally through the Services of Food Hygiene and Nutrition (SIAN) and the Veterinary Services (SV) of local health authorities' (ASL) Prevention Departments. Laboratory investigations are performed by Environmental Protection Regional Agencies (ARPA), the ASL Laboratory and Institutes Experimental Animal (CBM).

The Ministry of Agriculture, Food and Forestry collaborates with the Central Inspectorate for the quality control of food products (ICQRF), which is the official organ of ministerial responsibility to prevent and punish food relating fraud and mistaken agriculture technics (feed, seed, fertilizers and pesticides).

The Ministry of Economy and Finance acts in synergy with Customs Agency, Chemical Laboratories of Customs and Financial Guard and is responsible for control and supervision of food in relation to the prevention and repression of tax fraud, with possible health implications.

An annual report is provided to Parliament to inform about the results of supervisory activities and analytical control on food and drinks in Italy and it is carried out by all central government departments and local deputies to this activity. This ensures a constant monitoring for health protection in order to stop the trend of fraud and adulteration throughout the country. The report was implemented since 1986 as a result

of fraudulent use of methanol in wine which caused, in Lombardia, Liguria and Piemonte, the death of 19 people and serious injuries to 15 others. According to art. 6 Legge n. 462/1986, amendments of Decreto n. 282/1986 on urgent measures for the prevention and suppression of food adulteration, the Ministry of Health defines a systematic program of measures aimed to food security, providing the directives to the competent services of the central and peripheral surveillance and repression.

In EU countries the official control of foodstuffs is harmonized by CE Regulation n. 882/2004, that provides a Multi-Year National Plan of Integrated Control (MANCP), according to which the competent authorities prepare special programs to define the nature and frequency of inspections that need to be regularly carried out within the manufacture, packaging, administration and marketing phases.

Article 2 of Regulation 882/2004 defines the meaning of "official control", that is the one performed within the established parameters. Article 8 indicates that official controls, done by the competent authorities, should be carried out in accordance with documented procedures. Therefore, Member States should develop programs that define the nature and frequency of the inspections, that need to be regularly performed during a given period. Currently, in Italy, DPR 14 July 1995 summarizes the ways to direct and coordinate the states / provinces through uniform criteria related to official control of food and drink. According to Art. 1, paragraph 3, an additional program for the controls for residues of pesticides, hormones and veterinary drugs, with its own rules, was established.

The monitoring concerns all products and food additives, as well as material intended to come into contact with, marketed in the country (produced in Italy or imported) or for export and is focused on all production stages (processing, storage, transportation, distribution and marketing).

Pursuant to art. 10, official controls include the following activities:

A) Examination of all the control systems put in place by operators and the results obtained;
B) Inspections

- condition and cleanliness of plant and equipment of the premises, facilities and means of transport;

- semi-finished products and finished products and raw materials and ingredients used in their preparation or material intended to come into contact with;
- processes and chemicals used for pest control;
- technological processes of food production;
- presentation, labeling and storage conditions.

C) Sampling and subsequent analysis of the samples. The analytical tests are performed by the laboratories of ASL, ARPA, IZS, ICQRF and other laboratories indicated by public authorities.

D) Consideration of written material or other document in the possession of the head of the inspected.

E) Analysis of control systems adopted by the enterprise (HACCP), including the training of personnel.

## 2.2. WHO food safety

A large and growing problem of public health is represented by diseases caused by contaminated food. Most countries have good systems for reporting cases of foodborne illnesses which have documented a significant increase over the past decades.

The incidence of diseases caused by microorganisms in food consists of pathogens such as *Salmonella, Campylobacter jejuni*, Enterohaemorrhagic *Escherichia coli* and parasites like *Cryptosporidium, Cryptospora, Trematodes*.

About 1,8 million children in the developing world (excluding China) died from diarrheal diseases in 1998, driven by microbiological agents, mostly from contaminated food and water. In the United States, about 76 million cases of foodborne diseases, resulting in 325.000 hospitalizations and 5.000 deaths, are estimated to occur each year. There are only limited data on the economic consequences of food contamination and foodborne illnesses.

United States studies in 1995 estimated that the annual cost of 12 million cases of food-borne diseases caused by seven pathogens was U.S.D. 635 billion. Medical costs and the weight of five foodborne outbreaks in England and Wales in 1996 were estimated at UK £

300-700.000.000. The cost of the approximately 500 cases per day of food poisoning in Australia was calculated at AU $ 2,6 billion a year.

The increased incidence of foodborne illnesses due to microbiological hazards is the result of a variety of factors, all associated with our rapidly changing world. Demographic profiles are being edited, with an increasing proportion of people who are more susceptible to microorganisms in food. The increased incidence of foodborne illness is linked to changing lifestyles: modifications in agricultural practices, wider distribution systems for food, increasing preference of meat and poultry in developing countries, need to have an intensive production that meets the needs of a growing demand, a dense food distribution often in a short time; all these economic dynamics will seriously increase the risk of producing food that can host less common pathogens.

The intensive animal husbandry technologies, introduced to minimize production costs, have led to the emergence of new zoonotic diseases able to affect humans. Issues related to the proper and safe disposal of animal manure can represent (e.g. poultry breeding) a growing food security problem. In addition, the use of manure as a fertilizer is linked to high microbiological risks due to often pathogenic microorganisms.

Changes in consumption patterns, such as a preference for fresh and minimally processed foods, increasingly longer interval between processing and consumption of food and the increasing prevalence of eating food prepared outside home, all contribute to the higher incidence of foodborne illnesses.

The emergence of new pathogens and pathogens not previously associated with food is a public health problem. *E. coli* O157: H7 was identified for the first time in 1979 and subsequently caused illnesses and deaths (particularly among children), because of its presence in beef, unpasteurized apple cider sprouts, milk, lettuce, medical grass and others.

*Salmonella Typhimurium* DT104 has developed resistance to five antibiotics commonly prescribed and is a major concern in many countries because of its rapid spread during the 1990's.

These changes in microbiological hazards in foods have been recognized by the World Health Organization and the Codex Alimentarius. The 22nd Session of the Codex Alimentarius Commission and the 45th of the Codex Executive Committee requested FAO and WHO to convene an international advisory panel of experts similar to the Joint Expert

Committee on Food Additives (JECFA) and the Joint Meeting on Pesticide Residues (JMPR) on the microbiological aspects of food security to deal with, in particular, the microbiological risk assessment. The results of these risk assessments provide the scientific basis for measures to reduce illnesses from microbiological hazards in foods.

Effective management of microbiological hazards is enhanced through the use of tools such as microbiological risk assessment (MRA) and Hazard Analysis and Critical Control Point (HACCP) systems. Such microbiological risk assessment provides an understanding of the nature of the hazard and is a tool for setting priorities for action.

HACCP is a tool for process control, through the identification of critical control points. The ultimate goal is to improve public health and both MRA and HACCP are means to that aim.

Every year millions of people become ill and thousands die from a preventable foodborne. The WHO has developed a protocol for simple understanding based on **five keys** to give a message of global health that explains the basic principles that every individual should know all over the world to ensure safe food handling and prevent diseases with food origin. The Empowerment Website Project has been translated into 82 languages; Five Keys to Safer Food message form the basis for health promotion campaigns and educational programs in over 100 countries.

The protocol consists of a series of preliminary information to be disclosed, the illustration of five simple keys to understanding of good practice to be taken with food.

The protocol for food safety education always includes two guide columns: one contains the basic information that should be presented to all audiences; the second column contains additional information that need not to be disclosed to the public, but can be used for the trainer to answer questions that may arise in the specific meeting. For some sections, the manual also provides "Considerations and suggestions for the trainer", consisting of variations useful to adapt the material for different audiences and different places.

The core messages of the Five Keys to Safer Food are:

- keep clean;
- separate raw and cooked;
- cook thoroughly;
- keep food at safe temperatures;

- use safe water and raw materials.

In conclusion, dedicated solely to the training period there is a questionnaire to be completed by the trainer disciplinary and a questionnaire dedicated to those who participated in the training in order to evaluate the effectiveness of information.

In conclusion of the training is also provided the use of a glossary full of useful information to clarify doubts about the nomenclature used in the encounter specialized training. The project includes the existence of a poster in brief visual impact that summarizes the five keys for understanding the problem food safety.

## 2.3. Zoonoses and food-borne outbreaks in the EU: 2010 EFSA-ECDC report

Zoonoses are infections and diseases naturally transmissible directly or indirectly, for example via contaminated foodstuffs, between animals and humans. The severity of these diseases in humans varies from mild symptoms to life-threatening conditions. In order to prevent zoonoses from occurring, it is important to identify which animals and foodstuffs are the main sources of infections. For this purpose, information aimed to protect human health is collected and analyzed from all European Union Member States.

The main pathogens described in EFSA-ECDC Zoonoses 2010 report were *Salmonella* (30,5%), viral agents (15%) and *Campylobacter* (8,9%). As in previous years, the main sources of outbreaks were eggs and egg products, responsible for 154 episodes, followed by mixed feeds and buffet (different types of foods potentially involved), vegetables, pork and derived products. Worthy of note is the increase in outbreaks caused by foods of plant origin in 2010 (61 outbreaks), compared to 2009 (21 outbreaks).

The European Union (EU) system for the monitoring and collection of information on Zoonoses is based on the Zoonoses Directive 2003/99/CE, which obligates EU Member States to collect relevant and, where applicable, comparable data on zoonoses, zoonotic agents, antimicrobial resistance and food-borne outbreaks. In addition, Member States should assess trends and sources of these agents as well as outbreaks in their territory, transmitting an annual report to the European Commission (CE), covering the data

collected. The European Food Safety Authority (EFSA) is assigned the tasks of examining these data and publishing the EU Summary Report.

The human data analyses in the EU Summary Report for 2010 were realized by the Food- and Water-borne Diseases and Zoonoses programme at ECDC and were based on the data submitted to the European Surveillance System (TESSy), hosted at ECDC. TESSy is a software platform that has been operational to collect data on 49 infectious diseases since April 2008. Both aggregated and case-based data were reported to TESSy. Although aggregated data did not include individual case-based information, both reporting formats have been used to calculate country-specific notification rates and trends in diseases. Data on human zoonoses cases were received from all 27 Member States and additionally from two non Member States: Iceland and Norway. Switzerland sent its data on human cases directly to EFSA. The European member states in 2010 were: Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Spain and Sweden. The population density of states is shown in the Table 1 and, thanks to the chart below, it is possible to understand most populated States:

| Country | 2010 |
|---|---|
| **Austria** | 83.753 |
| **Belgium** | 108.399 |
| **Bulgaria** | 75.637 |
| **Cyprus** | 8.031 |
| **Czech Republic** | 105.068 |
| **Denmark** | 55.347 |
| **Estonia** | 13.401 |
| **Finland** | 53.514 |
| **France** | 647.163 |
| **Germany** | 818.023 |
| **Greece** | 113.051 |
| **Hungary** | 100.143 |
| **Ireland** | 44.679 |
| **Italy** | 603.403 |
| **Latvia** | 22.484 |

| | |
|---|---|
| **Lithuania** | 33.290 |
| **Luxembourg** | 5.021 |
| **Malta** | 4.130 |
| **Netherlands** | 165.750 |
| **Poland** | 381.673 |
| **Portugal** | 106.377 |
| **Romania** | 214.622 |
| **Slovakia** | 54.249 |
| **Slovenia** | 20.470 |
| **Spain** | 459.890 |
| **Sweden** | 93.407 |
| **United Kingdom** | 620.080 |
| **EU total** | 5.011.057 |
| **Norway** | 48.582 |
| **Switzerland** | 79.060 |

**Table 1 Human population in the UE (value x100) in 2010.**

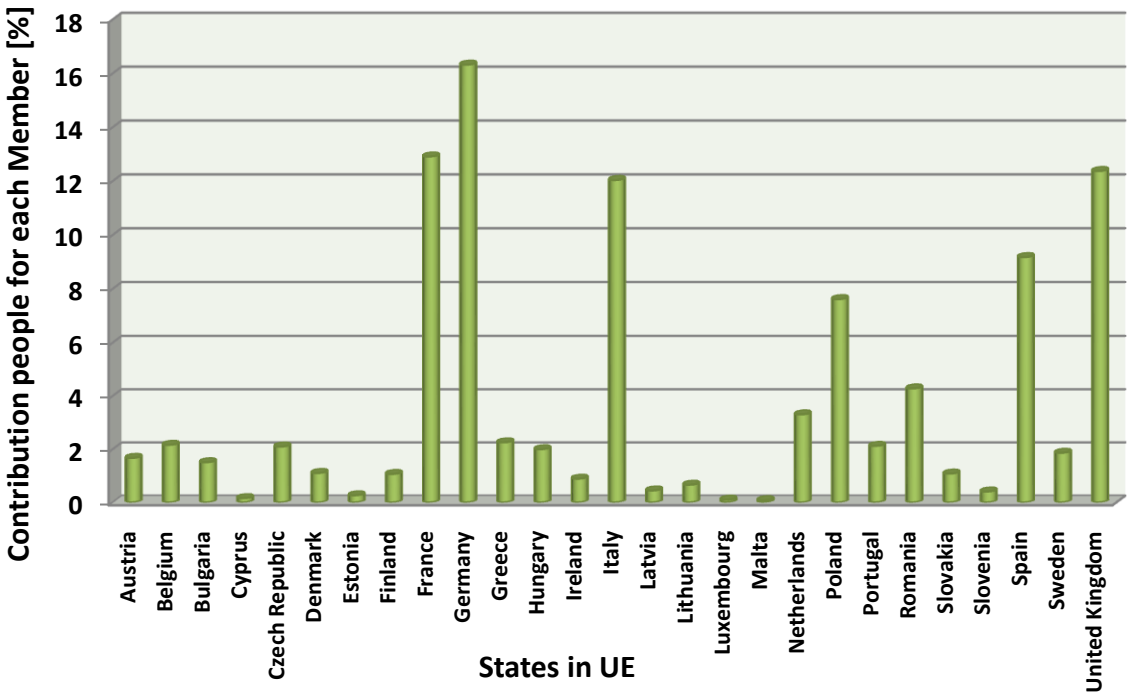



**Chart 1 People percentage contribution in each EU Member State.**

In 2010 data were collected on a mandatory basis for the following eight Zoonotic agents: *Salmonella,* thermophilic *Campylobacter, Listeria monocytogenes,* verotoxigenic

*Escherichia coli, Mycobacterium bovis, Brucella, Trichinella* and *Echinococcus*; data were reported on the following agents and Zoonoses*: Yersinia, rabies , Toxoplasma, Cysticerci, Francisella* and Q fever. Data on *Staphylococcus* and antimicrobial resistance in indicators *E. coli* and Enterococci isolates were also submitted. Furthermore, data on certain other microbiological contaminants in foodstuffs are: histamine, Staphylococcal enterotoxins and *Enterobacter sakazakii* (*Cronobacter spp.*), for which food safety criteria are set down in EU legislation.

### 2.3.1. Salmonellosis

In 2010, 99.020 confirmed cases of human Salmonellosis in Europe.

| Country | 2010 | | |
|---|---|---|---|
| | Cases | Confirmed cases | Confirmed cases/ 100.000 |
| **Austria** | 2.179 | 2.179 | 26,0 |
| **Belgium** | 3.169 | 3.169 | 29,2 |
| **Bulgaria** | 1.217 | 1.153 | 15,2 |
| **Cyprus** | 137 | 136 | 16,9 |
| **Czech Republic** | 8.456 | 8.209 | 78,1 |
| **Denmark** | 1.608 | 1.608 | 29,1 |
| **Estonia** | 414 | 381 | 28,4 |
| **Finland** | 2.422 | 2.422 | 45,3 |
| **France** | 7.184 | 7.184 | 11,1 |
| **Germany** | 25.306 | 24.833 | 30,4 |
| **Greece** | 300 | 299 | 2,6 |
| **Hungary** | 6.246 | 5.953 | 59,4 |
| **Ireland** | 356 | 349 | 7,8 |
| **Italy** | 2.730 | 2.730 | 4,5 |
| **Latvia** | 951 | 881 | 39,2 |
| **Lithuania** | 1.962 | 1.962 | 58,9 |
| **Luxembourg** | 211 | 211 | 42,0 |
| **Malta** | 160 | 160 | 38,7 |
| **Netherlands** | 1.447 | 1.447 | 13,6 |
| **Poland** | 9.732 | 9.257 | 24,3 |
| **Portugal** | 207 | 205 | 1,9 |
| **Romania** | 1.291 | 1.285 | 6,0 |
| **Slovakia** | 5.171 | 4.942 | 91,1 |
| **Slovenia** | 363 | 363 | 17,7 |
| **Spain** | 4.420 | 4.420 | 38,4 |
| **Sweden** | 3.612 | 3.612 | 38,7 |

| | | | |
|---|---|---|---|
| **United Kingdom** | 9.670 | 9.670 | 15,6 |
| **EU Total** | 100.921 | **99.020** | **21,5** |

**Table 2 Reported human Salmonellosis cases and notification rates for 2010.**

It was afterwards evidenced a decline of 8,8% compared to 2009. Every 10.000 inhabitants the rate of response was equal to 21,5 cases with a mortality equal to 0,13%. Indeed the serotype most frequently recognized as responsible in cases of human disease is *S. Enteritidis* with a 45% response followed by *S. Typhimurium* with 22,4%. Age groups most affected by Salmonellosis are children between the ages of 0 and 14 years with a peak in the end of the summer and early autumn.

| Serovar | Phagetypes | Outbreaks | | Human cases | | |
|---|---|---|---|---|---|---|
| | | N | % of EU total | N | Hospitalised | Deaths |
| **S. Enteritidis** | Unspecified | 182 | 53,4 | 2.068 | 486 | 2 |
| | PT 4 | 11 | 3,2 | 138 | 22 | 1 |
| | PT 8 | 4 | 1,2 | 30 | 12 | 0 |
| | PT 21 | 4 | 1,2 | 82 | 17 | 0 |
| | PT 2 | 6 | 1,8 | 88 | 14 | 0 |
| | PT 6 | 2 | 0,6 | 25 | 4 | 0 |
| **S. Typhimurium** | Unspecified | 36 | 10,6 | 948 | 150 | 2 |
| | Other | 1 | 0,3 | 172 | 0 | 0 |
| | DT 10 | 1 | 0,3 | 7 | 0 | 0 |
| | DT 120 | 1 | 0,3 | 20 | 0 | 0 |
| | DT 41 | 1 | 0,3 | 9 | 0 | 0 |
| | DT 8 | 2 | 0,6 | 116 | 23 | 1 |
| | DT 104 | 1 | 0,3 | 44 | 11 | 0 |
| | Not Typeable | 1 | 0,3 | 42 | 10 | 0 |
| **S. Typhimurium, monophasic** | DT 193 | 2 | 0,6 | 43 | 8 | 1 |
| | Unspecified | 1 | 0,3 | 2 | 2 | 0 |
| **S. Newport** | | 2 | 0,6 | 16 | 0 | 0 |
| **Salmonella spp.** | | 62 | 18,2 | 542 | 112 | 1 |
| **S. Mbandaka** | | 2 | 0,6 | 161 | 33 | 0 |
| **S. Infantis** | | 5 | 1,5 | 201 | 8 | 0 |
| **S. Virchow** | | 1 | 0,3 | 3 | 0 | 0 |
| **S. Montevideo** | | 1 | 0,3 | 4 | 1 | 0 |
| **S. Ohio** | | 1 | 0,3 | 4 | 0 | 0 |
| **S. Paratyphi B var, Java** | | 2 | 0,6 | 132 | 17 | 0 |
| **S. Saintpaul** | | 1 | 0,3 | 5 | 1 | 0 |
| **S. Choleraesuis** | | 1 | 0,3 | 15 | 15 | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **S. Dublin** | | 1 | 0,3 | 3 | 0 | 0 |
| **S. group D** | | 1 | 0,3 | 39 | 9 | 0 |
| **S. group D1** | | 1 | 0,3 | 3 | 3 | 0 |
| **S. Bareilly** | | 1 | 0,3 | 241 | 32 | 1 |
| **S. Kottbus** | | 1 | 0,3 | 4 | 0 | 0 |
| **Other Serovars** | | 2 | 0,6 | 5 | 4 | 0 |
| **EU Total** | | 341 | 100 | 5.212 | 994 | 9 |

**Table 3 *Salmonella* serovars reported for strong evidence food-borne outbreaks in the EU, 2010.**

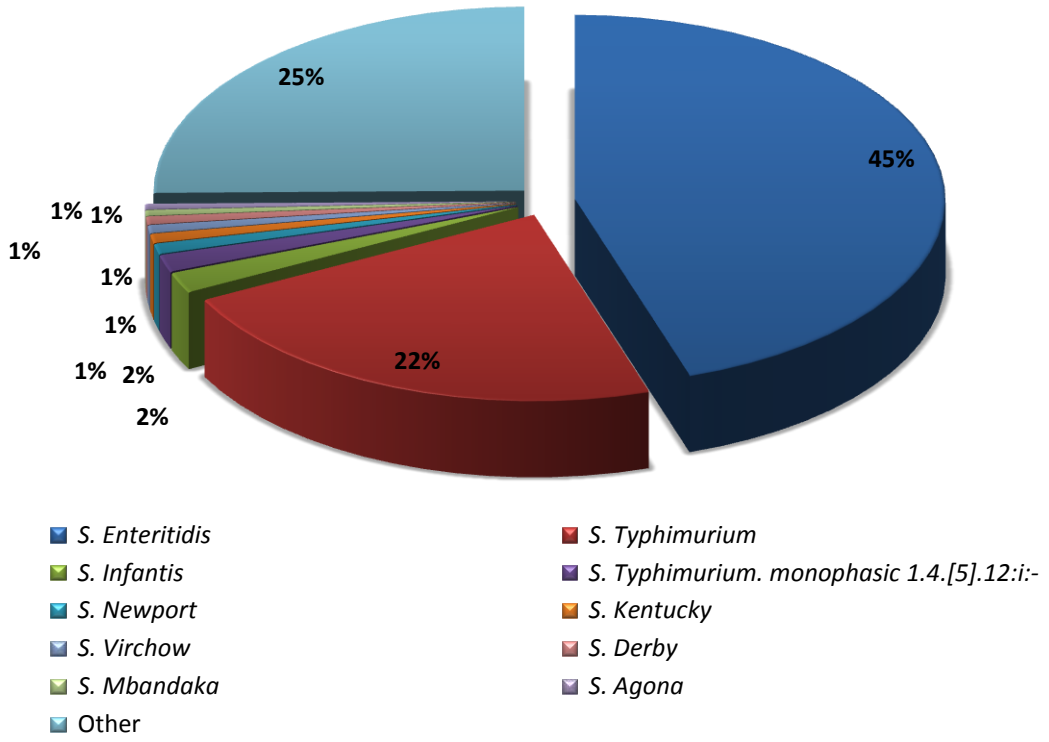



**Chart 2 Distribution of confirmed Salmonellosis cases in humans by serovar (10 most frequent serovars), TESSy 2010.**

Human *S. Enteritidis* cases are most commonly associated with the consumption of contaminated eggs and poultry meat, while *S. Typhimurium* cases are mostly associated with the consumption of contaminated pig, poultry and bovine meat. The number of human cases of *Salmonella spp.* in 27 Member States of the European Union continued to decline from 17,4% in 2009 to 8,8% in 2010.

In 2010, 62 deaths due to non-typhoidal Salmonellosis were reported.

In Germany there has been a decline in confirmed cases of 51,5%. Despite the decrease in different countries, 16 States have reported an increase in cases of human Salmonellosis

in 2010 compared to 2009. The largest increases were reported by Slovakia to 24,1% (760 cases) and 23,3% (736 cases) in Poland.

In the five years 2006-2010, the tendency of a statistically significant decrease was calculated in the European Union for the first time.

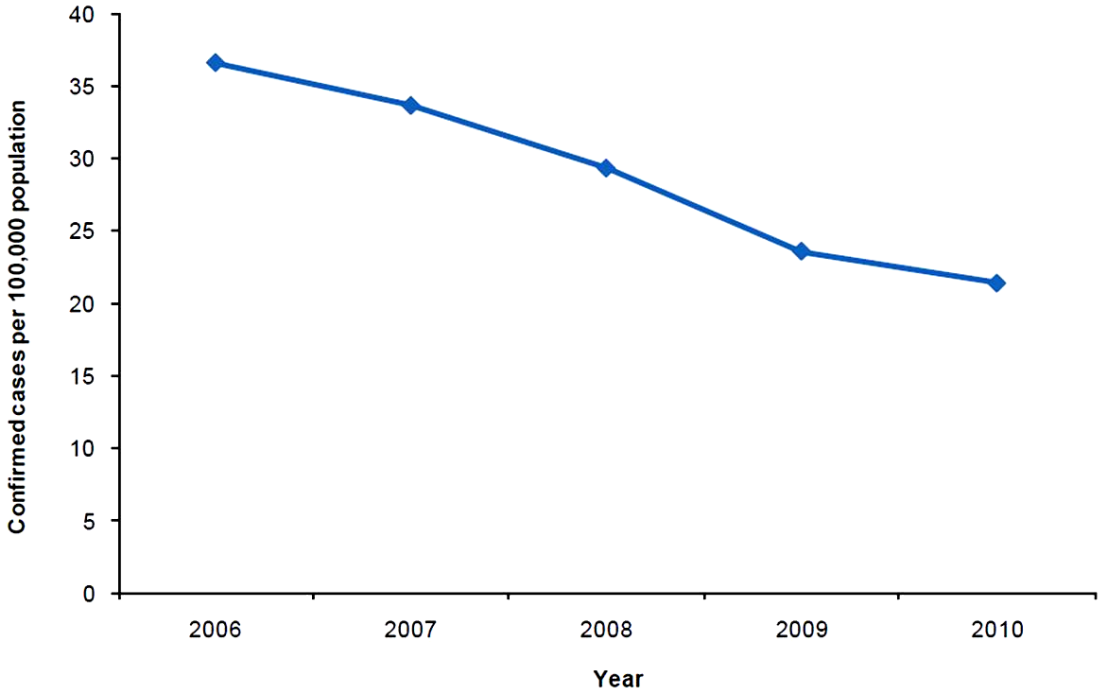



**Chart 3 Trend in reported confirmed cases per 100,000 population of human Salmonellosis in the EU, 2006-2010.**

Within the same period, the largest average annual drop of 25,0% of reported cases have been reported in the Czech Republic, while the highest average annual increase in the number of cases, 24 %, was observed in Malta, followed by Romania.

A very important case of Salmonellosis, defined as national or travel-associated disease (diagnosed once back from a foreign travel), remained at the level of 2009 (10,5% versus 10,9%). The proportion of confirmed cases with unknown origin are slightly less (26,0%) in 2010 compared to the previous year (27,1%). As before, three of the four Nordic countries - Finland, Sweden and Norway - continued to have the highest proportion of imported cases of Salmonellosis (83,8%, 73,8% and 65,5%, respectively), while the infections seem to be mainly domestically acquired in most other countries.

***Salmonella in food***

Salmonella criteria, laid down by Regulation (CE) No. 2073/2005, have been applied since 1 January 2006. The criteria were modified by Regulation (CE) No. 1441/2007, which came into force in December 2007. In 2008, 2009 and 2010 the highest levels of non-compliance of the genus *Salmonella spp.* for food of meat were recorded.

| Food categories | Total single samples | | | Total batches | | |
|---|---|---|---|---|---|---|
| | Sample weight | N | % non-compliant | Sample weight | N | % non-compliant |
| **Minced meat and meat preparations intended to be eaten raw** | 25 g | 3.373 | 1,8 | 25 g or not stated | 1.184 | 0,3 |
| **Minced meat and meat preparations from poultry intended to be eaten cooked** | 25 g or not stated | 2.458 | 5,3 | 10 g or 25 g or not stated | 8.248 | 0,9 |
| **Minced meat and meat preparations from other species than poultry intended to be eaten cooked** | 10 g or 25 g or various or not stated | 9.467 | 2,8 | 10 g or 25 g or not stated | 29.205 | 0,4 |
| **Mechanically separated meat** | 10 g or 25 g | 242 | 0 | 10 g or 25 g or 100 g or 200 g or not stated | 1.639 | 4,3 |
| **Meat products intended to be eaten raw** | 25 g | 164 | 0 | 25 g | 1.368 | 0 |
| **Meat products from poultry meat intended to be eaten cooked** | 25 g or various or not stated | 3.991 | 0,5 | 25 g or not stated | 4.407 | 1,2 |
| **Gelatine and collagen** | 25 g | 327 | 0 | 25 g | 87 | 0 |
| **Cheeses, butter and cream made from raw or low heat-treated milk** | 25 g or various | 1.685 | 0,1 | 25 g or not stated | 9.278 | <0,1 |
| **Milk and whey powder** | 25 g | 304 | 0 | 25 g or not stated | 3.011 | 0 |
| **Ice-cream** | 25 g or not stated | 11.245 | 0 | 25 g or not stated | 2.513 | 0 |
| **Egg products** | 25 g or not stated or various | 853 | 0,7 | 25 g or not stated | 1.282 | 0,3 |
| **RTE foods containing raw egg** | 25 g | - | - | 25 g | - | - |
| **Cooked crustaceans and molluscan shellfish** | 25 g | 80 | 0 | 25 g or not stated | 405 | 0,5 |
| **Live bivalve molluscs and** | 25 g | 201 | 1,5 | 25 g or not | 243 | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **live echinoderms, tunicates and gastropods** | | | | stated | | |
| **Sprouted seeds (RTE)** | 25 g | 130 | 0,8 | 25 g | - | - |
| **Pre-cut fruit and vegetables (RTE)** | 25 g | 1.975 | <0,1 | 25 g or not stated | 1.202 | 0 |
| **Unpasteurised fruits, vegetables and juices (RTE)** | 25 g | 25 | 0 | 25 g | 308 | 0 |
| **Dried infant formulae, and dried dietary foods for medical purposes2 and dried follow-on formulae** | 25 g | 873 | 0 | 25 g | 64 | 0 |

**Table 4 Compliance with the food safety *Salmonella* criteria laid down by EU Regulations 2073/2005 and 1441/2007, 2010.**

The highest level of non-compliance with the 5,3% of each sample was recorded by the minced chicken and meat preparations of poultry eaten after cooking. Minced meat and meat preparations of animal species other than poultry intended to be eaten cooked came in second place with 2,8% of individual samples that are positive for *Salmonella spp.*.

Of particular relevance, because of the risk they pose to human health, the results of *Salmonella spp.* in ready (like minced) meat, meat preparations intended to be eaten raw and shoots, with 1,8%. In other food categories, the level of non-compliance was usually very low and, in general, the level of non-compliance in 2010 is comparable to the results of previous years.

***Meat and meat products***

The highest positivity for cases of *Salmonella spp.* are to be ascribed to the fresh meat of chicken and turkey with respectively rates of 4,8% and 9,0%.

The global presence of *Salmonella spp.* in fresh turkey meat has increased compared to previous years.

| Country | Sample unit | Sample weight | 2010 | |
|---|---|---|---|---|
| | | | N | % pos |
| **At slaughter** | | | | |
| **Belgium** | Single | 1 g | 395 | 3 |
| **Cyprus** | Batch | 25 g | 184 | 0 |
| **Czech Republic** | Batch | 25 g | 725 | 7 |
| **Denmark** | Batch | 60 g | 346 | 0,3 |

| | | | | |
|---|---|---|---|---|
| **Estonia** | Batch | 25 g | 51 | 0 |
| **France** | Batch | 25 g | 67 | 10,4 |
| **Germany** | Single | 25 g | - | - |
| **Greece** | Single | 25 g | - | - |
| **Hungary** | Single | 25 g | 538 | 24 |
| **Ireland** | Single | Various | 430 | 6,3 |
| | Batch | Various | - | - |
| **Latvia** | Single | 10 g | - | - |
| **Poland** | Batch | 25 g | 272 | 11,4 |
| **Romania** | Batch | 25 g | 561 | 5,2 |
| **Spain** | Single | 25 g | 171 | 5,3 |
| **At processing/cutting plant** | | | | |
| **Austria** | Single | 25 g | - | - |
| **Belgium** | Single | 25 g | - | - |
| | Batch | 25 g | 358 | 5,9 |
| **Cyprus** | Batch | 25 g | 80 | 43,8 |
| **Czech Republic** | Batch | 25 g | 272 | 12,9 |
| **Estonia** | Batch | 25 g | 47 | 0 |
| **Finland** | Single | 25 g | 753 | 0 |
| **Germany** | Single | 25 g | 111 | 6,3 |
| **Greece** | Single | 25 g | - | - |
| **Hungary** | Single | 25 g | 273 | 20,5 |
| **Poland** | Single | 25g/300g | 35 | 8,6 |
| | Batch | 25 g | 530 | 11,9 |
| | Batch | 200g/500g | 55 | 9,1 |
| **Portugal** | Single | 25 g | 216 | 1,4 |
| **Romania** | Batch | 25 g | 73 | 0 |
| **Slovenia** | Single | 25 g | 100 | 2 |
| **Spain** | Single | 25 g | 63 | 7,9 |
| **Austria** | Single | 25 g | 372 | 5,6 |
| **Belgium** | Single | 25 g | - | - |
| | Batch | 25 g | 418 | 4,8 |
| **Bulgaria** | Batch | 25 g | 8677 | <0,1 |
| **Czech Republic** | Single | 27 g | - | - |
| **France** | Single | 25 g | 330 | 1,2 |
| **Germany** | Single | 25 g | 713 | 9 |
| | Single | 25 g | - | - |
| **Greece** | Single | 25 g | 28 | 0 |
| **Hungary** | Single | 25 g | 117 | 29,1 |
| **Latvia** | Single | 10g/25g | 75 | 5,3 |
| **Lithuania** | Single | 25 g | 26 | 23,1 |
| **Luxembourg** | Single | 25 g | 88 | 2,3 |
| **Netherlands** | Single | 25 g | 1092 | 4,7 |

| | | | | |
|---|---|---|---|---|
| | - | 25 g | - | - |
| **Portugal** | Batch | 25 g | 25 | 0 |
| **Romania** | Single | 25 g | - | - |
| | Batch | 25 g | 39 | 0 |
| **Slovakia** | Single | 25 g | - | - |
| **Slovenia** | Single | 25 g | - | - |
| **Spain** | Single | 25 g | 108 | 2,8 |
| **Sampling level not stated** | | | | |
| **Austria** | Single | 25 g | - | - |
| **Hungary** | Batch | 25 g | - | - |
| **Italy** | Batch | 25 g | - | - |
| | Batch | - | - | - |
| **Single** | - | | 277 | 4,3 |
| **Slovakia** | Batch | 25 g | - | - |
| **EU Total** | Total | | 21539 | **4,8** |
| | Single | | 6311 | 7,2 |
| | Batch | | 15228 | 3,8 |

**Table 5 *Salmonella* in fresh broiler meat at slaughter, processing/cutting level and retail 2010.**

| Country | Sample unit | Sample weight | 2010 | |
|---|---|---|---|---|
| | | | N | % pos |
| **At slaughter** | | | | |
| **Czech Republic** | Batch | 25 g | 255 | 3,9 |
| **Germany** | Single | 25 g | - | - |
| **France** | Batch | 25 g | 30 | 16,7 |
| **Hungary** | Single | 25 g | 489 | 14,5 |
| **Poland** | Batch | 25 g | 997 | 10,3 |
| **Cutting and processing plant** | | | | |
| **Finland** | Single | 25 g | 287 | 0 |
| **Germany** | Single | 25 g | 253 | 19 |
| **Hungary** | Single | 25 g | 331 | 6,9 |
| **Poland** | Batch | 10 g/25 g | - | - |
| **Slovenia** | Single | 25 g | 49 | 0 |
| **Spain** | Single | 25 g | - | - |
| **At retail** | | | | |
| **Austria** | Single | 25 g | 41 | 14,6 |
| **Bulgaria** | Batch | - | 46 | 0 |
| **France** | Single | 25 g | 242 | 8,7 |
| **Germany** | Single | 25 g | 942 | 5,9 |
| **Germany** | Single | 25 g | - | - |

| | | | | |
|---|---|---|---|---|
| **Hungary** | Single | 25 g | 106 | 7,5 |
| **Luxemburg** | Single | 25 g | - | - |
| **Netherlands** | Single | 25 g | 153 | 3,3 |
| **Romania** | Batch | 25 g | - | - |
| **Slovenia** | Single | 25 g | - | - |
| **Spain** | Single | 25 g | - | - |
| **Sampling level not stated** | | | | |
| **Italy** | Single | - | 108 | 29,6 |
| **Hungary** | Batch | 25 g | - | - |
| **Total (11 MSs in 2010)** | Total | | 4329 | 9 |
| **Single** | Single | | 3001 | 9 |
| **Batch** | Batch | | 1328 | 8,9 |

**Table 6 *Salmonella* in fresh turkey meat 2010.**

By a percentage equal to 0,9%, *Salmonella spp.* strains was detected in fresh pork; 0,2% of fresh beef sampling units were positive.

| Country | Sample unit | Sample weight | 2010 | |
|---|---|---|---|---|
| | | | N | % pos |
| **At slaughterhouse** | | | | |
| **Belgium** | Single | 600 $cm^2$ | 743 | 8,9 |
| **Czech Republic** | Batch | 100 $cm^2$ | 5718 | 0,4 |
| **Denmark** | Single | 300 $cm^2$ | 22485 | 1,2 |
| **Estonia** | Single | 1,400 $cm^2$ | 607 | 3,6 |
| **Finland** | Single | 1,400 $cm^2$ | 6559 | 0 |
| **Germany** | Single | 10 g | 4787 | 1 |
| **Hungary** | Single | - | - | - |
| **Latvia** | Single | - | - | - |
| **Poland** | Batch | 100 $cm^2$ | - | - |
| | Batch | 400 $cm^2$ | - | - |
| | Batch | 25 g | 9093 | 0,3 |
| **Portugal** | Single | 100 $cm^2$ | - | - |
| | Batch | - | - | - |
| **Romania** | Batch | 25 g | 1005 | 2,1 |
| | Batch | 400 $cm^2$ | - | - |
| **Spain** | Single | 25 g | 179 | 7,3 |
| **Sweden** | Single | 1,400 $cm^2$ | 5906 | 0 |
| **Norway** | Single | 1,400 $cm^2$ | 1811 | 0 |
| **At cutting/processing plants** | | | | |
| **Belgium** | Single | 25 g | - | - |

| | | | | |
|---|---|---|---|---|
| | Batch | 25 g | 297 | 1,7 |
| **Estonia** | Single | 25 g | 358 | 1,4 |
| **Finland** | Single | 25 g | 1529 | 0 |
| **Germany** | Single | 25 g | 593 | 2 |
| **Greece** | Single | 25 g | - | - |
| **Hungary** | Single | 25 g | - | - |
| **Ireland** | Single | 25 g | 25 | 0 |
| | Single | Various | - | - |
| **Lithuania** | Single | 25 g | - | - |
| **Poland** | Batch | 200 g | 46 | 0 |
| **Portugal** | Single | 25 g | 58 | 10,3 |
| **Romania** | Batch | 25 g | 98 | 2 |
| **Slovenia** | Single | 25 g | 292 | 0 |
| **Spain** | Single | 25 g | 48 | 10,4 |
| | At retail | | | |
| **Austria** | Single | 10 g/25 g | 1001 | 1,2 |
| **Bulgaria** | Batch | - | 4003 | 0 |
| **France** | Single | 25 g | 211 | 2,8 |
| **Germany** | Single | 25 g | 2154 | 2 |
| | Single | 25 g | - | - |
| **Greece** | Single | 25 g | - | - |
| **Hungary** | Single | 25 g | - | - |
| **Italy** | Single | 25 g | - | - |
| **Netherlands** | Single | 25 g | 642 | 0,5 |
| **Romania** | Batch | 25 g | 27 | 18,5 |
| | Single | 25 g | - | - |
| **Spain** | Single | 25 g | 111 | 9 |
| **United Kingdom** | Single | - | - | - |
| **Sampling level not stated** | | | | |
| **Hungary** | Batch | 25 g | - | - |
| **Italy** | Single | 25 g | - | - |
| | Single | - | 355 | 3,9 |
| | Batch | 25 g | - | - |
| | Batch | - | - | - |
| **Slovakia** | Batch | 25 g | - | - |
| **Sweden** | Single | - | 75 | 0 |
| **Total (17 MSs in 2010)** | | | 69005 | **0,9** |

**Table 7 *Salmonella* in fresh pig meat, at slaughter, cutting/processing level and retail 2010.**

| Country | Sample unit | Sample weight | 2010 | |
|---|---|---|---|---|
| | | | N | % pos |
| **At slaughterhouse** | | | | |
| **Czech Republic** | Batch | 100 $cm^2$ | 5053 | 0,3 |
| **Denmark** | Single | 300 $cm^2$ | 766 | 0,3 |
| **Estonia** | Single | 1,400 $cm^2$ | 286 | 0 |
| **Finland** | Single | 1,400 $cm^2$ | 3169 | 0 |
| **Germany** | Single | 10 g | 752 | 0,3 |
| **Hungary** | Single | 400 $cm^2$ | - | - |
| **Italy** | Single | - | 31 | 3,2 |
| **Latvia** | Single | - | - | - |
| **Poland** | Batch | 400 $cm^2$ | 463 | 0 |
| | Single | 400 $cm^2$ | 74 | 0 |
| **Portugal** | Batch | - | - | - |
| **Romania** | Batch | 400 $cm^2$ | - | - |
| | Batch | 25 g | 645 | 0 |
| **Spain** | Single | 25 g | 104 | 3,8 |
| **Sweden** | Single | 1,400 $cm^2$ | 361 | <0,1 |
| **Norway** | Single | 1,400 $cm^2$ | 1626 | 0 |
| **At processing/cutting plants** | | | | |
| **Estonia** | Single | 25 g | 183 | 0 |
| **Finland** | Single | 25 g | 1905 | 0 |
| **Germany** | Single | 25 g | 204 | 0,5 |
| **Hungary** | Single | 25 g | - | - |
| **Ireland** | Single | 25 g | 62 | 0 |
| **Poland** | Batch | 100 $cm^2$ | - | - |
| **Portugal** | Single | 25 g | 55 | 0 |
| **Romania** | Batch | 25 g | 98 | 0 |
| **Slovenia** | Single | 25 g | 291 | 0 |
| **Spain** | Single | 25 g | - | - |
| | At retail | | | |
| **Austria** | Single | 10 g/25 g | - | - |
| **Bulgaria** | Batch | - | 107 | <0,1 |
| **Germany** | Single | 25 g | 620 | 0,6 |
| **Greece** | Single | 25 g | - | - |
| **Hungary** | Single | 25 g | - | - |
| **Italy** | Single | - | 29 | 0 |
| **Luxembourg** | Single | 25 g | 48 | 0 |
| **Netherlands** | Single | 25 g | 667 | 0,7 |
| **Romania** | Batch | 25 g | - | - |
| **Romania** | Single | 25 g | - | - |

| | | | | |
|---|---|---|---|---|
| **Slovenia** | Single | 25 g | - | - |
| **Spain** | Single | 25 g | 88 | 2,3 |
| **United Kingdom** | Single | - | - | - |
| **Sampling level not stated** | | | | |
| **Hungary** | Batch | 25 g | - | - |
| **Italy** | Batch | 25 g | 44 | 0 |
| **Italy** | Single | - | 170 | 0 |
| **Slovakia** | Batch | - | - | - |
| | Single | 25 g | - | - |
| | Batch | 25 g | - | - |
| **Sweden** | Single | - | 87 | 0 |
| **Total (15 MSs in 2010)** | | | 34236 | **0,2** |
| | | | | |

**Table 8 *Salmonella* in fresh bovine meat, at slaughter, cutting/processing level and retail 2010.**

*Salmonella spp.* has been found in a very small percentage of table eggs (0,3%), lower than 2009 (0,5%).

In vegetables, fruits and herbs, 0,6% of the units tested were reported as positive.

| Country | Description | Sample unit | Sample weight | N | % pos |
|---|---|---|---|---|---|
| **Fruit** | | | | | |
| Italy | - | Single | - | 29 | 0 |
| At processing plant | | Batch | - | 121 | 0 |
| Products, at processing plant | | Batch | - | 32 | 0 |
| Products | | Single | - | 45 | 0 |
| **Vegetables** | | | | | |
| Italy | At processing plant | Batch | - | 91 | 0 |
| At retail | | Single | - | 57 | 0 |
| | | Single | - | 570 | 0,2 |
| Products | | Single | - | 94 | 2,1 |
| Sweden | | Single | - | 45 | 0 |
| **Fruit and vegetables** | | | | | |
| Bulgaria | Pre-cut | Batch | - | 107 | 0 |
| Germany | Pre-cut | Single | 25 g | 622 | 0,2 |
| Hungary | Pre-cut, RTE | Single | 25 g | 134 | 0 |
| Ireland | At retail | Single | Various | 222 | 0 |
| Products, at retail | Single | Various | 154 | 0 | |

| | | | | | |
|---|---|---|---|---|---|
| Portugal | Pre-cut, RTE | Batch | 25 g | 165 | 0 |
| Romania | Pre-cut, RTE, at processing plant | Batch | 25 g | 76 | 0 |
| Pre-cut, RTE, at retail | Batch | 25 g | 463 | 0 | |
| Slovakia | Pre-cut, RTE, at retail | Batch | 25 g | 132 | 0 |
| Slovenia | - | Batch | 25 g | 30 | 0 |
| Pre-cut, RTE, at retail | Single | 25 g | 100 | 0 | |
| Pre-cut, RTE | Batch | 25 g | 30 | 0 | |
| **Seeds, dried** | | | | | |
| Ireland | At retail | Single | Various | 341 | 0,9 |
| Seeds, sprouting | | | | | |
| Germany | RTE | Single | 25 g | 65 | 1,5 |
| Hungary | RTE | Single | 25 g | 65 | 0 |
| Salads | | | | | |
| Austria | RTE, at retail | Single | 25 g | 61 | 0 |
| | RTE, at retail, containing mayonnaise | Single | 25 g | 28 | 0 |
| Czech Republic | RTE, at processing plant | Batch | 25 g | 125 | 0 |
| | RTE, at retail | Batch | 25 g | 48 | 0 |
| Estonia | RTE, at processing plant | Single | 25 g | 38 | 0 |
| | RTE, at retail | Single | 25 g | 62 | 0 |
| Slovenia | RTE, at retail | Single | 25 g | 127 | 0 |
| Spain | RTE | Single | 25 g | 752 | 0 |
| **Herbs and spices** | | | | | |
| Austria | At processing plant | Single | 25 g | 36 | 0 |
| | At retail | Single | 25 g | 57 | 0 |
| Ireland | At retail | Single | Various | 28 | 3,6 |
| Netherlands | At retail | Single | 2,5 g | 392 | 2,3 |
| | At retail | Single | 25 g | 2098 | 1,6 |
| Slovakia | At retail | Batch | 25 g | 47 | 0 |
| Slovenia | Dried, non-irradiated, At retail | Single | 25 g | 44 | 0 |
| **Nuts and nut products** | | | | | |
| Ireland | At retail | Single | Various | 579 | 0 |
| **Total (15 MSs)** | | | | 8312 | **0,6** |

**Table 9 *Salmonella* in vegetables, fruit and herbs, 2010.**

In other food categories, the proportion of units not in accordance with the criteria was very low, except for the percentage of positive in live bivalve molluscs (1,5% of individual samples).

***Salmonella serovars in food***

The serotypes more frequently found were *S. Typhimurium* and *S. Derby* in pork, *S. Typhimurium* and *S. Dublin* in beef; *S. Typhimurium* Monophasic was the most commonly reported serotype in pork and beef.

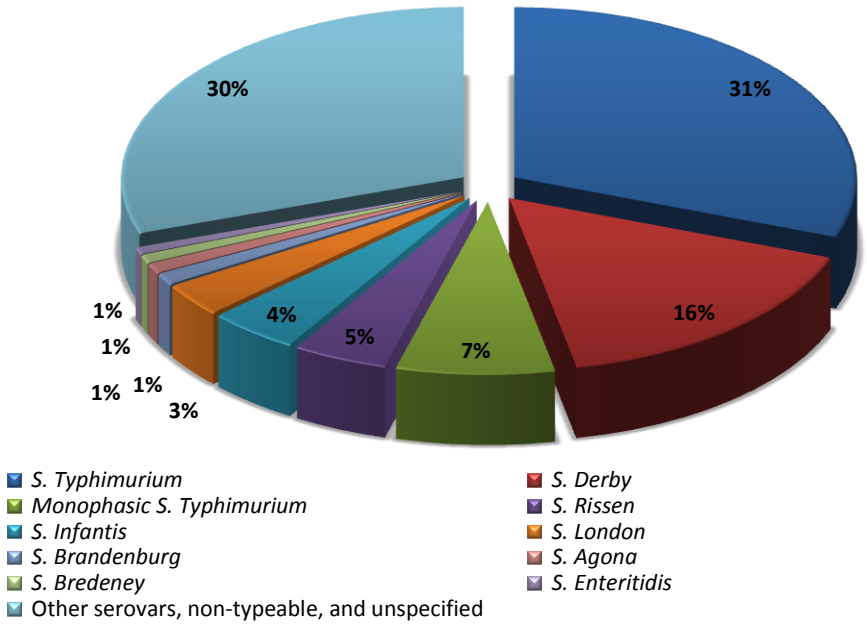



**Chart 4 Distribution of most common *Salmonella* serovars in pigs, 2010.**

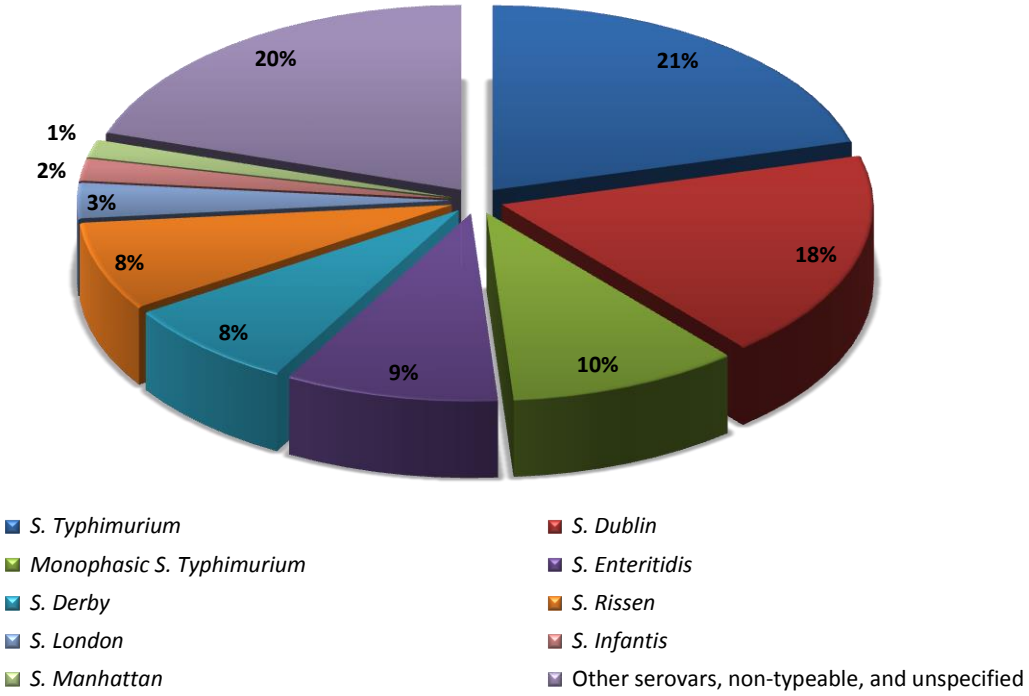



**Chart 5 Distribution of most common *Salmonella* serovars in bovine meat, 2010.**

From 2009 to 2010, European Union Member States increased from 18 to 20; the States aimed to the decreasing, compared to 2009, of the *Salmonella* species detection percentage to ≤ 1% in breeding flocks *Gallus gallus*. The reduction in the percentage considered five target serotypes: *S. Enteritidis, S. Typhimurium, S. Hadar, S. infantis, S. Virchow*. Data from 2010 highlighted an estimated rate of detection of the *Salmonella* five target serotypes of 0,7%, 1,2% in 2009. Only five EU countries have not reached this percentage goal, evidencing rates between 1,3 and 2,5%. Neverthless, this still represents a decrease, compared to 2009 (2,7%).

For turkey farms, 2010 was the first year for Member States to implement the objectives of reducing *Salmonella* (≤ 1% for *S. Enteritidis* and *S. Typhimurium*). All 13 States that have reported data on turkey breeding farms already achieved the goal, with a prevalence of 0,3% of two target serotypes. A further 20 Member States achieved the goal of turkey breeding farms for fattening before slaughter, with only one state not achieving the goal.

In 2010, 10 members of the European Union have contributed to the definition of the most frequent serotypes in poultry meat.

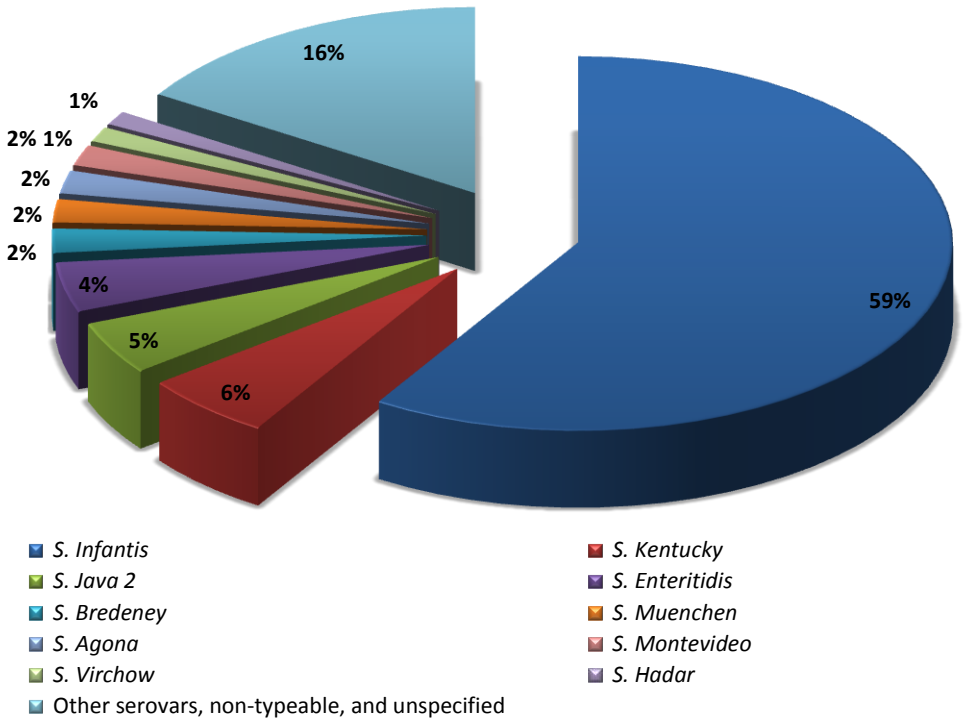



**Chart 6 Distribution of the 10 most common *Salmonella* serovars in broiler meat, 2010.**

Confirming the data of 2008 and 2009, *S. Infantis* is the most frequently serotype observed in chicken with a response rate of 58,9%, a result which is due to the origin of isolates from Hungary, where this serotype is dominant (almost in 96,0% of cases *Salmonella* serotype is *S. infantis*); moreover, in Austria and Romania more than 70% of all reported *Salmonella* in chicken meat are *S. Infantis*. The second most frequently observed serotype is *S. Kentucky* with a rate of 5,7%, mainly from isolates originating from Ireland with a percentage of 69,6%. The third most frequently reported serotype is *S. Java* with 4,6% with a very high prevalence in the Netherlands (53,5%) and in Germany (20,7%). The fourth most common serotype, *S. Enteritidis*, emerged with a percentage of 4,3%. The Czech Republic reported the highest percentage for *S. Infantis* and *S. Agona* (21,8%), while Italy recorded 16,4% of the isolates of *S. Muenchen* and 14,2% of *S. Montevideo*.

In 2009, the fifth most frequently observed serotype was *S. Typhimurium* although it was not classified in the 2010 top 10. This change may be due to reduction of *S. Typhimurium* in Germany, which weighed the majority of *S. Typhimurium* isolated in 2009 (32 isolates), none in 2010. In Germany, an active monitoring program for *Salmonella* in chicken meat

was performed in 2009. In 2010, the scientific contributions useful to define the most frequent serotypes were 2189, compared to the 1349 in 2009.

Regarding pork, 12 Member States most frequently reported *Salmonella* serotypes.

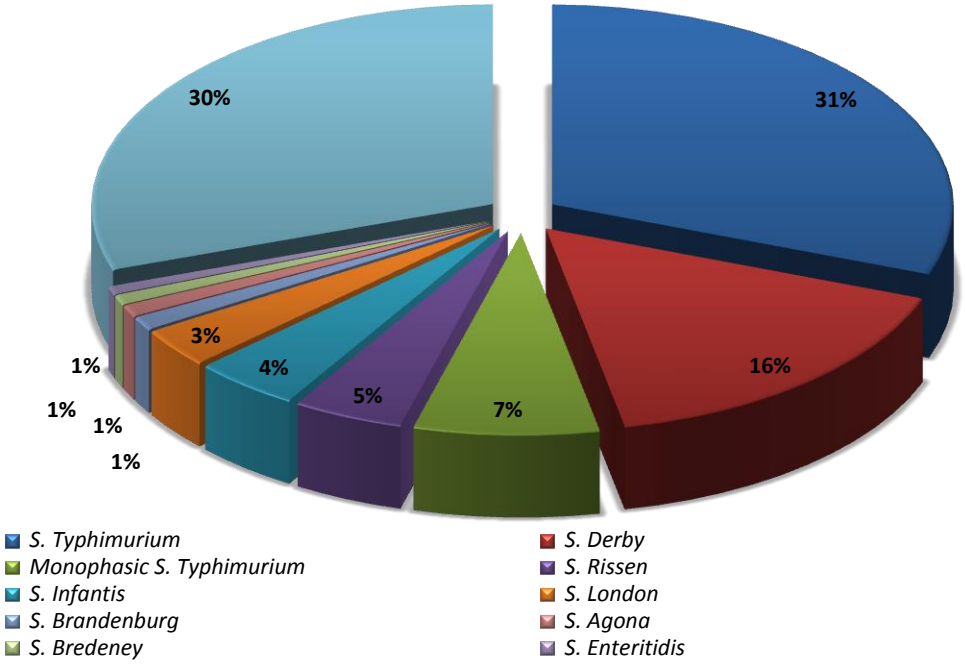



**Chart 7 Distribution of the 10 most common *Salmonella* serovars in pig meat in 2010.**

As in 2008 and 2009, *S. Typhimurium* and *S. Derby* were the most frequently isolated serotypes with respective percentages of 30,7% and 16,2%. On the other hand, Greece and Italy constitute an exception, since the most common serovar was *S. Rissen*. The third most reported serotype is *S. Typhimurium* Monophasic with a rate of 7,4%, but this contribution is available in only five states. The tenth most common serovar, *S. Enteritidis*, have been reported from Poland (10%) and Hungary (4%). Compared to 2009, *S. Agona* (which is often related to feed contamination) and *S. Bredeney* have replaced *S. Manhattan* and *S. Livingstone* in the top 10.

Regarding beef, in 2010, Member States which have contributed to the survey were seven, with very limited contributions.

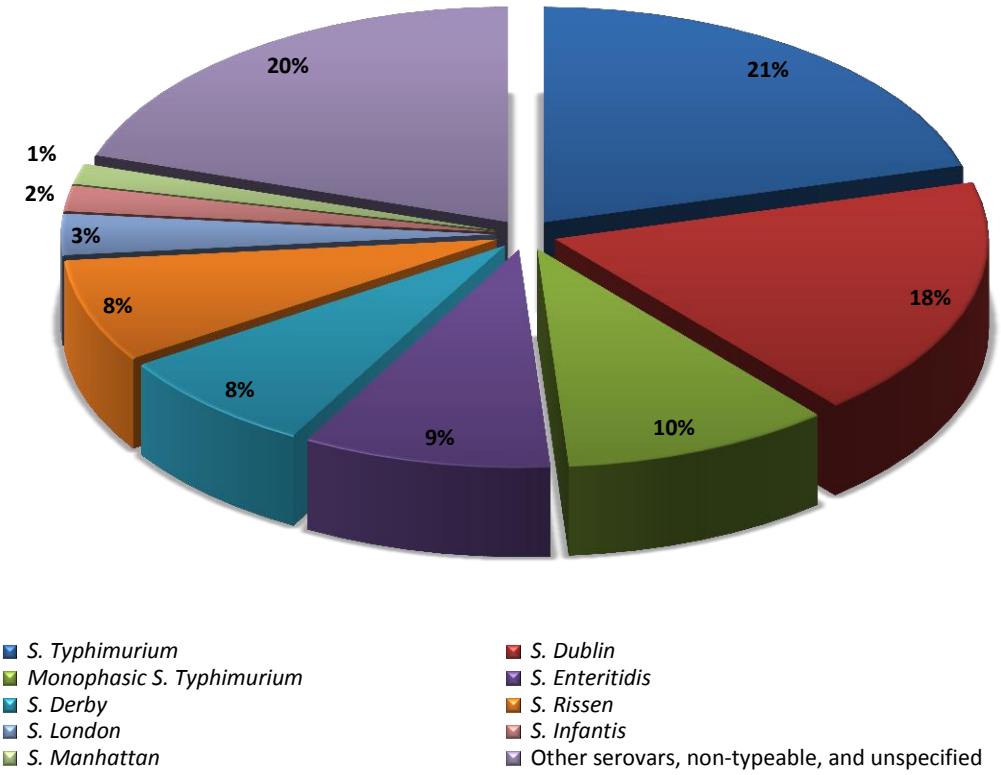



**Chart 8 Distribution of the 10 most common *Salmonella* serovars in bovine meat in 2010.**

Even in 2010, as reported in 2007-2009, *S. Typhimurium* (20,8%) and S. Dublin (18,1%) were the most frequently isolated serotypes in this type of meat. Despite this hierarchy, three States have reported the presence of *S. Dublin:* in Ireland, 69,2% of cases showed the presence of this serotype. The third most commonly reported serotype from four states has been *Monophasic S. Typhimurium*, with a percentage of 10,0% and it was the predominant serotype reported from Germany.

*Salmonella enteritidis* serotype was the most frequently isolated in Czech Republic, such as *S. Rissen* was in Italy, which, however, was also the only country bringing the information. *S.Bredeney, S.Bovismorbificans, S. Newport, S. Mbandaka* and *S. Tennessee* were ranked from tenth to fourteenth in beef.

Considering an overall picture of the most frequent serotypes besides their location or the type of food, as amended between 2009 and 2010, results bring out *S. Kentucky* (associated more with chicken) as the most observed isolate, followed by *S. Typhimurium* monophasic (which is mainly found in pigs), *S. Agona* and *S.Mbandaka,* all related to poultry and cattle; *S. Bovismorbificans* (porcine origin) emerged as fifth most common

isolate, preceding *S. Hadar,* typical of poultry (especially chickens and ducks) and *S. Saintpaul,* typical of turkeys. *S. Kentucky* (turkey and chicken meat), *S. Agona* (cattle, pork and chicken), *S. Mbandaka* (cattle and *Gallus gallus*) were respectively classified from eight to tenth.

Although some serotypes did not make the list, those still appear to be particularly common in some countries. As in 2008 and 2009, a high percentage of S. *Infantis* in chicken was reported by Hungary (96,0%), which was reflected in flocks of *Gallus gallus* in previous years, but no report of this serotype from *Gallus gallus* was provided in 2010.

*S. Infantis* has also been reported to account for 15,3% turkey meat, 10,0% pork and 8,3% beef from Hungary.

### 2.3.2. The Campylobacteriosis

The Campylobacteriosis in humans is caused by thermotolerant species of the genus *Campylobacter spp.*. Campylobacteriosis is an illness caused by an infectious dose also generally low. The species most commonly associated with human infection are *C. jejuni*, followed by *C. coli* and *C. lari*. The infection has an incubation period that ranges from two to five days. The symptoms may be more or less severe: diarrhea, abdominal pain, fever, headache and nausea.

Infections usually are self-limiting and tend to last a few days. There could be rare extra intestinal infections that can cause harmful effects such as arthritis and neurological disorders.

*C. jejuni* is strongly associated with the Guillain-Barré syndrome, resulting in paralysis that can lead to respiratory failure and severe neurological dysfunction and even death. The populations of thermotolerant *Campylobacter spp.* are widespread in nature. The main reservoirs are the alimentary tract of wild and domestic birds and mammals. They are prevalent in food animals, such as poultry, cattles, pigs and sheeps.

Bacteria of the genus *Campylobacter* may be present in different food matrices including meat, raw milk and dairy products, less frequently in fish and fishery products, molluscs and fresh vegetables. Among the sporadic human cases, contact with live poultry, consumption of chicken meat, drinking water from untreated sources of water and contact with pets and other animals have been identified as the main sources of

infection. Cross-contamination during the preparation of food at home has been described as an important transmission path. Raw milk and contaminated drinking water have been the cause of large outbreaks.

*Campylobacter* in 2010 has once again established itself as the most reported gastrointestinal pathogen in humans in the European Union since 2005, confirming this trend.

The number of confirmed cases of human Campylobacteriosis in the EU was 6,7% in 2010 (higher than 2009). The increase was also reflected on the overall rate of Campylobacteriosis notification in the European Union, from 45,6 per 100.000 population in 2009 to 48,56 per 100.000 inhabitants in 2010. In 2010, 266 deaths have been reported due to Campylobacteriosis.

| | | 2010 | | |
|---|---|---|---|---|
| **Country** | Report Type1 | Cases | Confirmed cases | Confirmed cases/ 100,000 |
| **Austria** | C | 4.405 | 4.405 | 52,6 |
| **Belgium** | C | 3.031 | 3.031 | 27,96 |
| **Bulgaria** | A | 6 | 6 | 0,08 |
| **Cyprus** | C | 55 | 55 | 6,85 |
| **Czech Republic** | C | 21.164 | 21.075 | 200,58 |
| **Denmark** | C | 4.037 | 4.037 | 72,94 |
| **Estonia** | C | 197 | 197 | 14,7 |
| **Finland** | C | 3.944 | 3.944 | 73,7 |
| **France** | C | 4.324 | 4.324 | 6,68 |
| **Germany** | C | 65.713 | 65.110 | 79,59 |
| **Greece** | | - | - | - |
| **Hungary** | C | 7.201 | 7.201 | 71,91 |
| **Ireland** | C | 1.662 | 1.660 | 37,15 |
| **Italy** | C | 457 | 457 | 0,76 |
| **Latvia** | C | 1 | 1 | 0,04 |
| **Lithuania** | C | 1.095 | 1.095 | 32,89 |
| **Luxembourg** | C | 600 | 600 | 119,51 |
| **Malta** | C | 204 | 204 | 49,4 |
| **Netherlands** | C | 4.322 | 3.983 | 46,21 |
| **Poland** | C | 375 | 367 | 0,96 |
| **Portugal** | | - | - | - |
| **Romania** | C | 179 | 175 | 0,82 |
| **Slovakia** | C | 4.578 | 4.476 | 82,51 |
| **Slovenia** | C | 1.022 | 1.022 | 49,93 |

| | | | | |
|---|---|---|---|---|
| **Spain** | C | 6.340 | 6.340 | 55,14 |
| **Sweden** | C | 8.001 | 8.001 | 85,66 |
| **United Kingdom** | C | 70.298 | 70.298 | 113,37 |
| **EU Total** | | 213.211 | 212.064 | **48,56** |
| **Iceland** | C | 55 | 55 | 17,32 |
| **Liechtenstein** | - | - | - | - |
| **Norway** | C | 2.682 | 2.682 | 55,21 |
| **Switzerland** | C | 6.604 | 6.604 | 85,05 |

**Table 10 Reported Campylobacteriosis cases in humans and notification rates for 2010.**

The performance of Campylobacteriosis shows an increasing trend over the last five years (2006-2010), more evident since 2008.

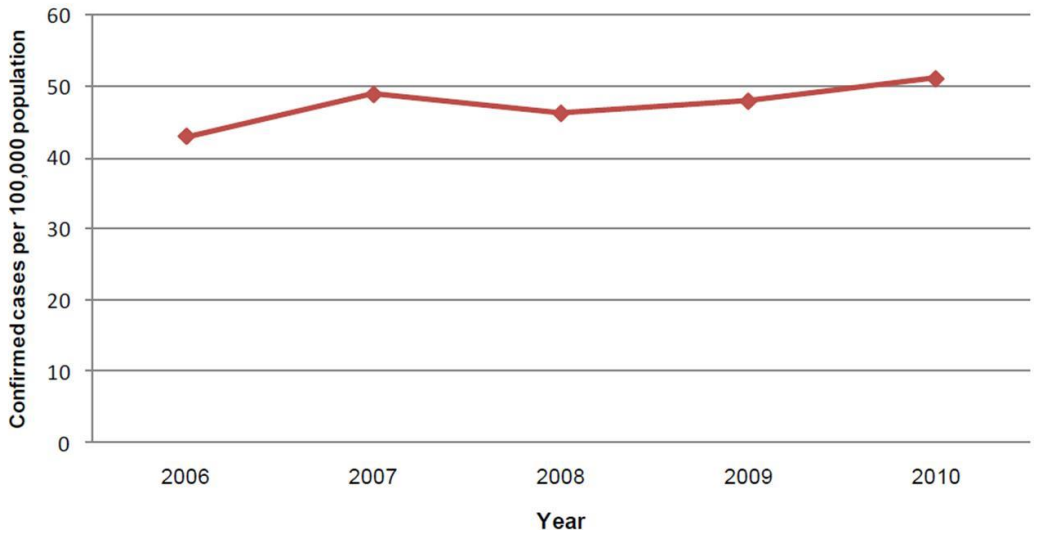



**Chart 9 Trend from 2005 of *Campylobacter* like a gastrointestinal pathogen in Humans in Europe.**

This trend was observed for 24 States of European Union: most significant findings are shown for Cyprus, Estonia, France, Luxembourg, Malta, Netherlands and Poland, with a statistically significant trend of reduction observed in Belgium and Bulgaria. As in previous years, children under five years of age had the highest notification rate in 2010. However, this rate was slightly lower than in 2009. Overall, notification rates for all ages kept on increasing. In 2010, the fatality rate was low, 0,22%. As in previous years, the highest rate number and notification of cases of *Campylobacter* in humans has been reported during the summer months, from June to August, gradually decreasing from September to

December. Examining data concerning responsibilities of the various *Campylobacter* species in relation to human diseases it is worth noting that in 2010 *C. jejuni* resulted with a 35,7% rate, *C. coli* stated a percentage equal to 2,3%. Other species reported included *C. lari* (0,22%) and *C. upsaliensis (*0,006%).

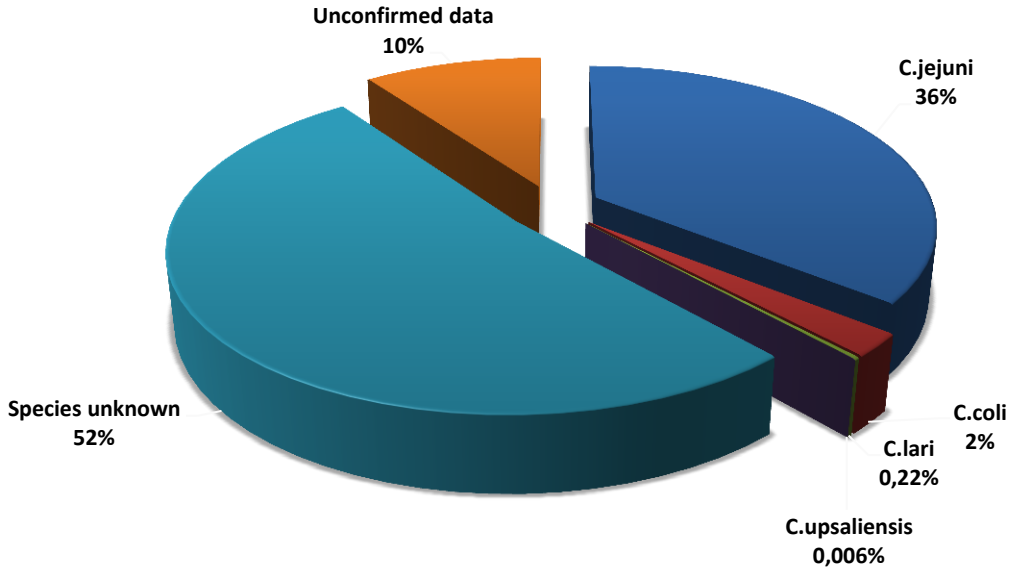



**Chart 10 Responsibilities of the various species of the genus *Campylobacter* in relation to human diseases.**

### *Campylobacter in food*

The presence of *Campylobacter* in broiler meat in 2010 evidences a percentage of 29,6%. The species of the genus are featured below:

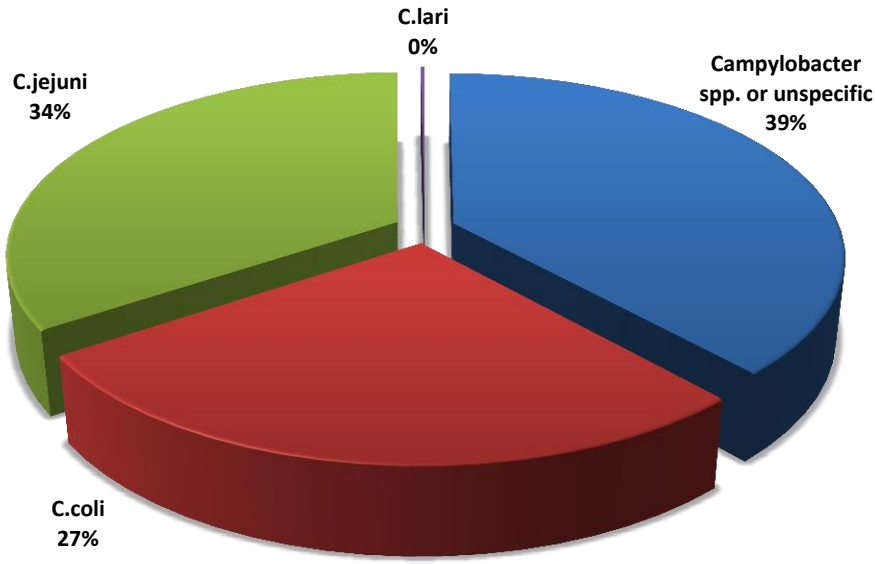



**Chart 11 Species distribution of Campylobacter isolates from fresh broiler meat, 2010.**

As in previous years, the percentage of samples of Campylobacter-positive broiler meat, at any level of sampling, varies greatly between Member States, even from 3,1% to 90,0%; in particular, seven Member States such as Austria, Hungary, Ireland, Luxembourg, Poland, Slovenia and Spain revealed very high percentages, above 50%.

| Country | Sample unit | Sample weight | 2010 | |
|---|---|---|---|---|
| | | | N | % pos |
| **At slaughter** | | | | |
| **Belgium** | Single | 1 g | 388 | 37,9 |
| **Denmark** | Single | 10 g/15 g | 1177 | 10,4 |
| **Estonia** | Batch | 1 g | 47 | 8,5 |
| **Hungary** | Single | 25 g | 170 | **54,1** |
| **Greece** | Single | 25 g | - | - |
| **Ireland** | Single | Various | 202 | **63,4** |
| **Italy** | Batch | Not indicated | 30 | 26,7 |
| **Poland** | Single | 400 $cm^2$ | 451 | **58,8** |
| **Romania** | Batch | 1 g | 225 | 40,4 |
| **Spain** | Single | 25 g | 139 | 44,6 |
| **At processing plants** | | | | |
| **Austria** | Single | 25 g | 30 | **90** |
| **Belgium** | Batch | 1 g | 358 | 8,9 |
| **Germany** | Single | 25 g | 107 | 47,7 |
| **Hungary** | Single | 25 g | 77 | 29,9 |
| **Poland** | Single | 10 g | 118 | **89** |
| **Portugal** | Single | 25 g | 108 | 19,4 |
| **Slovenia** | Single | 1 g | 100 | **79** |
| **Spain** | Single | 25 g | 178 | **74,7** |
| **At retail** | | | | |
| **Austria** | Single | 25 g | 324 | 3,1 |
| **Belgium** | Batch | 1 g | 439 | 12,1 |
| **Czech Republic** | Single | 25 g/27 g | - | - |
| **Denmark** | Single | 10 g/15 g | 767 | 46,2 |
| **France** | Single | 1 g | - | - |
| **Germany** | Single | 25 g | 681 | 28,5 |
| **Hungary** | Single | 25 g | 30 | 43,3 |
| **Latvia** | Single | 25 g | 50 | 10 |
| **Luxembourg** | Single | 10 g | 68 | **58,8** |
| **Netherlands** | Single | 25 g | 1023 | 9,9 |
| **Slovenia** | Single | 25 g | - | - |
| **Spain** | Single | 25 g | 126 | 25,4 |
| **Sampling level not** | | | | |

| | | | | |
|---|---|---|---|---|
| **stated** | | | | |
| **Italy** | Batch | Not indicated | - | - |
| | Single | Not indicated | - | - |
| **Total (16 MSs in 2010)** | | | 7413 | 29,6 |

**Table 11 *Campylobacter* in fresh broiler meat 2010.**

Observing *Campylobacter spp.* contamination trend in meat, it is worth a note that there is not a homogeneous supply of contributions from Member States of the European Union, which is why, given the number of samples tested and the highly variable methods sampling and testing, it is not properly possible to assess all the variables.

In particular, Austria, Belgium, Germany, Hungary, Poland and Spain have reported data from two or three stages of the food chain (slaughter, processing or retail).

A reduction in the presence of *Campylobacter spp.* in the food chains was primarily observed in Austria, Belgium and Germany. The presence of *Campylobacter spp.* is increasing in Hungary, Poland and Spain.

In 2010, seven States have helped to define the level of contamination of *Campylobacter spp.* even in turkey meat; 29,5% of positivity is due to the turkey meat, while 24,2% was isolated in other poultry meat samples.

| Country | Sample level | Sample unit | Sample Weight | N | % pos |
|---|---|---|---|---|---|
| **Turkeys** | | | | | |
| **Germany** | Slaughter | Single | 25 g | 359 | 68 |
| | Retail | Single | 25 g | 649 | 19,1 |
| **Hungary** | Slaughter | Single | 25 g | 69 | 26,1 |
| | Processing | Single | 25 g | 263 | 20,9 |
| | Retail | Single | 25 g | 68 | 22,1 |
| **Netherlands** | Retail | Single | 25 g | 135 | 6,7 |
| **Slovenia** | Processing | Single | 1 g | 49 | 10,2 |
| **Total turkeys (4 MSs)** | | | | 1.592 | **29,5** |
| **Other poultry** | | | | | |
| **Belgium (laying hens)** | Slaughter | Single | 1 g | 300 | 35,3 |
| **Hungary (ducks)** | Retail | Single | 25 g | 36 | 25 |
| **Hungary (geese)** | Slaughter | Single | 25 g | 123 | 8,1 |
| **Hungary (ducks)** | Slaughter | Single | 25 g | 167 | 18,6 |
| **Italy (unspecified)** | unspecified | Single | Not indicated | 40 | 12,5 |
| **Spain (unspecified)** | Retail | Single | 25 g | 46 | 23,9 |
| **Total other poultry (4 MSs)** | | | | 712 | 24,2 |

**Table 12 *Campylobacter* in fresh non-broiler poultry meat, 2010.**

These results indicate that poultry meat in general, not just chicken, represents an important vehicle for *Campylobacter spp.* because of infections in humans. Germany and Hungary are among the European Union member countries that have contributed most to the evaluation of samples of turkey in several stages of the production chain.

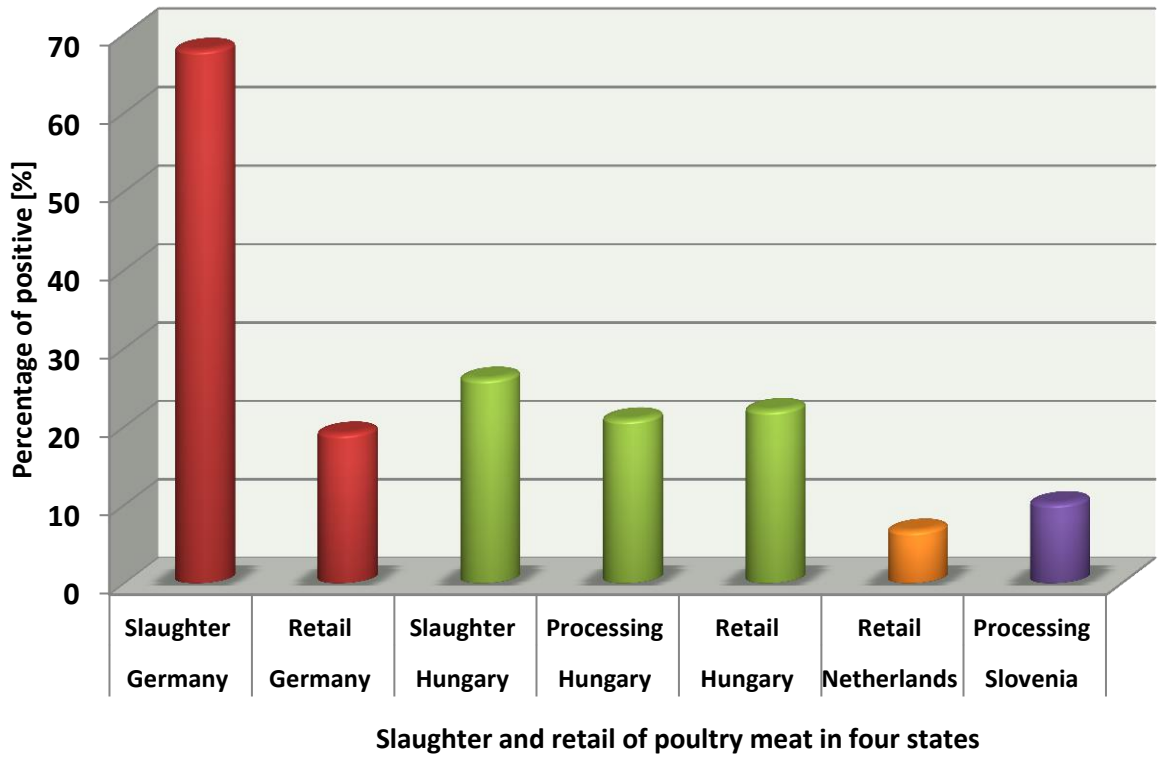



**Chart 12 *Campylobacter spp.* in fresh turkey meat, 2010.**

The percentage of positive samples recorded in retail phase, compared to the slaughter phase, considerably decreased in Germany, from 68,0% to 19,1%.

Instead, a not significant difference was observed between the proportions of positive samples at different stages in Hungary.

In the UK, the EFSA has developed a strategy for the reduction of cases of contamination of chicken meat from *Campylobacter*. This plan intends, within the period 2010-2015, to communicate the message that food produced or sold in the UK are safe to eat. The program includes a series of interventions designed to monitor various points along the food chain, from farm to table. In addition, it aims to reduce *Campylobacter* in chickens not only in terms of microbial loads, which now amounted to very high values of more than 1000 CFU/g, but also to break down the percentage value of the feedback from the

current 27% to a target of 10% in 2015. To achieve this goal a project has been designed in order to build a close collaboration between government and industry.

Data about pork samples provided by Member States which contributed to the monitoring in 2010, reported a low percentage of positivity for *Campylobacter spp.*.

| Country | Sample unit | Sample weight | 2010 | |
|---|---|---|---|---|
| | | | N | % pos |
| **Germany** | Single1 | 25 g | 174 | 1,7 |
| **Hungary** | Single | 25 g | 46 | 4,3 |
| **Latvia** | Single | 1 g | - | - |
| **Luxembourg** | Single | 10 g | - | - |
| **Netherlands** | Single | 25 g | 617 | 0 |
| **Spain** | Single | 25 g | 95 | 1,1 |
| **United Kingdom** | Single | Swab | - | - |
| **Total (4 MSs in 2010)** | | | 932 | **0,6** |

**Table 13 *Campylobacter* in fresh pig meat at retail 2010.**

An overall positive percentage of 0,6%, from 2008 to present, demonstrate that pork is rarely contaminated with *Campylobacter spp.*.

In 2010, Belgium, Germany, Hungary and Spain have reported data sampled at different stages of production. The presence of *Campylobacter spp.* at slaughter was 10,4%, at processing 0,4%. During slaughter stage, Spain has experienced a high percentage of positive samples (45,5%). Assessing processing stage, Germany produced 0,9% of positive results, Poland 27,9% and Portugal 1,7%.

Five Member States of the European Union have contributed communicating data on the presence of *Campylobacter spp.* in fresh bovine in 2010.

| Country | Sample unit | Sample weight | 2010 | |
|---|---|---|---|---|
| | | | N | % pos |
| **Germany** | Single | 25 g | 53 | **1,9** |
| **Hungary** | Single | 25 g | 70 | 0 |
| **Luxembourg** | Single | 10 g | 58 | 0 |
| **Netherlands** | Single | 25 g | 595 | **0,3** |
| **Spain** | Single | 25 g | 32 | 0 |
| **United Kingdom** | Single | | - | - |
| **Total (5 MSs in 2010)** | | | 808 | **0,4** |

**Table 14 *Campylobacter spp.* in fresh bovine meat at retail 2010.**

The response rate was equal to 0,4% of positive samples. Data mostly reflect the trend of previous years. The only States to report the positive results were Germany, with a market share of 1,9% and the Netherlands (0,3%). Poland was the only State which recorded a larger proportion of *Campylobacter spp.*, with a share of 15,0% during slaughter stage. Hungary reported slaughtering, processing and retailing data since 2010, describing a decrease of *Campylobacter spp.* from 2,2% at slaughter phase to 0,6% during transformation and retail.

For meat and ready to use products in 2010, *Campylobacter spp.* was isolated only from products based on chicken and turkey meat in Germany and Ireland.

| Country | Description | Sample unit | Sample weight | N | N pos | % pos |
|---|---|---|---|---|---|---|
| **Broiler meat** | | | | | | |
| **Germany** | Meat products at retail | Single | 25 g | 126 | 8 | **6,3** |
| **Ireland** | Meat products at processing | Single | 25 g | 50 | 0 | 0 |
| **Ireland** | Meat products at retail | Single | Various | 400 | 3 | **0,8** |
| **Slovakia** | Meat products at retail | Batch | 25 g | 34 | 0 | 0 |
| **Total broiler meat (3 MSs)** | | | | 610 | 11 | 1,8 |
| **Turkey meat** | | | | | | |
| **Germany** | Meat products at retail | Single | 25 g | 36 | 1 | **2,8** |
| **Ireland** | Meat products at processing | Single | 25 g | 29 | 0 | 0 |
| **Ireland** | Meat products at retail | Single | Various | 77 | 0 | 0 |
| **Total turkey meat (2 MSs)** | | | | 142 | 1 | 0,7 |
| **Pig meat** | | | | | | |
| **Ireland** | Meat products at processing | Single | 25 g | 116 | 0 | 0 |
| **Ireland** | Meat products at retail | Single | Various | 173 | 0 | 0 |
| **Total pig meat (1 MS)** | | | | 289 | 0 | 0 |
| **Bovine meat** | | | | | | |
| **Ireland** | Meat products at retail | Single | Various | 98 | 0 | 0 |
| **Total bovine meat (1 MS)** | | | | 98 | 0 | 0 |
| **Unspecified meat** | | | | | | |
| **Ireland** | Meat products at retail | Single | Various | 76 | 0 | 0 |
| **Total unspecified meat (1 MS)** | | | | 76 | 0 | 0 |

**Table 15 *Campylobacter spp.* in ready-to-eat meat products of meat origin, 2010.**

Both results were positive at a low positivity levels. *Campylobacter spp.* was not isolated from pork and beef.

In 2009, the most common species found in the contamination of meat were related to *C. jejuni*; unfortunately, almost half of *Campylobacter spp.* isolates were only reported as *Campylobacter spp*.. Only five Member States (Austria, Hungary, Ireland, Poland and Romania) have listed *C. coli* as the predominant specie by a total percentage equal to 45,9%, 59,3% in fresh poultry meat, while *C. jejuni* has been declared as the predominant specie (60,0%-62,0%) in three Member States, Germany, Luxembourg and Slovenia. *C. lari* was found in fresh poultry meat in Germany, Hungary and Romania.

The Campylobacteriosis results, since 2005, the most commonly emerged Zoonotic diseases in humans in the EU. In 2010, the number of reported cases of Campylobacter in the EU increased by 6,7%, compared to 2009. There has been a more evident growing trend in 2008. The reasons for this increasing trend are still not completely understood. One possible explanation for the continuing marked increase since 2008 may be linked to strong policies for the decrease of human Salmonellosis.

### 2.3.3. *Listeria monocytogenes*

*Listeria* genus comprises eight species: *Listeria monocytogenes*, in particular, is responsible for Listeriosis cases in humans species. The major medium of transmission to humans and animals is believed to be through the consumption of contaminated food or feed.

The forms of human Listeriosis often affect the fetus, children, the elderly and those with compromised immune systems. Symptoms vary, ranging from mild flu-like symptoms and diarrhea to life-threatening infections, characterized by septicemia and meningoencephalitis. In pregnant women, the infection can spread to the fetus, which can both be born severely ill or die in uterus, resulting in abortion. The disease is often severe and the outcome is often fatal. These organisms are among the most important causes of death from food-borne infections in industrialized countries.

In 2010, the 26 Member States that have reported 1601 confirmed human cases of Listeriosis.

| Country | 2010 | | | |
|---|---|---|---|---|
| | Report Type | Cases | Confirmed cases | Confirmed cases/ 100.000 |
| **Austria** | C | 34 | 34 | 0,41 |
| **Belgium** | C | 40 | 40 | 0,37 |
| **Bulgaria** | A | 4 | 4 | 0,05 |
| **Cyprus** | C | 1 | 1 | 0,12 |
| **Czech Republic** | C | 26 | 26 | 0,25 |
| **Denmark** | C | 62 | 62 | **1,12** |
| **Estonia** | C | 5 | 5 | 0,37 |
| **Finland** | C | 71 | 71 | **1,33** |
| **France** | C | 312 | 312 | 0,48 |
| **Germany** | C | 390 | 377 | 0,46 |
| **Greece** | C | 10 | 10 | 0,09 |
| **Hungary** | C | 20 | 20 | 0,2 |
| **Ireland** | C | 10 | 10 | 0,22 |
| **Italy** | C | 95 | 95 | 0,16 |
| **Latvia** | C | 7 | 7 | 0,31 |
| **Lithuania** | A | 5 | 5 | 0,15 |
| **Luxembourg** | U | 0 | 0 | 0 |
| **Malta** | C | 1 | 1 | 0,24 |
| **Netherlands** | C | 72 | 72 | 0,43 |
| **Poland** | C | 59 | 59 | 0,15 |
| **Portugal** | - | - | - | - |
| **Romania** | C | 6 | 6 | 0,03 |
| **Slovakia** | C | 5 | 5 | 0,09 |
| **Slovenia** | C | 11 | 11 | 0,54 |
| **Spain** | C | 129 | 129 | **1,12** |
| **Sweden** | C | 63 | 63 | 0,67 |
| **United Kingdom** | C | 176 | 176 | 0,28 |
| **EU Total** | | 1.614 | **1.601** | 0,35 |
| **Iceland** | C | 1 | 1 | 0,31 |
| **Liechtenstein** | - | - | - | - |
| **Norway** | C | 23 | 23 | 0,47 |
| **Switzerland** | C | 67 | 67 | 0,9 |

**Table 16 Reported Listeriosis cases in humans and notification rate for confirmed cases, 2010.**

Compared to 2009, there was a decrease of 3,2%; rates are mostly attributable to Finland with 1,33 cases per 100.000 inhabitants, followed by Denmark and Spain (1,12 cases per 100,000 inhabitants). Analyzing Listeriosis cases in Europe between 2006-2010, it is

evident a statistically significant upward trend in Austria, Latvia and Spain, and a relevant downward trend in Belgium, Czech Republic, Luxembourg and Slovakia.

The age distribution contemplates an increasing in the number of cases comprised in age range > 65 years (1,21 cases per 100.000 inhabitants) with a 60,2% percentage of all reported cases; 6,7% cases were detected in the age group 0-4 years and most of these cases were reported in children under the age of 1 year.

Transmission route was referred only for 8,26% of confirmed cases; within these, due to contamination in relation to food, there are 87 cases and 43 cases were associated with pregnancy. One case has been reported as transmission by contact with animals.

Among the cases of infection associated with the consumption of food, contaminated cheese was listed as the suspect vehicle. The outcome of the disease is known for the 1063 confirmed cases (66,3%). 17% confirmed cases of Listeriosis have seen death in children with high percentage, followed by the middle-aged and, to finish, the elderlies.

The criteria for *L. monocytogenes* is provided by Regulation No. (CE) 2073/2005: there is to underline that fishery products were listed as the category with the highest levels of non-compliance (4,9%).

| | | Absence in 25 g | | ≤100 cfu/g | |
|---|---|---|---|---|---|
| **Food category** | Sampling unit | Units tested | % in non-compliance | Units tested | % in non-compliance |
| **RTE food intended for infants and for medical purposes** | | | | | |
| **Processing plant** | Batch | 70 | 0 | - | - |
| **Retail** | Single | 746 | 0 | 41 | 0 |
| **Retail** | Batch | 446 | 0 | | - |
| **RTE products of meat origin other than fermented sausage** | | | | | |
| **Processing plant** | Single | 5.221 | 2,3 | - | - |
| | Batch | 12.684 | 1,7 | - | - |
| **Retail** | Single | - | - | 12.474 | 0,4 |
| | Batch | - | - | 3.577 | 0,6 |
| **RTE products of meat origin, fermented sausage** | | | | | |
| **Processing plant** | Batch | - | - | 36 | 0 |
| **Retail** | Single | - | - | 102 | 0 |
| | Batch | - | - | 33 | 0 |
| **Milk, RTE** | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| **At farm** | Single | 26 | 11,5 | - | - |
| **Processing plant** | Single | 401 | 0 | - | - |
| | Batch | 1.312 | **2,7** | - | - |
| **Retail** | Single | - | - | 238 | 0 |
| | Batch | - | - | 2.528 | 0 |
| **Soft and semi-soft cheeses, RTE** | | | | | |
| **At farm** | Batch | 34 | 8,8 | - | - |
| **Processing plant** | Single | 1.046 | 0,9 | - | - |
| | Batch | 2,91 | 1,5 | - | - |
| **Retail** | Single | - | - | 3.358 | **0,2** |
| | Batch | - | - | 3.783 | 0,8 |
| **Hard cheeses, RTE** | | | | | |
| **Processing plant** | Single | - | - | 366 | 0,3 |
| | Batch | - | - | 422 | 0 |
| **Retail** | Single | - | - | 1.375 | 0 |
| | Batch | - | - | 6.254 | 0 |
| **Other Dairy products, RTE** | | | | | |
| **At farm** | Batch | 65 | 6,2 | - | - |
| **Processing plant** | Single | 1.418 | 0 | - | - |
| | Batch | 1.378 | 0,8 | - | - |
| **Retail** | Single | - | - | 615 | **0,2** |
| | Batch | - | - | 978 | 0 |
| **Fishery products, RTE** | | | | | |
| **Processing plant** | Single | 612 | 9,6 | - | - |
| | Batch | 330 | 4,5 | - | - |
| **Retail** | Single | - | - | 3.442 | **1** |
| | Batch | - | - | 476 | 0 |
| **Other RTE products** | | | | | |
| **Processing plant** | Single | 243 | **4,9** | - | - |
| | Batch | 727 | 2,2 | - | - |
| **Retail** | Single | - | - | 9.786 | 0,1 |
| | Batch | - | - | 1.707 | 0,2 |

**Table 17 *L.monocytogenes* non compliance (%) in food.**

Samples of different fermented sausage meat or cheese and other dairy products encountered non-compliance ranges from 0% to 2,3%. Worrying results concern milk samples with a response rate of 2,7%, of which 32 raw milk positive samples for human consumption in the Czech Republic and three from pasteurized milk in Ireland.

Among the analyzed retail foods in 2010, the highest level of non-compliance to the criterion (≤ 100 CFU/g between individual samples taken at the point of sale) have been observed in fishery products (1%) and meat based products (0,4%). The non-compliance was also observed for soft and semi-soft cheeses (0,2%) and other dairy products (0,2%).

To evaluate the contamination of bovine meat products by *L. monocytogenes,* 17 Member States contributed, although the available data were lower than in previous years.

| Country | Sampling unit | Units tested presence | presence in 25 g | Units tested enumeration | > 100 cfu/g |
|---|---|---|---|---|---|
| | | N | % pos | N | % pos |
| **At processing/ cutting plant** | | | | | |
| **Czech Republic** | Batch | **814** | **0,1** | 185 | 0 |
| **Ireland** | Single | 52 | 0 | - | - |
| **Poland** | Batch | 40 | 0 | - | - |
| **Single** | Meat products | 105 | **2,9** | 40 | - |
| **At retail** | | | | | |
| **Austria** | Single | 256 | 5,9 | 255 | 0 |
| **Bulgaria** | Batch | - | - | 93 | 0 |
| **Ireland** | Single | 183 | 1,6 | 355 | 0 |
| **Netherlands** | Single | - | - | 31 | 0 |
| **Single** | | - | - | 1.209 | 0,2 |
| **Total (6 MSs)** | | **1450** | **1,5** | 2.168 | 0,1 |

**Table 18 *L. monocytogenes* in ready-to-eat products of bovine meat, 2010.**

In 2010, 1.450 samples were collected. The contamination by *L. monocytogenes* was detected in 25 g of 1,5% of these units. The highest presence of *L. monocytogenes* in production was recorded in samples of meat products from Poland by a percentage equal to 2,9%. Another significant contribution has to be referred to the Czech Republic, where 0,1% of the 814 batches tested meat products have revealed the presence of *Listeria monocytogenes*.

The findings relating to products derived from pork were made possible thanks to the collaboration of 16 Member States.

| | | Description | Units tested presence | presence in 25 g | Units tested enumeration | > detection but ,100 cfu/g | >100 cfu/g |
|---|---|---|---|---|---|---|---|
| **Country** | Sampling unit | | N | % pos | N | % pos | % pos |
| **At processing/ cutting plant** | | | | | | | |
| **Czech Republic** | Batch | - | 6.461 | 1,2 | 1.445 | 0 | 0 |
| **Denmark** | Batch | - | 44 | 4,5 | 110 | 0 | 0 |
| **Estonia** | Single | - | 114 | 7 | - | - | - |
| **Germany** | Single | Heat treated meat prod. | 86 | 3,5 | 64 | 0 | 0 |
| **Hungary** | Single | - | 107 | **13,1** | 43 | 16,3 | 0 |
| **Ireland** | Single | - | 131 | 0 | - | - | - |
| **Poland** | Batch | - | 2.913 | 2,3 | 806 | 0 | 0,6 |
| | Batch | Intended to be eaten raw | 115 | **31,3** | 70 | 0 | 0 |
| | Single | - | 3,1 | 1,9 | 1,04 | 0,2 | 0,5 |
| **Portugal** | Single | - | 122 | **9** | 122 | 7,4 | 1,6 |
| **Romania** | Batch | - | 26 | 7,7 | - | - | - |
| **Slovakia** | Batch | - | 243 | 0,8 | - | - | - |
| **At retail** | | | | | | | |
| **Austria** | Single | - | 348 | 2 | 347 | 0 | 0 |
| **Bulgaria** | Batch | - | 231 | 0 | 551 | 0 | 0 |
| **Czech Republic** | Single | - | 71 | 1,4 | 71 | 1,4 | 0 |
| | Batch | - | - | - | 180 | 0 | 0 |
| **Denmark** | Single | - | 40 | **5** | 76 | 0 | 0 |
| **Estonia** | Single | - | - | - | 36 | 0 | 0 |
| **France** | | RTE | 5.827 | 0,6 | 5.827 | <0,1 | <0,1 |
| **Germany** | Single | Heat treated meat prod. | 903 | 3,3 | 727 | 1,4 | 0,1 |
| **Greece** | Single | - | 36 | 0 | - | - | - |
| **Hungary** | Single | - | 125 | 3,2 | 33 | 0 | 3 |
| **Ireland** | Single | - | 213 | 1,4 | 718 | 0 | 0 |
| **Netherlands** | Single | - | 105 | **10,5** | 106 | 0 | 0 |
| | Single | Intended to be eaten raw | 219 | 11,4 | - | - | - |
| **Portugal** | Batch | - | - | - | 1.345 | 0 | 1,1 |
| **Romania** | Batch | - | 91 | 1,1 | - | - | - |
| **Sampling level not specified** | | | | | | | |
| **Spain** | Single | Unspecified RTE | 487 | 9,7 | 455 | 1,5 | 9,5 |
| **Total (16 MSs)** | | | **22.158** | **2** | 14.172 | 0,3 | 0,5 |

**Table 19 *L. monocytogenes* in ready-to-eat products of pig meat, 2010.**

Qualitative investigations were performed on 22.158 units, and *L. monocytogenes* was detected in 25 g of 2,0% samples. The proportion of units positive for *L. monocytogenes* varied from 0% to 31,3%. Poland was the country where there was, in terms of percentage, the major contamination of pork with a percentage that is around 31,3%, followed by Hungary (13,1%) and Portugal (9,0%).

Evaluating the levels of contamination referred to retail products, Netherlands and Denmark counted the highest rates. In the early stages, from manufacturing to retail sales, increases in the proportion of positive samples for *L. monocytogenes* were observed in Czech Republic, Denmark and Ireland, while decreases were observed in Germany, Hungary and Romania. Quantitative surveys about ready to eat food (pork) revealed low presence of units higher than 100 CFU/g of samples from Spain, Hungary and France. The investigation of the poultry has been conducted with the help of thirteen States.

| Country | | Description | Units tested presence | Presence in 25 g | Units tested enumeration | > detection but ,100 cfu/g | L, m, >100 cfu/g |
|---|---|---|---|---|---|---|---|
| | Sampling unit | | N | % pos | N | % pos | % pos |
| **At processing/ cutting plant** | | | | | | | |
| **Belgium** | Batch | Broiler meat products | 106 | 3,8 | 59 | 3,4 | 0 |
| **Czech Republic** | Batch | Broiler meat products | 542 | 0,6 | 226 | 0 | 0 |
| **Germany** | Single | Broiler meat products | 56 | 0 | 25 | 0 | 0 |
| **Hungary** | Single | Broiler meat products | 80 | 7,5 | 33 | 9,1 | 0 |
| | Single | Turkey meat products | 77 | 2,6 | - | - | - |
| **Ireland** | Single | Broiler meat products | 460 | 0 | - | - | - |
| | Single | Turkey meat products | 30 | 0 | - | - | - |
| | Single | Poultry meat, unspecified | 30 | 3,3 | - | - | - |
| **Poland** | Batch | Broiler meat products | 306 | 0 | - | - | - |
| | Single | Broiler meat products | 405 | - | 361 | 0,6 | 0 |
| | Batch | Turkey meat products | 70 | 0 | - | - | - |
| **Portugal** | Single | Broiler meat products | 36 | 2,8 | 36 | 2,8 | 0 |
| | Single | Turkey meat products | 34 | 11,8 | 34 | 11,8 | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Romania** | Batch | Broiler meat products | 235 | 0 | - | - | - |
| **Slovakia** | Batch | Broiler meat products | 40 | 0 | - | - | - |
| **At retail** | | | | | | | |
| **Bulgaria** | Batch | Broiler meat products | 89 | 0 | 310 | 0 | 0 |
| **Estonia** | Single | Broiler meat products | - | - | 42 | 0 | 0 |
| **Germany** | Single | Broiler meat products | 270 | 4,1 | 194 | 1,5 | 1 |
| **Hungary** | Single | Broiler meat products | 188 | 5,9 | 33 | 0 | 3 |
| | Single | Turkey meat products | 218 | 1,8 | - | - | - |
| **Ireland** | Single | Broiler meat products | 220 | 1,4 | 886 | 0,5 | 0,1 |
| | Single | Turkey meat products | 42 | 2,4 | 156 | 0 | 0 |
| **Netherlands** | Single | Broiler meat products | 45 | 4,4 | 49 | 0 | 0 |
| **Not specified** | | | | | | | |
| **Spain** | Single | Broiler meat products | 57 | 0 | - | - | - |
| **Total (13 MSs)** | | | **3.636** | 1,5 | **2.444** | 0,8 | 0,2 |

**Table 20 *L. monocytogenes* in ready-to-eat products of poultry meat, 2010.**

The contamination by *L. monocytogenes* in ready-to-use food was evaluated for chicken and turkey meat products. Of 3636 poultry meat based products tested, *Listeria monocytogenes* has been isolated with positivity from 0% to 7,5% for chicken meat and from 0% to 11,8% for turkey meat. Recorded increases on detection of *L. monocytogenes* within the production chain can be attributed to chicken meat in Germany and turkey meat for Ireland. Hungary has experienced a decrease in *L. monocytogenes* in chicken and turkey meat. Quantitative surveys were conducted on 2.444 ready for consumption poultry meat products, but only three states have reported retail samples with levels above 100 CFU/g. Data are included in 0,1%-3,0% range, and the highest percentage was reported by Hungary.

In turkey meat none of the samples contained levels above 100 CFU/g.

The report *L. monocytogenes* research assessed other foods such as cheese (of various kinds), fishery products and other ready for consumption foods.

Cases of food-borne Listeriosis in 2010 highlighted three cases of severe outbreaks caused by *Listeria* in two Member States. The vehicles identified were fish, meat mixture and a not specified source.

A wide range of different food products could be potentially contaminated by *L.monocytogenes*. In relation to human population health, foods that contain less than 100 CFU/g are considered to present a negligible microbiological risk and therefore EU criterion for *L. monocytogenes* in ready for consumption foods is set to ≤ 100 CFU/g.

### 2.3.4. *Escherichia coli* verotoxin-producing (VTEC)

The human pathogenic VTEC strains often possess critical virulence factors linked to the development of human disease. A large number of serogroups of *E. coli* have been recognized as verocytotoxin producers. Human VTEC infections are, however, more often associated to two different types of antigens, O and H, that define different serogroups. Between these, the O157:H7 and O157:H-serogroups (VTEC O157) are the most frequently strains associated with human disease.

The majority of Human VTEC infections reports evidence sporadic cases. The symptoms associated with VTEC infection in humans varies from mild to bloody diarrhea, which is often accompanied by abdominal cramps, usually without fever. VTEC infections can cause hemolytic uremic syndrome (HUS). HUS is characterized by acute renal failure, anemia and lowered platelet counts. HUS develops in approximately 10% of patients infected with VTEC O157 and is the leading cause of acute renal failure in young children. Human infection caused by strains of *E. coli* VTEC can be contracted through the consumption of contaminated food or water, by direct transmission from person to person or from infected animals to humans. Animals are the reservoir for VTEC and VTEC (including VTEC O157) have been isolated from many animal species from the gastrointestinal tract of healthy ruminants, including cows, goats and sheeps.

This condition shows the ability of these animals to contaminate the surrounding environment by expelling their own feces. This possibility means that even foods such as vegetables can be contaminated with VTEC or sources of drinking water. The way many VTEC serogroups that can be isolated from animals and food for infections in humans is, however, not yet clear.

The highest notification rate occurred in the age group 0-4 years, followed by children aged between 5 and 14 years.

The serotypes responsible for human infections are attributable to different antigen classes.

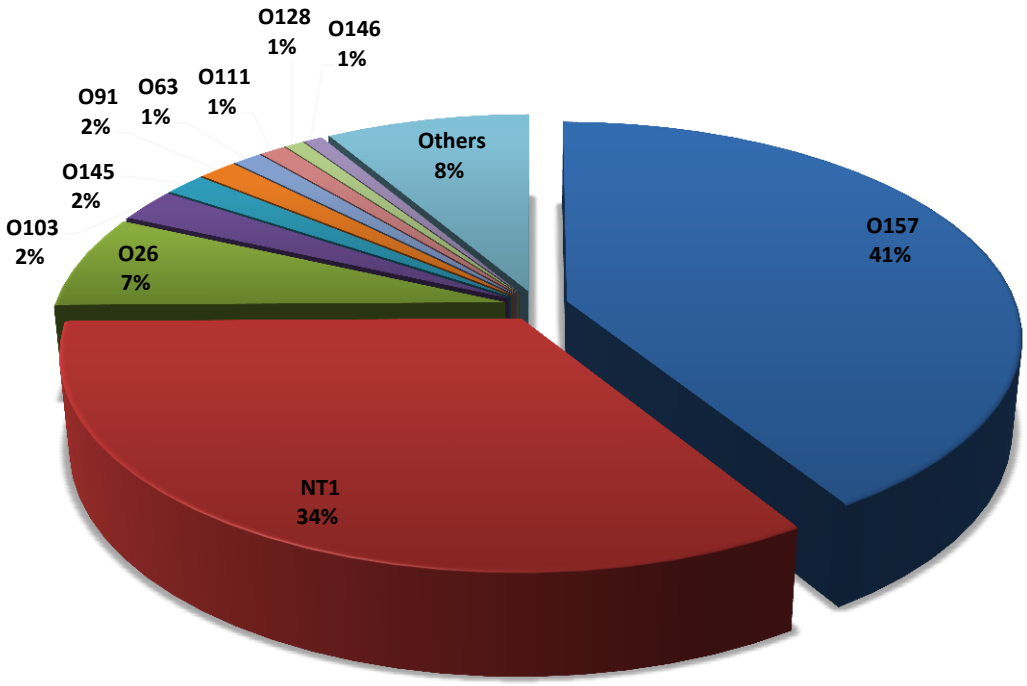



**Chart 13 Reported confirmed VTEC cases in humans by serogroup (top 10), 2010.**

Almost half of the O serogroups reported were O157 (41,1%). This represents a decrease of 18,8% compared to the reported cases associated with serogroup O157 in 2009. As in previous years, the highest percentage of O157 has been registered from United Kingdom and Ireland. Another serotype of public health importance, resulted in lower number in 2010, was VTEC O104. Two confirmed cases of VTEC O104 have been reported in Austria and one in Sweden. In addition, a confirmed case has been declared from Finland as travel-associated (Egypt).

In 2010, the number of human cases due to VTEC infection has increased by 12.0% compared to 2009.

| Country | 2010 | | | | 2009 |
|---|---|---|---|---|---|
| | Report Type1 | Cases | Confirmed cases | Confirmed cases/ 100,000 | Confirmed cases |
| **Austria** | C | 88 | 88 | 1,05 | 91 |
| **Belgium** | C | 84 | 84 | 0,77 | 96 |
| **Bulgaria** | U | 0 | 0 | 0 | 0 |
| **Cyprus** | U | 0 | 0 | 0 | 0 |
| **Czech Republic** | - | - | - | - | - |
| **Denmark** | C | 186 | 175 | 3,16 | 160 |
| **Estonia** | C | 5 | 5 | 0,37 | 4 |
| **Finland** | C | 21 | 21 | 0,39 | 29 |
| **France** | C | 103 | 103 | 0,16 | 93 |
| **Germany** | C | 1.317 | 1.304 | 1,59 | 878 |
| **Greece** | C | 1 | 1 | 0,01 | 0 |
| **Hungary** | C | 7 | 7 | 0,07 | 1 |
| **Ireland** | C | 199 | 197 | 4,41 | 237 |
| **Italy** | C | 41 | 31 | 0,05 | 51 |
| **Latvia** | U | 0 | 0 | 0 | 0 |
| **Lithuania** | C | 1 | 1 | 0,03 | 0 |
| **Luxembourg** | C | 7 | 7 | 1,39 | 5 |
| **Malta** | C | 1 | 1 | 0,24 | 8 |
| **Netherlands** | C | 478 | 478 | 2,88 | 313 |
| **Poland** | C | 4 | 3 | 0,01 | 0 |
| **Portugal** | -2 | - | - | - | - |
| **Romania** | C | 2 | 2 | 0,01 | 0 |
| **Slovakia** | C | 10 | 10 | 0,18 | 14 |
| **Slovenia** | C | 20 | 20 | 0,98 | 12 |
| **Spain** | C | 18 | 18 | 0,04 | 14 |
| **Sweden** | C | 334 | 334 | 3,58 | 228 |
| **United Kingdom** | C | 111 | 111 | 1,79 | 1.339 |
| **EU Total** | | **4.037** | 4 | 0,83 | **3.573** |
| **Iceland** | C | 2 | 2 | 0,63 | 8 |
| **Liechtenstein** | - | - | - | - | - |
| **Norway** | C | 50 | 50 | 1,03 | 108 |
| **Switzerland** | C | 31 | 31 | 0,4 | 42 |

**Table 21 VTEC infection, 2009-2010.**

2010 represents the third consecutive year that registered an increase of VTEC human infection in the EU. As in previous years, children under 4 years are the group at highest

risk of infection with VTEC O157. In 2010, the most commonly reported serotypes were O157: H7, followed by O157: H and O103: H2.

Many Member States only evaluate O157 strains and therefore the results do not represent the true situation.

Unfortunately, data are not comparable between the years and that is why it is not possible to make conclusions about trends. However, VTEC serogroups and human pathogens can be found from a wide range of animal species and different categories of foods. The levels of positive VTEC seem to vary greatly between Member States.

EFSA opinion on monitoring BIOHAZ VTEC37 currently considers the most effective serogroups for pathogenicity onset in humans: O26, O91, O103, O111, O145 and O157. Monitoring should be extended to include these serogroups in the future.

The joint EFSA-ECDC summarizes the results reported Shiga toxin/verotoxin-producing *E. coli* (STEC/VTEC) prevalence and incidence in humans, food and animals. In this report, a special mention was given to the strain STEC O104: H4, which was isolated as the causative agent for the largest outbreak ever recorded, occurred in Germany in May 2011. Before the year 2010, the serotype of the outbreak strain STEC O104: H4 was very rare and only a few case reports in humans have been diagnosed and reported. Furthermore, the serotype has never been registered in animals or foods.

***Foods of bovine origin***

Beef is considered the main responsible for food-borne infections VTEC to humans.

| | 2010 | | |
|---|---|---|---|
| **Country** | N | VTEC | VTEC O157 |
| | | % pos | % pos |
| **At slaughter, cutting/processing plant** | | | |
| **Belgium** | 913 | 0,7 | 0 |
| **Bulgaria** | - | - | - |
| **Czech Republic** | 632 | 0,5 | 0,5 |
| **Estonia** | 113 | 0 | 0 |
| **France** | - | - | - |
| **Germany** | 257 | **2,3** | 0,4 |
| **Hungary** | 319 | 0 | 0 |
| **Ireland** | 90 | 0 | 0 |

| | | | |
|---|---|---|---|
| **Poland** | - | - | - |
| **Romania** | 804 | 0 | 0 |
| **Slovenia** | - | - | - |
| **Spain** | 33 | 0 | 0 |
| **At retail** | | | |
| **Belgium** | - | - | - |
| **Bulgaria** | 428 | 0 | 0 |
| **France** | 2.476 | 0,2 | <0,1 |
| **Germany** | 491 | **2,2** | 0 |
| **Hungary** | 158 | 0 | 0 |
| **Ireland** | - | - | - |
| **Italy** | - | - | - |
| **Latvia** | - | - | - |
| **Luxembourg** | - | - | - |
| **Netherlands** | 818 | 0,1 | 0,1 |
| **Poland** | - | - | - |
| **Romania** | 472 | 0 | 0 |
| **Slovenia** | - | - | - |
| **Spain** | 92 | **5,4** | **1,1** |
| **United Kingdom** | - | - | - |
| **Level of sampling not specified** | | | |
| **Bulgaria** | 46 | 0 | 0 |
| **Germany** | 394 | **1,5** | 0,3 |
| **Hungary** | - | - | - |
| **Italy** | 30 | 0 | 0 |
| **Slovakia** | - | - | - |
| **Total (12 MSs in 2010)** | **8.566** | **0,5** | **0,1** |

**Table 22 VTEC in fresh bovine meat by country, year 2010.**

In 2010, 12 States underlined that they had investigated 8.566 units made from beef (from surveys of 25 or more samples) with a positive response of 0,5% for the VTEC-positive and VTEC O157-positive; the response rate is equal to 0,1%.

Member States who declared a match up of VTEC were Belgium, Germany and Spain, reporting proportions of positive units of 1,0% or higher. Compared to 2009, there has been an increase in the detection of VTEC-positive and VTEC O157 for bovine meat in 2010.

Additional serogroups of major importance to human health are VTEC O26, O91, O111, O103 and O145.

In 2010, serogroups O26 and O145 were detected in beef from France. Noteworthy the role of raw cow's milk as another VTEC risk related food.

Member States Bulgaria, Germany, Hungary and Slovakia have contributed to the data collection of VTEC in raw cow's milk survey: as a result, Germany registered a moderate rate (17,6%). This is substantially higher than that recorded in previous years by Germany; between strains, however, VTEC O157 never appeared until 2010.

Other foods involved in the risk of contamination by VTEC strains are those from sheeps and goats.

| Country | Description | Sample weight | 2010 | | |
|---|---|---|---|---|---|
| | | | N | VTEC | VTEC O157 |
| | | | | % pos | % pos |
| **Ovine** | | | | | |
| **At retail** | | | | | |
| **Bulgaria** | Fresh | - | 80 | 0 | 0 |
| **Germany** | Fresh | 25 g | 62 | **21** | 0 |
| **Netherlands** | Fresh | 25 g | 121 | 0 | 0 |
| **At slaughter, cutting/processing plant** | | | | | |
| **Ireland** | Carcass | - | - | - | - |
| **Poland** | Fresh | 1 g | - | - | - |
| **Spain** | Fresh | 25 g | - | - | - |
| **Not specified** | | | | | |
| **Bulgaria** | - | - | 44 | 0 | 0 |
| **Germany** | - | 25 g | 87 | **18,4** | 0 |
| **Italy1** | Fresh | 25 g | - | - | - |
| **Total (3 MSs in 2010)** | | | 394 | 7,4 | 0 |
| **Goat** | | | | | |
| **Spain** | Fresh | 25 g | - | - | - |

**Table 23 VTEC in fresh ovine and goat meat, 2010.**

Only Germany has reported positive samples for VTEC. The percentage of positive VTEC samples in Germany increased from 5,4% in 2007 to 21,0% in 2010 in retail food and from 7,3% to 18,4% in all investigations of surveillance. VTEC O157 was not detected in all samples of fresh sheep within the four investigations years.

Many States declared that there is a high risk of contamination by VTEC in pork.

Many investigations have been negative. Only six States have provided positive results, though the very low percentage. VTEC O157 was detected in three of these investigations and the highest percentage of positive samples for VTEC O157 was 1,2% (Spain).

The same trend is available for chicken meat samples: data show low rates of positivity for four States. Poultry samples were also investigated for VTEC and four States evidenced VTEC with a 0,1% frequency. VTEC O157 was detected in only one investigation from Spain.

Taking into account wild meat, data were provided by Austria and Germany: in particular, Germany weighed low values, together with the isolation of O157 serogroup. Less VTEC data were provided by other foodstuffs. For dairy products (except cheese and raw milk), only Spain has reported positive VTEC.

Positive VTEC samples were recorded on fruits, vegetables and juices and, with a lower frequency, on fishery products (4,2%).

The ECDC-EFSA 2010 report also includes findings on data processing for other Zoonoses of European importance including: *Yersinia, Mycobacterium bovis, Brucella, Trichinella, Echinococcus, Toxoplasma,* Rabies, Q fever, *Tularaemia, Cysticerci* and Tuberculosis.

The major causes of foodborne outbreaks involving human cases are *Salmonella*, viruses and bacterial toxins, that represent 73,8% of the outbreaks and 80,2% of human cases.

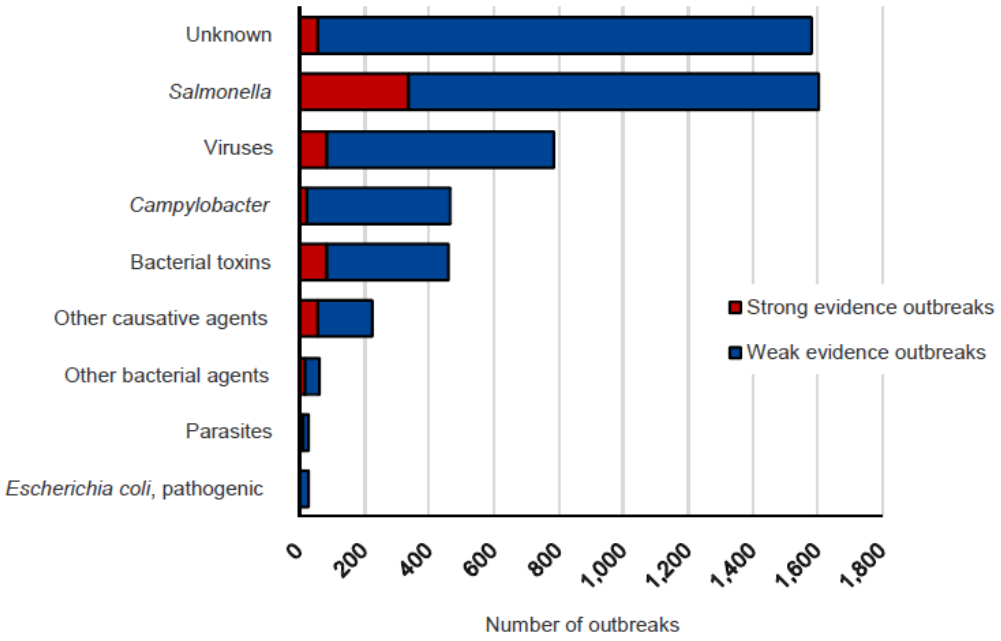



**Chart 14 Distribution of food-borne outbreaks in 2010.**

In addition, these outbreaks accounted for 86,2% admissions and 66,7% deaths related to outbreaks. However, *Brucella* outbreaks had the highest proportion of hospitalized cases, while outbreaks caused by *Clostridium botulinum* and *Listeria* had a high percentage of hospitalizations (95,2% and 84,6%, respectively). The setting of *Listeria* findings was provided in 96,4% of cases with the following frequencies: domestic (38,7%) of the outbreaks and restaurants/bars (30,8%), human cases (26,0%).

In 2010, most of the outbreaks have been associated with foods of animal origin. As in previous years, the most epidemic risky categories was tied to eggs and egg products, counting 154 outbreaks (22,1%). Mixed meals or buffets resulted the most common categories (13,9%), followed by vegetables, fruit juices, dairy products (8,7%), molluscs, crustaceans (8,5%).

Interestingly, the number of outbreaks caused by vegetables, fruit juices and related products increased compared to 2009 (21 outbreaks in 2009, 61 outbreaks in 2010). In 2010, these outbreaks were mainly caused by lettuce contaminated with Norovirus.

Food-borne viral infections are usually categorized as intermediate (one to three days) incubation period, causing diseases that are self-limiting in otherwise healthy individuals. Since most viruses are host specific, food-borne outbreaks caused by viruses are in most cases caused by contaminated food by infected food handlers. Calicivirus (including Norovirus) cause approximately 90% of epidemic non-bacterial outbreaks of gastroenteritis around the world and are responsible for many outbreaks of food-borne gastroenteritis. The virus is transmitted by food or water contaminated with human feces and person-to-person contact.

Rotavirus is the leading single cause of severe diarrhea in infants and young children. It is transmitted by the fecal-oral route. Infects cells, that line the small intestine, produce an enterotoxin, which induces gastroenteritis, resulting in severe diarrhea and sometimes death through dehydration.

However, in 2009, the number of outbreaks has increased by over 40% compared to 2007 and 2008, and the number of viral food-borne outbreaks reported in 2010 was higher than in previous years. Information on the food vehicle was provided for all of the 84 strong evidence outbreaks caused by Caliciviruses (including Norovirus). Vegetables and juices and other thereof products were the main implicated food vehicles in 2010 for Calicivirus, relating to 26 outbreaks (31,0 %).

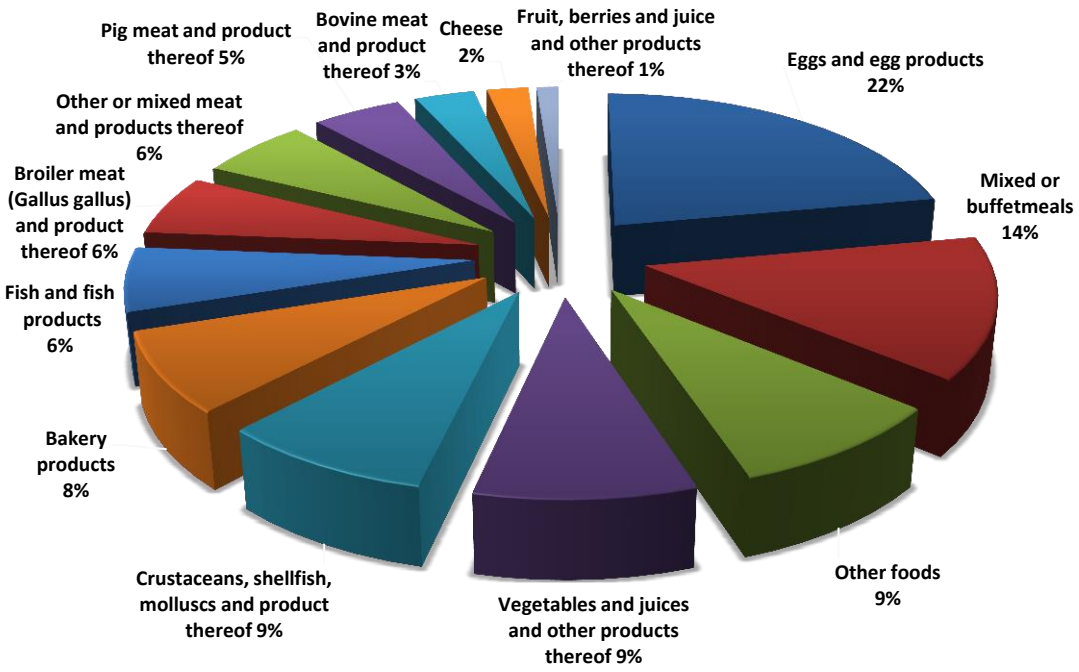



**Chart 15 Distribution of strong evidence outbreaks by food vehicle in the EU, 2010.**

In 2010, data related to the transmission of infections due to food contaminated by VTEC were received from 19 Member States and one non-member State of the European Union.

Analyzing data concerning food and animals, it is important to note that results from different surveys are not directly comparable because of differences in sampling strategies and methods of analysis used. In fact, the most widely used analytical method is proposed to detect only VTEC O157, while less investigations have been conducted to investigate other serotypes of VTEC.

## 2.4. The Annual Report PNI (Italy)

The Annual PNI (national integrated plan) Report for 2011, prepared in accordance with Regulation (CE) n.882/2004 (on official controls performed to ensure the compliance with feed and food law, animal health and animal welfare) and Regulation (CE) 654/2008 by the Ministry of Health as a national contact point, gathers the most important information on official controls performed during the year of reference for Food, Feed,

Health and animal Welfare, Healthcare plant, Byproducts, Zoonoses in humans and Environment.

Based on the principles of the CE Regulation, it is possible to compare the system of official controls to a complex organism, consisting of individual parts, all equally necessary for the overall proper functioning.

PNI 2011-2014 provides a description of the system and its components and the annual report illustrates the activities performed during a given calendar year.

The Annual Report provides a dual operational tool: to test the effectiveness and appropriateness of official controls carried out and to guide future work, in order to improve and rationalize the whole system of controls.

The breadth and variety of topics covered in the Annual Report by the PNI give rise to a large and complex document that can be accessed by different routes:

1. The report is divided into six chapters, according to a logical sequence of topics:

Chapter 1: the official control activities carried out in 2011.

Chapter 2: the non-conformities.

Chapter 3: the corrective actions that have resulted, in respect of operators and for the improvement of the system of official controls.

Chapter 4: the processation of the results to verify the operation of the control system.

Chapter 5: self-assessment and critical analysis of the system and results, both as to individual policy areas and in consideration of all the available information.

Chapter 6: changes to the PNI in 2011, considering special contingencies.

Food Hygiene and Nutrition Services and Veterinary Services of the ASL Prevention Departments checked a total of 358.196 units (plant and equipment of the buildings, facilities and means of transport). Operating subsidiary units amounted to 24,7% of those reported by the ASL Prevention Departments in Italy.

In 2011, audits were carried out in Italy by public laboratories (ARPA, Laboratory of Public Health of the ASL, instituted in some regions, and IZS). The analyzed samples, both of animal and vegetable origin, were a total of 117.185.

The first chapter of the report on the food's carried activity explores security and nutrition issues, quality merchandise, drinking water and minerals, import and trade and the activities conducted by the Police.

Within safety and nutrition, PNI emphasizes the following:

- Food of animal origin - Inspections and audits of regional and local authorities on the approved establishments
- Food of animal origin - recognition factories
- Food and beverage - Plan of supervision and control
- Celiac disease: survey of patients, restaurants census and training activities on the employed personnel
- Export food - Authorization and monitoring of establishments and microbiological tests
- Pesticides: Official controls on food residues of plant origin
- EFSA flow: zoonotic agents in food
- Raw milk: direct selling companies and vending machines - official controls to verify the microbiological criteria
- *Listeria monocytogenes* in certain categories of foodstuffs ready for consumption at the retail level
- Guides to good practice, hygiene in the food industry (Validation)
- Mussels alive (MBV) - Control of farms - Classification and monitoring of production and housing areas
- GMO National Plan of official control on the presence of genetically modified organisms in food
- National Residues Plan
- Products intended for particular nutritional uses (dietetic and early childhood)
- Nutritional Supplements: food containing vitamins and minerals as additives
- Ionizing radiation - Control of food and treated food ingredients
- *Trichinella* in meat - Official Controls

In 2011 all the countries examined a total of 228.622 farming for which requirements are in accordance with Reg. (CE) 853/2004, Allegato III. The data received showed that the

highest number of hits was performed in slaughterhouses and sectioning red meat with a total of 91.230 accesses, while the highest average number of controls per year is reported in white meat slaughterhouses (reaching around 44) and the lowest average number of checkings per year refers to the farming processing frogs' legs and snails.

In 2011 there has been an increase in the number of evaluations in factories, the biggest of which occurred in the area of red meat abattoirs, slaughterhouses and white meat. On the other hand a lower examination rate was reported for milk and derived products, in minced meat, meat preparations and rendered animal fat.

The detection of EFSA zoonotic agents flow in food depends on production, processing, storage or distribution from: slaughterhouses, canteens, caterings, restaurants, production plants, retail stores, cold storages. In the table below food samples of meat, fish, milk, eggs and all their derivates were collected (provided by the Regions within the information system SINZOO).

| Type of food | units sampled | positive units | % |
|---|---|---|---|
| **prepared meat** | 2264 | 66 | 2,91% |
| **fresh shellfish** | 4721 | 17 | 0,36% |
| **dairy** | 10913 | 3 | 0,03% |
| **frozen products derived from the processing of milk** | 1954 | 0 | 0,00% |
| **eggs and egg products** | 3448 | 2 | 0,06% |
| **fishery products** | 3005 | 1 | 0,03% |
| **meat products** | 12553 | 162 | 1,29% |
| **fresh Meat** | 5207 | 66 | 1,26% |
| **minced meat, mechanically separated** | 1376 | 20 | 1,45% |
| **entrailes** | 917 | 11 | 1,20% |
| **butter and other fats and oils** | 330 | 0 | 0,00% |
| **curd** | 108 | 0 | 0,00% |
| **crustaceans** | 34 | 0 | 0,00% |

**Table 24 *Salmonella spp.* in different types of food.**

The results of the samples for the detection of *Salmonella spp.* evidenced the following serotypes:

**Pig meat:** *S. Rissen, S. give, S. infantis, S livingstone, S. derby, S. tiphymurium*

**Beef:** *S. hadar and S. derby*

**Poultry:** *S. hadar, S. enterica, S. newport, S. schwarzengrud, S. thompson, S. saintapaul*

**Sheep and goat meat:** *S. derby*

**Solipeds:** *S. tiphymurium*.

In shellfish, *S. tiphymurium* has been mainly isolated (3 cases), followed by *S. indian, S. cerro, S. enteriditis, S. derby, S. rissen, S. liverpool*. In fishery products *S. tilburg* was isolated.

The greatest number of positive units, in relation to the number of samples carried out, has affected the preparations of meat of avian origin.

Regarding eggs and egg products, *S. braenderup* and *S. enteriditis* were found positive in *Gallus gallus* eggs.

*Listeria monocytogenes* was found purely in meat and poultry; in raw food the response was limited to the avian origin offal.

| Type of food | units sampled | positive units | % |
|---|---|---|---|
| **prepared meat** | 1500 | 73 | 4,86 |
| **fresh shellfish** | 42 | 0 | 0 |
| **dairy** | 22092 | 191 | 0,86 |
| **eggs and egg products** | 159 | 0 | 0 |
| **fishery products** | 3369 | 391 | 11,6 |
| **meat products** | 9962 | 247 | 2,48 |
| **fresh Meat** | 3965 | 58 | 1,46 |
| **minced meat, mechanically separated** | 676 | 27 | 3,99 |
| **entrailes** | 863 | 134 | 16,45 |
| **butter and other fats and oils** | 492 | 1 | 0,2 |

**Table 25 *Listeria monocytogenes* in different types of food.**

*Yersinia spp.* was only isolated in dairy products: in particular, the most frequently isolated serotype was *Yersinia enterocolitica*.

| Type of food | units sampled | positive units | % |
|---|---|---|---|
| **prepared meat** | 41 | 0 | 0,00% |
| **dairy** | 70 | 1 | 1,42% |
| **eggs and egg products** | 1 | 0 | 0,00% |
| **fishery products** | 21 | 0 | 0,00% |
| **meat products** | 126 | 0 | 0,00% |
| **fresh Meat** | 124 | 0 | 0,00% |
| **minced meat, mechanically separated** | 12 | 0 | 0,00% |
| **entrailes** | 38 | 0 | 0,00% |
| **curd** | 2 | 0 | 0,00% |

**Table 26 *Yersinia enterocolitica* in the different type of food.**

The research shows the performance of the samples for the detection of *Staphylococcus spp.*; milk samples and products containing pork were positive to staphylococcal enterotoxin.

| Type of food | units sampled | positive units | % |
|---|---|---|---|
| **prepared meat** | 276 | 0 | 0,00% |
| **fresh shellfish** | 1 | 0 | 0,00% |
| **dairy** | 2109 | 140 | 6,63% |
| **eggs and egg products** | 5 | 0 | 0,00% |
| **fishery products** | 81 | 0 | 0,00% |
| **meat products** | 141 | 0 | 0,00% |
| **fresh Meat** | 195 | 0 | 0,00% |
| **minced meat, mechanically separated** | 9 | 0 | 0,00% |
| **entrailes** | 52 | 0 | 0,00% |
| **butter and other fats and oils** | 48 | 0 | 0,00% |
| **curd** | 125 | 0 | 0,00% |

**Table 27 *Staphylococcus spp.* in different types of food.**

VTEC *Escherichia coli* was isolated in meat products, fresh meat, butter and other fats and oils.

| Type of food | units sampled | positive units | % |
|---|---|---|---|
| **prepared meat** | 566 | 3 | 0,53% |
| **fresh shellfish** | 17 | 0 | 0,00% |
| **dairy** | 3467 | 0 | 0,00% |
| **frozen products derived from the processing of milk** | 18 | 0 | 0,00% |
| **eggs and egg products** | 1 | 0 | 0,00% |
| **fishery products** | 105 | 0 | 0,00% |
| **meat products** | 583 | 0 | 0,00% |
| **fresh meat** | 469 | 2 | 0,42% |
| **minced meat, mechanically separated** | 171 | 1 | 0,58% |
| **entrailes** | 18 | 0 | 0,00% |
| **butter and other fats and oils** | 149 | 5 | 3,35% |
| **curd** | 64 | 0 | 0,00% |

**Table 28 VTEC in different types of food.**

*Campylobacter spp.* isolates (*C.jejuni C. thermophilic, C. coli and C.jejuni)* were found in dairy and fresh meat.

| Type of food | units sampled | positive units | % |
|---|---|---|---|
| **prepared meat** | 294 | 0 | 0,00% |
| **dairy** | 4444 | 10 | 0,22% |
| **eggs and egg products** | 3 | 0 | 0,00% |
| **fishery products** | 20 | 0 | 0,00% |
| **meat products** | 345 | 0 | 0,00% |
| **fresh meat** | 321 | 8 | 2,49% |
| **minced meat, mechanically separated** | 108 | 0 | 0,00% |
| **entrailes** | 43 | 0 | 0,00% |
| **fresh shellfish** | 47 | 0 | 0,00% |
| **crustaceans** | 3 | 0 | 0,00% |

**Table 29 *Campylobacter spp.* in different types of food.**

Evaluation for algal biotoxins in isolated shellfish did not provide any positivity.

*Echinococcus* was investigated in 10.721.924 samples collected in slaughterhouses: 12.631 resulted positive for *Echinococcus granulosus* (3471 in cattles, 5912 in sheeps and goats, 47 in boars, 54 in pigs) and one for *E. multilocularis* (sheep and goats).

*Trichinella spiralis* were tested too: 9.700.380 slaughterhouse units between solipeds, wild boars and pigs were examined , with no positive results.

Controls on Cysticercosis responsible *Taenia* Genus were conducted: from the 5.385.804 units (cattle and pigs) collected, 252 units (cattle) emerged positive for *Taenia saginata*.

Data analysis reports that:

- a significant percentage (20-30%) of the outbreaks, epidemics encountered, do not allow to identify the food vehicle of disease;
- most of the reported cases is mainly associated with bacterial species, although an higher number were estimated in outbreaks of viral etiology;
- new diagnostic techniques will allow in the future a more precise characterization of the epidemic viral etiology;
- it would be useful to improve the national and international network of information flows in order to have a clearer epidemiological picture, which is essential for the planning of preventive strategies.

# 3. SCOPE OF WORK

The purpose of this research was to perform a hygiene monitoring on food matrices such as: chicken, turkey, beef hamburger, pork and I and IV "gamma" vegetables.

Food matrices listed above were investigated for the detection of common microorganisms mentioned in CE Regulation 1441 December 2007.

The study also focused on the evaluation of the so called "emerging pathogens", microorganisms which should not be investigated according to law, but that started to be frequently isolated in food matrices attracting the attention on their potential danger.

Examined bacteria were: *Escherichia coli, E. coli O157:H7, Aeromonas spp., Campylobacter spp.,* Mesophilic*,* Sulphite-reducing *Clostridia, Clostridium difficile, Salmonella spp., Lysteria spp. and Lysteria monocytogenes, Clostridium perfrigens, Staphylococcus spp. and Staphylococcus aureus, Enterococcus spp.* .

Further studies consisted on performing biochemical investigations on isolated strains.

The problem of microbial biofilms has attracted a wider attention lately, partly thanks to the fact that these have been discovered to be ubiquitous in nature, industrial settings and clinics. The interest in clinical research on biofilms has been remarkable and this is not surprising since there are known health implications of biofilm formation in the development and chronicity of certain diseases and in the conduct of surgical equipment and hospital care. Noteworthy the difficulty of eradicating biofilm, issue which led the biological research to a technical-engineering level through the planning of new materials and products that may prevent its formation.

Depending on the microbial species and type of antimicrobial and experimental system, bacteria involved in biofilm formation can be up to a thousand times more resistant to antimicrobial stress than the ones of the same species not protected by biofilms. Understanding the nature of the increase of antibiotic resistance is a central goal of much of the basic and clinical research.

The role of biofilms in the pathogenesis is of fundamental importance as regards the oral tract infection and colonization of permanent medical devices; for many other infections, especially of chronic type, the bond between it and the pathology is less clear.

The strains of *Campylobacter spp., S. aureus, Salmonella spp.* and *C.perfringens* were tested in order to evaluate their ability to form biofilms and adhere to plastic materials. This feature has been assessed following the Stepanovich et al. protocol (2004).

The hydrophobic nature of the bacterial cell surface can be considered a virulence factor; hydrophobic strains have increased invasiveness regard to cells and tissues of those hydrophilic (Kjelleberg S. et al., 1980). The hydrophobicity of the cell surface is a measure of the tendency of a cell to interact with a surface (tissue and/or mucosal).

One of the most commonly used tests which measure this tendency is the MATH, Microbial Adhesion to Hydrocarbons, (M. Rosenberg et al., 1980). The test involves contact between a suspension of microbial cells and small volumes of hydrocarbon and subsequently measures the removal of cells from the aqueous phase of the same hydrocarbon: a cell suspension is stirred for two minutes in the presence of p-xylene (or hexadecane, n-octane); the evaluation is realized through the observation of the degree of hydrophobicity of the cells on the basis of the decrease in turbidity of the aqueous phase.

The cell surface of Gram-negative bacteria, provided with lipopolysaccharides, is usually hydrophilic. The acquisition of hydrophobicity by these cells may be associated with a mutation of the polysaccharide layer or with the production of fimbrial adhesins (AL Lobashevskiĭ, 1992.)

A direct relationship exists between capacity, for bacteria, to synthesize extracellular enzymes and their virulence; although it has not been demonstrated that the single enzyme is the only factor responsible for the virulence of bacteria (SC Edberg et al., 1996), there is no doubt that they play a key role in the more or less marked pathogenicity.

Within the investigations conducted on enzymes, the ability of selected strains to synthesize protease was tested; substrates used were:

- ✓ skim milk, casein and gelatin;
- ✓ collagenase, Hide Azure powder, collagen type I;
- ✓ mucinase, with the mucin of type II and III;
- ✓ the activity of elastin with bovine neck ligament elastin;
- ✓ the activity of the lecithinase using egg yolk emulsion;
- ✓ hemolytic activity through the use of blood agar;

- ✓ amylase with Starch;
- ✓ chitinase with chitin;
- ✓ DNase.

Enzymes such as DNase, gelatinase and proteases are known to affect the cellular components such as nucleic acids and proteins; therefore, the ability in their synthesis, by bacterial strains, is definitely a promoter of virulence factor.

Because a microbe is virulent it is necessary to produce more than an extracellular enzyme and this condition is important but not exclusive and sufficient to ensure its virulence (Edberg et al., 1996).

The European Food Safety Authority (EFSA) has issued an opinion on the consequences to public health arising from the presence of strains of methicillin-resistant *Staphylococcus aureus* (MRSA) in animals and foods. Methicillin-resistant *Staphylococcus aureus* is a bacterium that has developed the ability to survive the most common antibiotics and is responsible for difficult to treat infections in humans.

The Scientific Panel on Biological Hazards (BIOHAZ) found that, although the foods may be contaminated with MRSA, there is currently no evidence that eating such foods or manipulating them can lead to an increased risk for man to become a healthy carrier of the bacterium or to be infected.

The group also concluded that, in the case of high prevalence of MRSA in animals grown for human food, the people who have contact with live animals, such as farmers, veterinarians, are at greater risk of remaining population.

The scientific opinion of EFSA BIOHAZ, which has also helped the European Centre for Disease Prevention and Control (ECDC), was conducted in conjunction with the work done by the European Medicines Agency (EMEA) related to other aspects of MRSA. The EMEA has published a discussion paper on the use of antimicrobials in livestock and companion animals on the risk of MRSA infection.

# 4. MATERIALS AND METHODS

Samples of meat and vegetables foods were purchased at retailers specifically identified in shops of the historical center of Naples (area between the Decumani), close to University “Federico II”, located in Via Mezzocannone. The samples were kept cold within transport to the laboratory (taking care of shortening the times as much as possible).

The analysis were conducted using standardized procedures for each analyte by weighing and homogenizing sample aliquots to obtain the desired solution.

Afterwards, sub-aliquots were inoculated in liquid and solid enrichment growth media to allow subsequent identification and enumeration of metabolic germs.

The standardized methods used are listed below:

| | |
|---|---|
| **Preparation of test samples** | UNI EN ISO 6887-2 (specific rules for meat) |
| **Mesophilic microorganisms** | UNI EN ISO 4833:2004 |
| ***Escherichia coli*** | UNI 10980:2002 |
| **Sulphite-reducing Clostridia** | Internal method |
| ***Aeromonas spp.*** | Internal method |
| ***Listeria spp.*** | UNI EN ISO 11290-1/2:2005 |
| ***Listeria monocytogenes*** | UNI EN ISO 11290-1/2:2005 |
| ***Campylobacter spp.*** | ISO 10272-1:2006 |
| ***Clostridium difficile*** | Aliberti, Dumontet, Normann, Krovacek 2009 |
| ***Salmonella spp.*** | UNI EN ISO 6579:2004 |
| **Enterococcus spp.** | UNI EN 15788:2009 |
| ***Staphylococcus spp.*** | UNI EN ISO 6888-1:2004 |
| ***Staphylococcus aureus*** | UNI EN ISO 6888-1:2004 |
| ***Clostridium perfringens*** | UNI EN ISO 7937:2005 |
| ***Escherichia coli O157: H7*** | Technical details for isolation and identification Biolife |

## 4.1. Mesophilic microorganisms

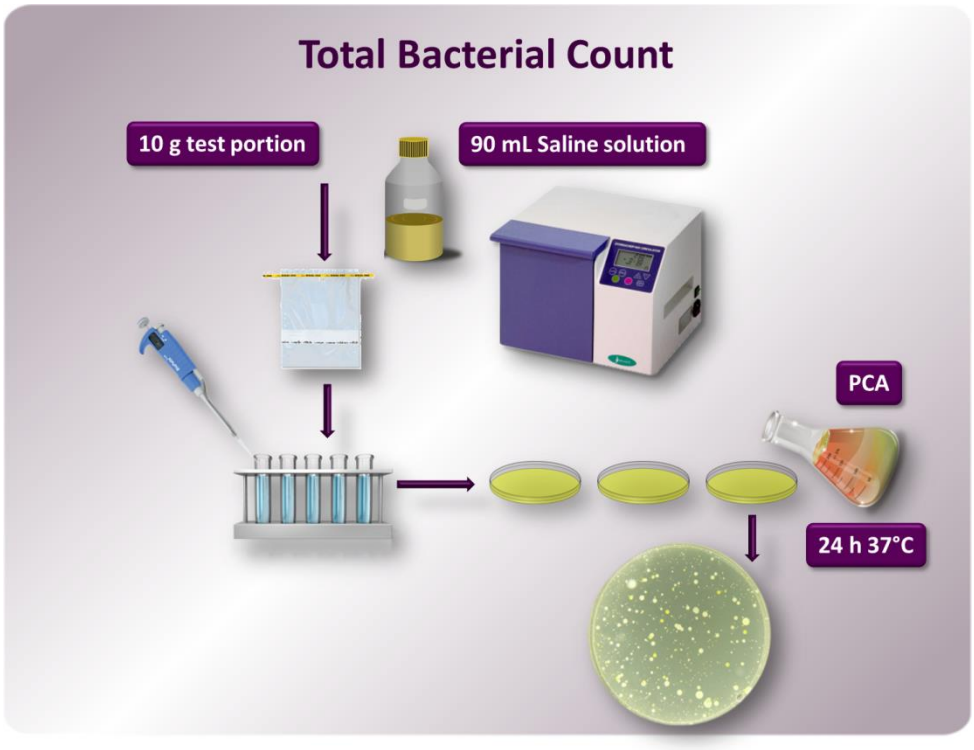



**Image 1 Flow chart of microbiological technique for TBC detection.**

Total bacterial count (TBC) protocol consists on adding an aliquot of 10 g of meat and 90 mL of sterile saline solution to a Stomacher bag. After homogenization with Stomacher, 1 mL from the sample is included in PCA agar (Oxoid), using the pour plate method; the PCA, Plate Count Agar, contains tryptone, glucose, yeast extract and agar and is used for the total counts of aerobic bacteria and facultative anaerobes in different matrices. The PCA plates are incubated at 37° C for 24 hours. The sequence of preparation of the aqueous suspension is similar for the investigation of *Escherichia coli* and Sulphite-reducing Clostridia.

## 4.2. *Escherichia coli*

Escherichia coli is a Gram-negative bacterium and is the best known Genus *Escherichia* specie. The Genus counts 171 serotypes, each characterized by the different combinations of antigens O, H, K, F.

*E.coli* is one of the main species inhabiting the lower intestine of warm-blooded animals (birds and mammals, including humans) and the principal faecal contamination indicator (along with Enterococci).

The bacterium is a rod-shaped, Gram-negative, aerobic and facultatively anaerobic, non-spore-forming microorganism, which grows at a temperature of 44,5° C, lactose-fermenting, indole-positive in tryptophan containing media, beta-D-glucuronidase-positive. Literature reports that the presence of beta-D-glucuronidase has been highlighted in the 94 to 99.5% of the biotypes of *Escherichia coli*, with the exception of serotypes O157: H7.

### 4.2.1. Method

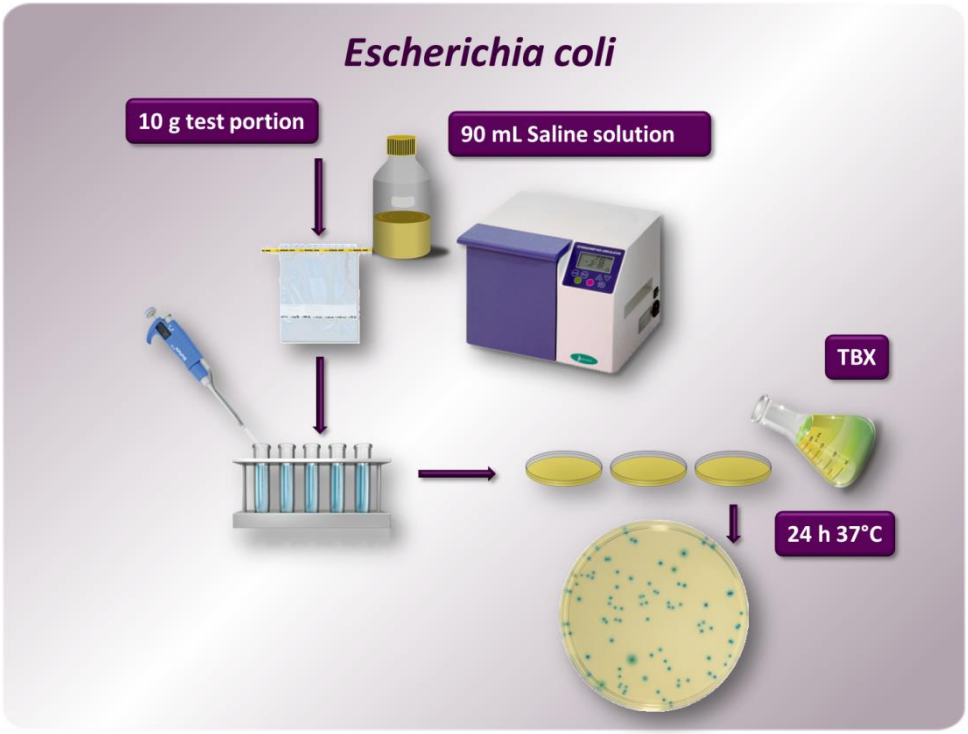



**Image 2 Flow chart of microbiological technique for *E.coli* detection.**

To determine the contamination due *Escherichia coli,* an aliquot of 10 g of meat and 90 mL of sterile saline solution are added to a Stomacher bag. After homogenization with Stomacher, 1 mL from the sample is included in TBX agar (Oxoid), using the pour plate method.

Plates are incubated at 44 ° C for 24 hours. The TBX, Tryptone Bile X-glicoronide Medium, contains tryptone, bile salts, agar and X-glucuronide. Most strains of *E.coli* differ from

other coliforms for the presence of the enzyme glucuronidase. The chromogen X-glucuronide is insoluble and accumulates in cells, the enzyme cleaves the bond between the chromophore and the glucuronide, giving, as a result, characteristic blue/green colonies.

## 4.3. *Aeromonas spp.*

According to Kirov and Sanderson, 1995 and Isonhood and Drake, 2002 species belonging to the Genus *Aeromonas* have been recognized as pathogens that can cause a variety of serious infections in humans; extraintestinal infections can cause bacteremia, meningitis, pulmonary infections.

*Aeromonas spp.* can play a significant role in the "summer-diarrhea," a well known problem that affects mainly children under five years of age, the elderly and travelers. The responsibility of these bacteria in infections associated with the consumption of contaminated food is not firmly established, but the Genus *Aeromonas spp.* has the potential to emerge as an important food-borne pathogen.

The most important pathogens are *A. hydrophila*, *A. caviae*, and *A. veronii* biovar *sobria.*

The organisms are ubiquitous in fresh and brackish water. Two major diseases are associated with *Aeromonas*: gastroenteritis and wound infections, with or without bacteremia. Gastroenteritis typically occurs after ingestion of contaminated food or water, while wound infections result by exposure to contaminated water.

### 4.3.1. Method

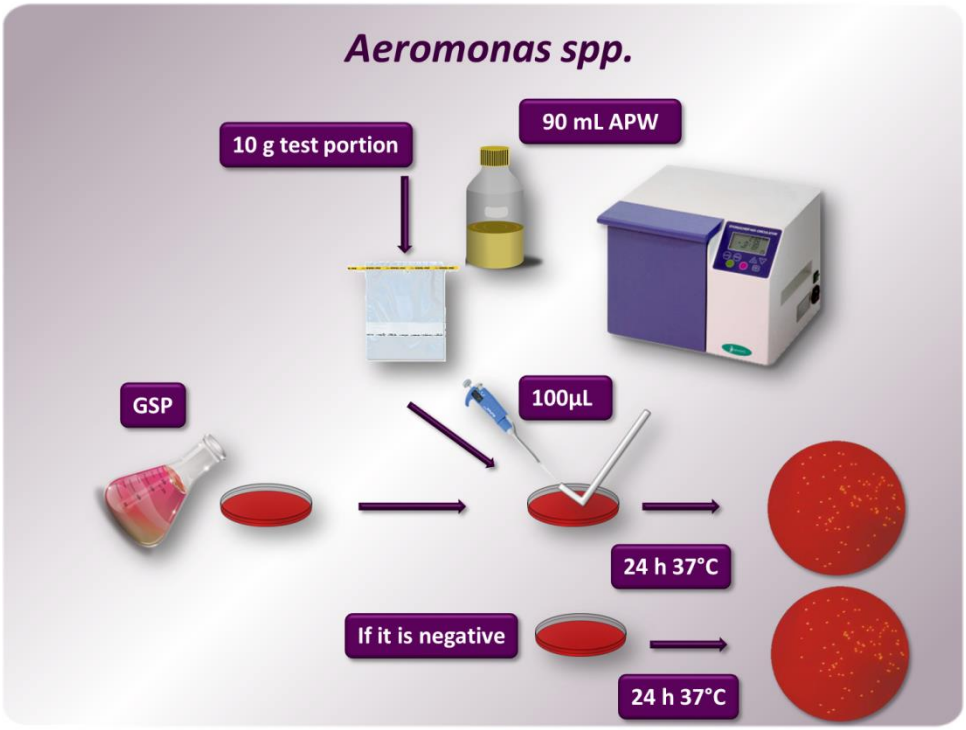



**Image 3 Flow chart of microbiological technique for *Aeromonas spp.* detection.**

In order to proceed to the isolation of *Aeromonas spp.*, an aliquot of 10 g of meat and 90 mL of Alkaline peptone water (enrichment broth consisting of peptone and sodium chloride) are added to a Stomacher bag. After homogenization with Stomacher, 100 µL from the sample are introduced in GSP agar (Oxoid), using the spread plate method.

GSP agar contains sodium L-glutamate, starch, potassium dihydrogen phosphate, magnesium sulfate, phenol red and agar. Indeed, Penicillin G (62.6 mg / L) is added to the medium to inhibit the growth of Gram-positive organisms and Fungi.

Stomacher bags and plates are afterwards incubated at 37° C for 24 hours. After incubation it is possible that there are no colonies on the agar: in this case it is necessary to perform a qualitative survey through a streak from the enrichment broth on a new GSP plate and check any eventual growth after further 24 hours. The glutamate and starch are the only sources of nutrients in the culture medium. Starch is degraded by *Aeromonas spp.* producing acid that causes the color change of phenol from red to yellow. The colonies present, therefore, a characteristic yellow color.

## 4.4. *Listeria spp.* and *L. monocytogenes*

*L. monocytogenes* is a rod-shaped, Gram-positive bacterium; infections in humans are caused in 98% of cases by serovars 1/2a, 1/2b and 4b. Listeriosis is a bacterial disease that usually arises as meningoencephalitis and or septicemia in newborns and adults. Onset may be sudden with fever, intense headache, nausea, vomiting, meningeal irritation, delirium and coma may be registered. This infection was infrequently diagnosed, with an incidence in the United States, cases of hospitalization, 1/200000 inhabitants. In Europe it is often associated with the consumption of unpasteurized milk or milk products.

The main sources of infection are soil, fodder, water, mud and silos. The seasonal habit of storing grain in silos increases the incidence of Listeriosis in animals. Other reservoirs are infected domestic and wild mammals, poultry and man. Unlike other pathogens, *Listeria* can multiply in foods, even if refrigerated. Outbreaks of Listeriosis have been reported in association with the consumption of raw milk or contaminated soft cheeses, vegetables and meat.

Listeriosis (*Listeria monocytogenes* infection) was added to the list of nationally notifiable diseases in 2001. To improve surveillance, the Council of State and Territorial Epidemiologists has recommended that all *L. monocytogenes* isolates have to be forwarded to state public health laboratories for subtyping through the National Molecular Subtyping Network for Foodborne Surveillance Disease (PulseNet).

All States disposed regulations requiring health care providers to report cases of Listeriosis and public health officials to interview all people with Listeriosis promptly using a standard questionnaire about high risk foods. The *Listeria* Initiative is an enhanced surveillance system that aids in investigations of Listeriosis outbreaks and clusters. States participating in the *Listeria* Initiative conduct prompt interviews of patients using the Listeria Case Report, a standardized questionnaire developed to collect detailed information about food exposures.

When PulseNet, the National Molecular Subtyping Network for Foodborne Disease Surveillance, identifies a cluster of *Listeria monocytogenes* isolated from patients with the same molecular pattern, epidemiologists can conduct a preliminary case-control analysis

by comparing responses to food exposures reported on the *Listeria* Case Report from patients in the cluster to those from patients outside the cluster.

### 4.4.1. Method


**Image 4 Flow chart of microbiological technique for *Listeria spp.* and *L.monocytogenes* detection.**

The evaluation of *Listeria spp. and Listeria monocytogenes* consists on adding an aliquot of 10 g of meat and 90 mL of One Broth-Listeria Base (Oxoid) to a Stomacher bag.

One Broth-Listeria Base (Oxoid) is composed of peptone, mix of carbohydrates and salt mix, which is then added according to a specific supplement. After homogenization, the bag is incubated at 30 ° C for 24 hours. Using the spread plate method, 100 µL from the sample are introduced in Chromogenic Listeria Agar Base (OCLA, Oxoid) plates, a selective medium that uses the chromogen X-glucoside for presumptive identification of *Listeria spp.*.

*Listeria* cleaves X-glucoside thanks to its ß-glucosidase, common to all species of the Genus. Other organisms that possess this enzyme, such as Enterococci, are inhibited by the agents within the selective medium: lithium chloride, polymyxin B and nalidixic acid,

while amphotericin inhibits the growth of yeasts and molds that can be present in the sample.

*Listeria monocytogenes* and other species are additionally differentiated thanks to their ability to produce enzymes phospholipase PI-PLC and PC-PLC, which hydrolyze phosphatidylinositol or lecithin in the middle, producing an opaque white halo around the colony. This shape was useful to *Listeria spp.* identification.

OCLA is enriched with OCLA (ISO) Selective Supplement (SR0226, Oxoid) and OCLA Differential Supplement (SR0244, Oxoid), following the manufacturer's instructions, according to the formulation described by Ottaviani and Agosti (ALOA) ISO 11290 -1.

This formulation incorporates ALOA phosphatidylinositol so that phosphatidylinositol phospholipase C (PI-PLC), produced by *Listeria monocytogenes,* may be detected.

Both PI-PLC and PC-PLC are associated with the virulence of *Listeria* and, therefore, the identification of one of the enzymes represents a useful indicator of pathogenicity.

## 4.5. *Campylobacter spp.*

Campylobacter is a Gram negative, narrow, curved rod. It only grows with reduced oxygen levels, that is in microaerobic conditions, requiring 3-5% oxygen and 2-10% $CO_2$ to ensure maximum growth. Campylobacter is motile due to the presence of flagella that confer the ability of a screw motion. This way, the penetration of the mucus layer that covers the intestinal epithelium is facilitated, consequently allowing the colonization of the intestinal tract. The penetration of the protective barrier, represented by mucus, is made easier for bacterium through the action of chemotactic factors.

At first the bacterium, thanks to a variety of adhesins but, most of all, of flagella, is able to attach to epithelial cells and is internalized by a phagocytic-type mechanism.

Several toxic factors have been described: an enterotoxin similar to cholera toxin and several cytotoxins.

Although several studies have attempted to correlate the in vitro production of toxins to clinical expression, none of them demonstrated the presence of free toxins in stool samples from patients with C*ampylobacter* enteritis.

The *Campylobacter* are part of the normal intestinal flora of a wide variety of animals both wild and domesticated. Microorganisms can contaminate animal carcasses at different stages of slaughter and poultry meat is the most frequently and heavily contaminated; the bacterium may also be find in not pasteurized cow's milk and is present in sewage and surface waters untreated.

There are thermo-tolerant strains which can, usually, grow at temperatures above 37° C, up to 42-43° C.

Listed below there are the most involved species in human intestinal infectious diseases:

- *Campylobacter jejuni subspecies jejuni*
- *Campylobacter jejuni subspecies doylei*
- *Campylobacter coli*
- *Campylobacter lari*
- *Campylobacter upsaliensis*
- *Campylobacter mucosalis*

The symptoms and signs of infection by C*. jejuni/coli* are not so unique and it can be therefore differentiated from those caused by other enteric pathogens.

Once ingested, the bacterium, after the gastric barrier, reaches the ileum and colon, where it multiplies and causes a non-specific acute inflammatory reaction with neutrophils, monocytes and eosinophils, degeneration of glands, microabscesses of the crypts and mucosal lesions. This framework is associated with the pathologic finding of erythrocytes and leukocytes in the stool (in over 75% of cases).

In general, diarrhea and fever are symptoms common to all bacterial enteritis but, while in mild cases the symptoms are indistinguishable from a viral gastroenteritis, in more serious cases, severe forms of colitis very similar to ulcerative colitis or Crohn's disease have been described.

Bacteria of the genus *Campylobacter spp.* are considered the most common cause of zoonoses in Europe and in the world, although their incidence is not well known to the general public, unlike other pathogenic bacteria such as *Salmonella spp.* and *Escherichia coli* real-cytotoxic. In the U.S.A., from about 5,2 million clinical cases of food-related diseases annually, an estimated 2,4 million are due to *Campylobacter*.

In 2010, *Campylobacter* has continued to be the most commonly reported gastrointestinal bacterial pathogen in humans in the European Union since 2005. The number of confirmed cases of human Campylobacteriosis in the EU increased by 6,7% in 2010, compared to 2009. The growth was also reflected as an increase in the overall rate of notification of Campylobacteriosis in the EU, from 45,6 per 100.000 population in 2009 to 48,6 per 100.000 population in 2010. In 2010, 266 deaths have also been reported due to Campylobacteriosis.

The notification rate of confirmed cases of Campylobacteriosis in the EU showed a significant increasing trend in the last five years (2006-2010), more evident since 2008. By country, a statistically significant increase in Campylobacteriosis notification rates (2006-2010) was observed in Cyprus, Estonia, France, Luxembourg, Malta, Netherlands and Poland; with a statistically significant trend of decrease observed in Belgium and Bulgaria.

### 4.5.1. Method

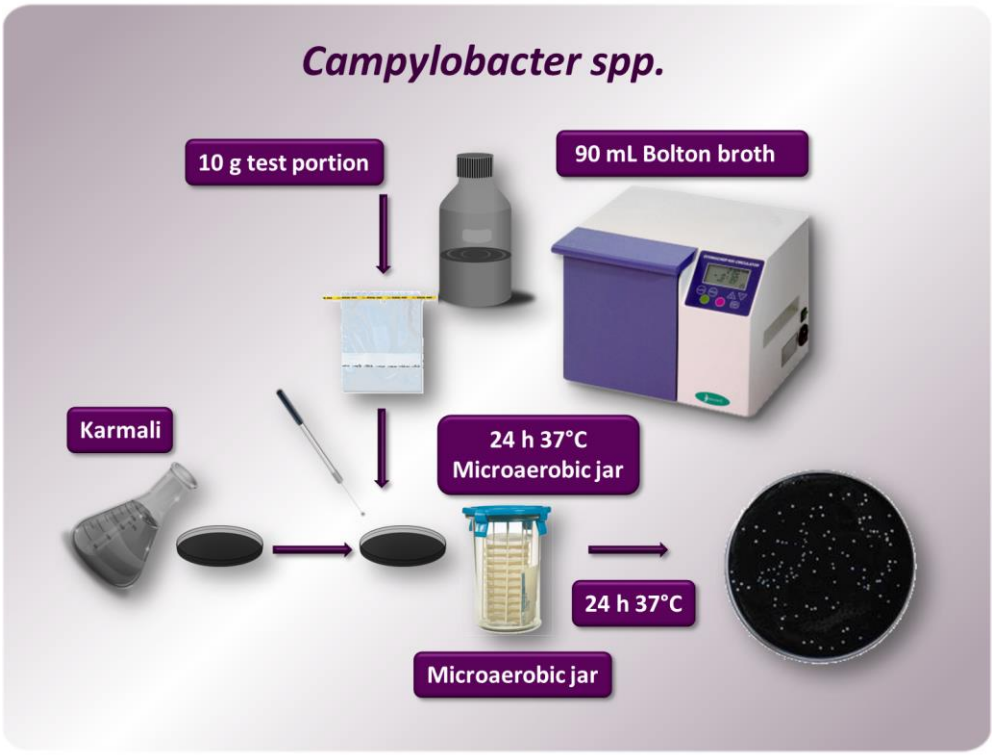



**Image 5 Flow chart of microbiological technique for *Campylobacter spp.* detection.**

To allow the isolation *Campylobacter spp.*, an aliquot of 10 g of meat and 90 mL of Bolton Selective Enrichment Broth (Oxoid) are added to a Stomacher bag.

Bolton Selective Enrichment Broth (Oxoid) consists of meat peptone, lactalbumin idrosilata, yeast extract, sodium chloride, alpha-ketoglutaric acid, sodium pyruvate, sodium metabisulfate, sodium carbonate, hemin. The selective enrichment broth was formulated to revitalize damaged cells and encourage their multiplication.

The broth is then enriched with laked horse blood supplement and Bolton Broth Selective Supplement (Oxoid SR0183).

Bolton Broth Selective Supplement contains vancomycin (an antibiotic that acts against Gram-positive, inhibiting them), cefoperazone (mainly against Gram-negative), trimethoprim (against a wide variety of Gram-negative and Gram-positive) and cicloheximide (an antifungal).

Homogenized Stomacher bags are incubated in jar at microaerophilic conditions (5% $O_2$), at 37° C for 4 hours and then moved at 42°C for another 24 hours.

Transition temperature is important since, as the majority of species of the genus *Campylobacter spp.* are thermotolerant, it increases the selective pressure on the microorganisms competitors.

After the incubation, using a loop, the sample is surface plated on the selective medium Campylobacter Agar Base (Karmali, Oxoid) through streak method.

Campylobacter Agar Base (Karmali, Oxoid) contains Columbia agar base, activated charcoal and hemin. To the soil supplement Oxoid SR0167 is added: it is composed by sodium pyruvate, cefoperazone, and vancomycin cicloheximide.

After incubation at 42° C for 48 h in a microaerobic jar, grown *Campylobacter* colonies should result gray, flat and swarming. Suspect colonies are subjected to biochemical tests as a confirmation; oxidase and catalase are performed: the bacterium is positive for both.

Oxidase test evaluates the presence of cytochrome oxydase enzyme through the use of strips (Oxoid) distributed in commerce. A positive reaction is evident when a blue-violet color develops within a few seconds.

Catalase reaction test consists on picking up a colony and suspending it on a drop of hydrogen peroxide. The appearance of bubbles indicates positivity. At this point it is possible to proceed with the identification through testing multiple metabolic wells (API Campy system Bio-Merieux).


**Image 6 Api Campy Biomerieux.**

## 4.6. *Salmonella spp.*

In the recently proposed nomenclature, *S.typhi* is called *S.enterica*. Numerous *Salmonella* serotypes are pathogenic for both animals and humans; in the majority of countries with a surveillance system for *Salmonella*, the serovar *Typhimurium* (S.enterica) and *Salmonella enterica* subspecies *enterica* serovar *enteritidis* are the most commonly isolated.

Salmonellosis has spread all over the world, although the best surveillance system is correctly realized in North America and Europe.

Salmonellosis is classified as a food relate disease. Only a small proportion of cases are clinically diagnosed and in industrialized countries it is estimated that only 1% of clinical cases is notified. Every year, in the U.S., about 5 million cases of Salmonellosis are registered. USA experienced an epidemic, involving 25.000, cases caused by water of a non-chlorinated aqueduct; the largest single outbreak affected 285.000 people and its source resulted incorrectly pasteurized milk. Infection reservoirs are a wide variety of domestic and wild animals, including chickens, pigs, cattle, rodents, iguanas, turtles, chickens, dogs and cats.

Man can also constitute a reservoir of infection. Transmission route is by ingestion of the microorganisms present in foods due to fecal contamination. Recently, numerous outbreaks have been attributed to consumption of contaminated vegetables during fertilization and not properly washed before use.

Strains able to cause typhoid and paratyphoid fever in human are *S.enterica enterica Typhi (S.typhi) and S.Paratyphi A and Paratyphi B*.

The annual incidence of typhoid fever is estimated at about 17 million with approximately 600.000 deaths. The majority of cases occur in developing countries. The number of sporadic cases per year in the USA amounts to less than 500. Paratyphoid fever occurs sporadically.

### 4.6.1. Method

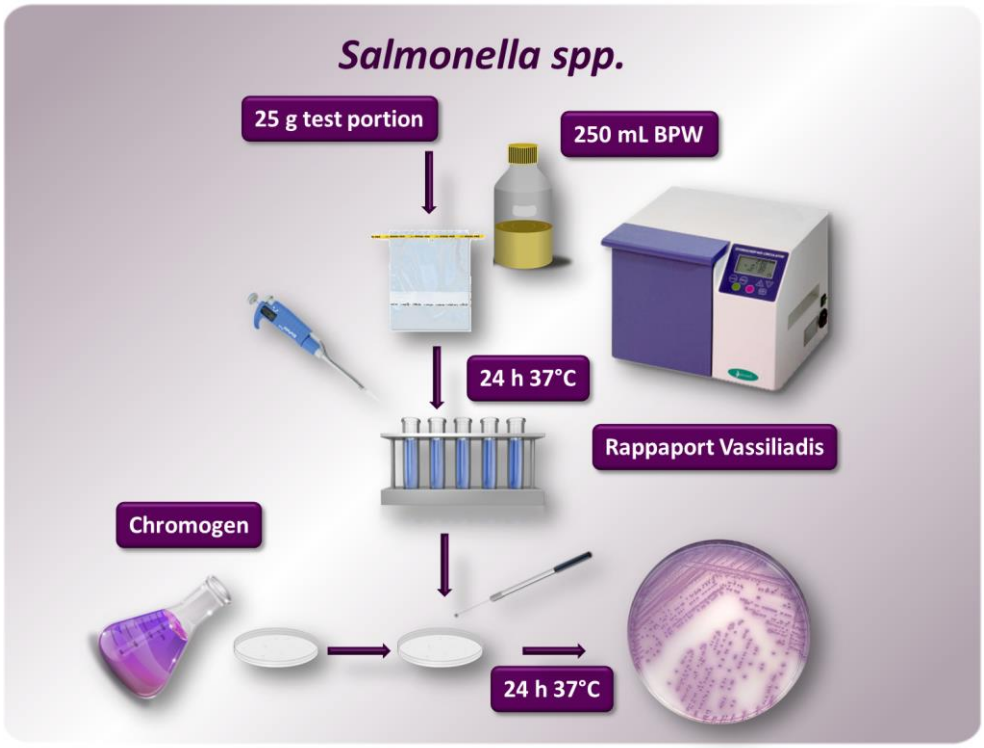



**Image 7 Flow chart of microbiological technique for *Salmonella spp.* detection**

According to *Salmonella spp.* isolation protocol, an aliquot of 25 g of meat and 250 mL of Buffered Peptone Water (Oxoid) must be added to a Stomacher bag. BPW enrichment broth consists of peptone, sodium chloride, sodium phosphate dibasic and potassium phosphate monobasic: this represents a pre-enrichment phase.

Homogenized Stomacher bags are incubated at 37 ° C for 24 hours. 100 µL from the samples are then added to 10 mL of Rappaport-Vassiliadis broth (Oxoid), a selective enrichment medium.

The tryptone contained in Rappaport Vassiliadis Broth provides the carbon and the nitrogen necessary for growth. The magnesium chloride increases the osmotic pressure in the agar. The Malachite Green inhibits organisms other than Salmonella. The low pH of the medium (5.1 ± 0.2), malachite green and the high concentration of magnesium chloride, which increase the osmotic pressure, are selective for the Salmonella spp. One proceeds to incubation at 41.5 ° C ± 0.5 ° C for 24 hours in order to optimize the isolation of Salmonella spp. .

After the incubation, using a loop, the sample is surface plated on the medium selective XLD (Oxoid), containing xylose, L-lysine, lactose, sucrose, sodium chloride, yeast extract, sodium deoxycholate, sodium thiosulfate, Fe -ammonium citrate, phenol red, agar (final pH 7.4 + / - 0.2).

Plates are incubated for 24 hours at 37 °C. Through a sterile loop, sample is even streaked on an additional medium, suitable for the isolation and identification of *Salmonella spp.*, including *S. typhi*: Salmonella Chromogenic Agar (Oxoid). This is the only medium on the market allowing the determination of lactose-positive strains of *Salmonella*. The selectivity of the medium is ensured by a mixture of inhibitory substances comprising a cephalosporin mainly active in the suppression the growth of *Pseudomonas spp.*, From sodium deoxycholate and sodium cholate, active in the suppression of Gram positive bacteria and some Gram-negative, tergitol 4, inhibitor especially of *Proteus spp..*

The differentiation between *Salmonella* and no Salmonella strains is possible through:

- the presence in plates of a chromogenic substrate which acts on a specific esterase of *Salmonella* with release of a metabolite-magenta-colored red;
- the presence of a chromogenic derivative glucopiranosidico on which acts the beta glucosidase with release of a metabolite-green-blue.

Chromogenic Salmonella Agar is used to determine Salmonella spp, with high sensitivity, so that all the plates not showing typical colonies (red-magenta) could be eliminated. The plates with growth characteristics (red-magenta) must be subjected to biochemical and serological confirmation.

A wide range of Salmonella flagella antigens have been used to produce polyvalent antisera in rabbits. *Salmonella spp.* antibodies present in the antisera include : a, b, c, d, eh, Fg, gp, gms, gq, gst, i, k, lv, lw, mt, r, y, z, enz15, enx, 1.2, 1.5, 1.6, 1.7, z4, z6, z10, z23, z24, z29, z38 and gpu.

The purified antibodies are used to sensitise the latex particles. Presumptive *Salmonella spp.* colonies can be taken from an agar plate, mixed with the latex test reagent and agglutination will occur if *Salmonella spp.* is present.

## 4.7. *Staphylococcus spp* and *Staphylococcus aureus*

*S. aureus,* belonging to the family of *Micrococcaceae,* occurs in aggregates of Gram-positive cocci. Currently 32 species of the Genus *Staphylococcus* are known and some of these are pathogenic to humans and animals.

*Staphylococcus aureus* is a microorganism capable of synthesizing numerous toxins able to favoring the colonization of the host organism from sites of infection. However, the interest of the microorganism in the context of food safety, resides in its ability to synthesize thermostable toxins in contaminated food, denominated "staphylococcal enterotoxins" (ES), which, when present in sufficient quantity, give rise to a common form of food poisoning whose name is "staphylococcal intoxication" (IS). The main characteristic of this is to be a thermostable enterotoxin, therefore the normal cooking treatments are not able to turn it off. Five different enterotoxins, designated as ABCD and E, have been currently identified.

The toxin A is the most implicated in cases of intoxication (about 80%), while E is very rare.

The symptoms occur rapidly after ingestion of contaminated food, usually between 2 and 6 hours and include nausea, headache, severe and frequent abdominal pain and diarrhea;fever is rare.

All this results in about 24 hours, but may persist for a feeling of exhaustion in the days immediately following. Particularly susceptible to dehydration as children and the elderly should be considered at higher risk. Treatment is symptomatic and supportive is based on the control of fluid and electrolyte.

The staphylococcal intoxication is extremely widespread and very common. The true incidence is, however, very difficult to assess because many cases are not reported: there are known only ones which lead to hospitalization and involve a large number of people, while cases contracted at the household level remain unknown.

### 4.7.1. Method

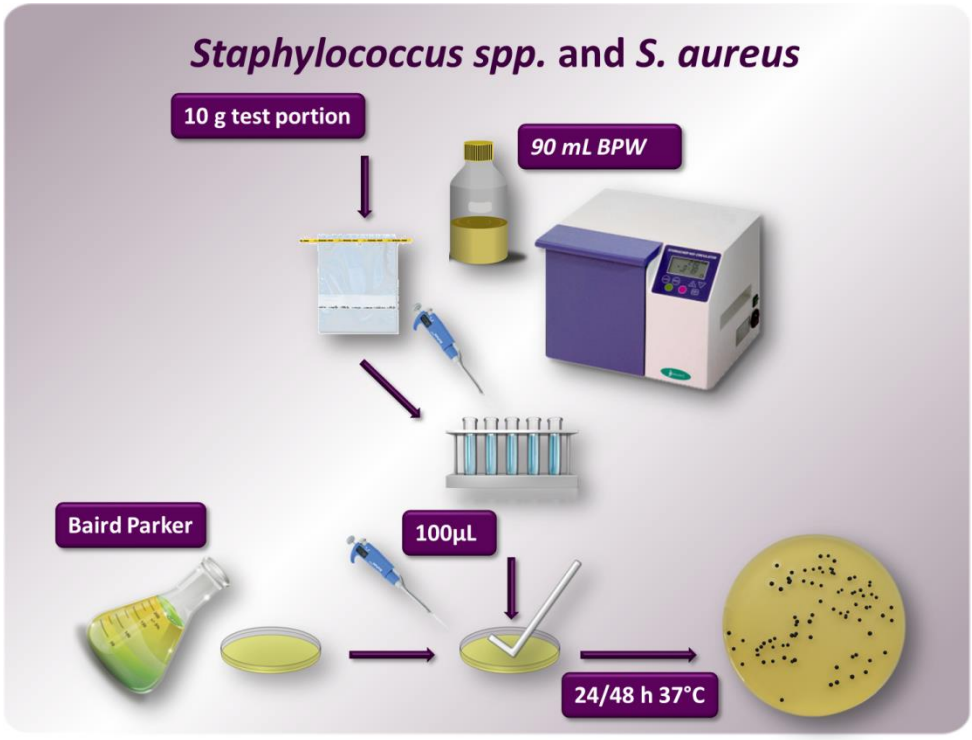



**Image 8 Flow chart of microbiological technique for *Staphylococcus spp.* and *S.aureus* detection**

For the detection of *Staphylococcus spp* and *Staphylococcus aureus,* an aliquot of 10 g of meat and 90 mL of Buffered Peptone Water (Oxoid) must be added to a Stomacher bag.

After homogenization with Stomacher, 100 µL from the sample are introduced in Baird Parker (Oxoid), using the spread plate method. Selective medium, with the precautions of asepsis, is supplemented with Egg Yolk Tellurite Emulsion (Oxoid).

Baird Parker Agar is a selective and diagnostic recommended by ISO 6888-1 and the FDA (Food and Drug Administration) for the isolation of coagulase positive Staphylococci from foods. It contains enzymatic hydrolyzate of casein, meat extract, yeast extract, sodium pyruvate, glycine, lithium chloride, agar. Lithium chloride and potassium tellurite is inhibitory to contaminating flora, glycine and sodium pyruvate facilitate the development of staphylococci, the reduction of tellurite to tellurium (black) and clarification of the egg yolk allows the presumptive identification of colonies on Baird Parker Egg Yolk Tellurite Agar, the formation of an opaque halo on Baird Parker RPF Agar allows the definitive identification of coagulase positive staphylococci.

The medium also allows the growth of some strains of *Streptococci, Micrococci, Corynebacteria* and *Enterobacteria*, though not developing the typical reaction; some

yeasts, fungi and bacilli may also be present, easily distinguishable by the morphology and gray colonies.

It is necessary to incubate at 37 °C for 48 hours, marking on the bottom of the plates, the presence of typical colonies. A second reading after a further 24 hours of incubation is needed, keeping on marking additional typical colonies and counting the total. Plates at two dilutions, containing up to 150 typical colonies and / or atypical, must be counted.

Typical colonies of *S. aureus* are black, shiny, convex, 1-1.5 mm in diameter after 24 hours of 1.5-2.5 mm after 48 hours, surrounded by a halo of egg clarification that can, however, appear partially opaque. After 48 hours, colonies of *S. aureus* always present a double halo, an inner opaque and an outer transparent. It should be borne in mind that *S. aureus* can grow with atypical colonies, similar to previous ones, but not alone. These colonies should be considered suspect and counted separately. Finally, the suspect colonies of *S. aureus* undergo an Immunoassay investigation with Latex test (Oxoid) for presumptive identification. The test takes advantage of blue latex particles coated with fibrinogen of porcine origin and rabbit IgG that include specific polyclonal antibodies directed against capsular polysaccharides of *S. aureus*.

## 4.8. Clostridia genus

Clostridia are rod, Gram-positive, motile and non-motile, generating oval or spherical endospores. These bacteria do not possess a capsule, excepting *C. perfringens* and a few other species. Nearly all species of the Genus *Clostridium* are attaining butyric fermentation with production of large amounts of gases (mainly $CO_2$ and $H_2$), yield mixtures of organic acids and alcohols from carbohydrates and peptones. Many may be saccharolytic, or proteolytic, neither or both. *Clostridia* may metabolize carbohydrates, alcohol, amino acids, purines, sterols or other organic compounds. Some species fix atmospheric nitrogen and are sulfite-reducers. Catalysis is usually negative, although traces of catalase could be found in some strains. Many species are obligate anaerobes, although some tolerate oxygen, some others grow but not produce spores in presence of air at atmospheric pressure. The majority of species grows rapidly at pH 6,5-7 and at a

temperature between 30° C and 37° C. The temperature range for optimum growth is from 15° C to 69°C (Bergey's Manual, 1986).

*Clostridia* are ubiquitous microorganisms, being natural inhabitants of the soil, intestine of living organisms. The most prominent feature is the possibility that they produce endospores that are more resistant than vegetative forms to heat, dehydration and other chemical and physical destructive agents. The endospores have the function of allowing the bacterium to face more or less long periods where adverse environmental conditions would lead to death of vegetative forms (Bergey's Manual, 1986).

Some species produce exotoxins and are pathogenic to humans, the infectious dose is known to be $10^8$-$10^9$ CFU/g (Bitton, 1984; Pahren, 1987). With the exception *of C. botulinum and C. difficile*, respectively responsible for Botulism and pseudomembranous Colitis in humans and animals, other species are associated to the most infections of wounds, among them the most important are: *C. tetani*, head of the Tetanus; *C. perfringens, C. novyi, C. septicum, C. histolyticum, C. bordelli,* often associated with gas gangrene, *C. botulinum* and *C. perfringens* cause serious phenomena linked to consumption of contaminated food.

Other Clostridial species are rarely associated with infections while others, such as *C.bifermentans* and *C. sporogenes*, are considered nonpathogenic.

## 4.9. Sulphite-reducing Clostridia

### 4.9.1. Method

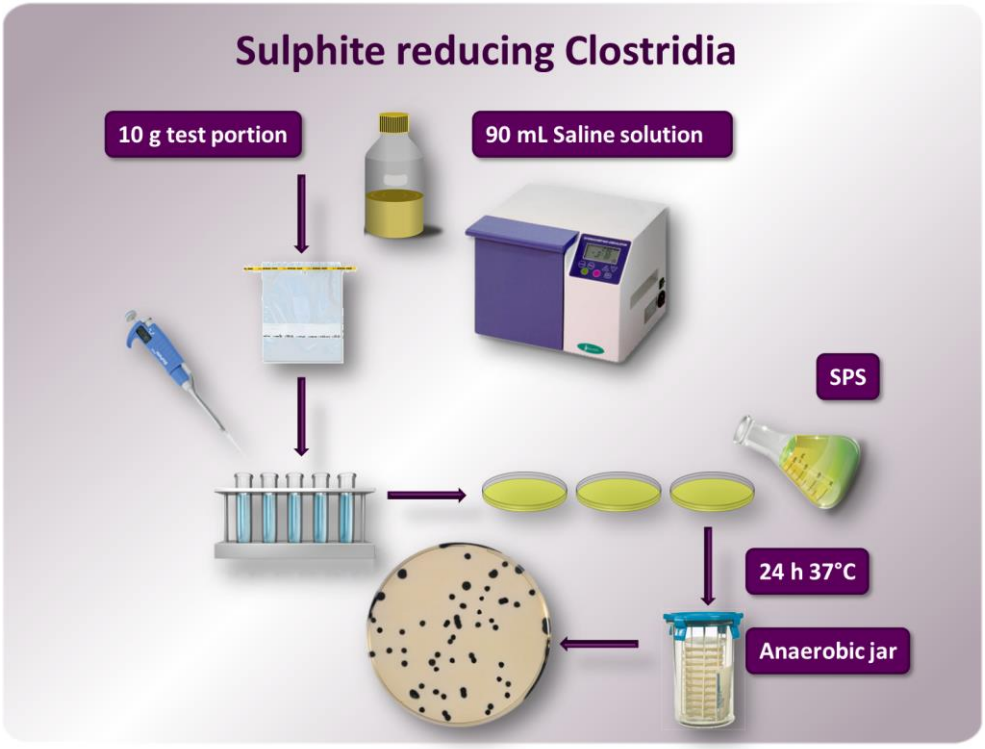



**Image 9 Flow chart of microbiological technique for Sulphite reducing Clostridia detection.**

The procedure for the detection of Sulphite-reducing Clostridia suggests the pour plating method after dilution. The selective medium for research is the SPS (Oxoid). The plates of SPS are incubated at 37 ° C for 48 hours under anaerobic conditions.

The selective medium SPS, Sulphadiazine Sulphite Polymyxin Agar, contains tryptic hydrolyzate of casein, yeast extract, sodium sulfite, ferric citrate, polymyxin B sulfate, sulfadiazine, agar.

The polymyxin B sulfate and sulfadiazine inhibit the majority of Gram-negative and Gram-positive bacteria. The sulphite-reducing Clostridia reduce sulfite to sulfide which reacts with iron ions to form a black precipitate of iron sulfide. Consequently, the colonies on the ground appear black.

## 4.10. *Clostridium difficile*

Some strains are equipped with a polysaccharide capsule that serves as an anti-phagocytic factor.

Although the fimbriae are not present in the majority of Gram-positive (but are virulence factors of many Gram-negative bacteria), their presence has been detected in *C. difficile*.

Many studies have confirmed the production of isoenzymes (hyaluronidase, gelatinase and collagenase) responsible for the destruction of connective tissue.

For many enteropathogenic bacteria, the ability to adhere to the intestinal mucosa is a prerequisite for colonization. The highly virulent strains of *C. difficile* attack the intestinal mucosa in an invasive way; the adhesion and colonization among the various strains is partly due to the production of toxins.

The toxins of *C. difficile* are among the largest bacterial toxins known. The toxin A has a molecular mass of 308 kDa and toxin B of 270 kDa. The tcdA and tcdB are encoded by the same locus.

This pathogen is responsible for many nosocomial diseases which may be asymptomatic or lead to diarrhea of varying severity (up to the most severe cases of Pseudomembranous Colitis), in most cases as a result of a broad-spectrum antibiotic therapy (cephalosporins, penicillin, clindamycin, ampicillin) that has undermined the intestinal flora. Usually, the infection is contracted in a hospital setting for direct contact with the patient or with contaminated objects.

In addition, *C. difficile* was isolated even outside of the gastrointestinal tract, in cases of chronic arthritis, osteomyelitis and in an immunocompetent child with a liver abscess.

Currently there is no objective evidence indicating *C. difficile* as a pathogen carried by food, but recent changes in the epidemiology and the emergence of the pathogen have necessitated his research even in the food. The most recent studies conducted in Canada (2007) have demonstrated the presence of the germ in 12 of 60 samples of meat bought in a period of ten months. It should be noted that among those isolates, 11 were toxigenic. Similar results have also been obtained in the USA, but in Europe there is no record evidencing either the presence or the production of toxins by *C. difficile* in meat.

The first study on *Clostridium difficile* dates back to 1935 when Hall and O'Toole isolated this organism from the intestines of sick children, and named it "*Bacillus difficilis"*. Its name derives from the fact that it initially grew very slowly in culture, in fact, early isolation attempts were difficult. The two researchers found out that the bacteria produced toxins lethal to pigs and rabbits after subcutaneous injection. Subsequently, in 1978, studies were carried out on the connection between *C. difficile*, diarrhea associated with antibiotic use and Pseudomembranous Colitis (PMC) in humans. *C. difficile* is now recognized as the major cause of these types of diseases in humans.

Since 2003, in North America, particularly in Canada and the United States have been reported increasing rates of *C. difficile*, with a period of severe and higher mortality, associated with the birth of a new strain: the virulent strain ribotype 027, which subsequently has been recognized as a cause of epidemic in many European countries.

The ribotype 027 strain presents a deletion in the gene that results in tcd increased production of toxins. However, in Belgium, Netherlands, Ireland and Scotland ribotype 078, which has a mechanism of overproduction of toxins similar to type 027, has been identified as responsible for the increase of the disease associated with the organism.

According to official WHO data, the last five years described an increase in hospitalizations for *C. difficile* infection in many European countries: Austria has gone from 0,19 per 1000 inhabitants in 2001 to 0,24 in 2006, Finland by 0,24 in 2002 to 0,31 in 2006, Norway in 2002 from 0,15 to 0,21 in 2005 and the UK from 0,1 in 2000 to 0,17 in 2005.

The data of the European Centre for Disease Prevention and Control show an increase in the prevalence of nosocomial cases attributable to this pathogen from 0,039% in 1999 to 0,122% in 2007.

In particular, in England, between 1990 and 2004, there has been an increase in cases, especially in subjects aged between 60 and 64 years, although a growth was also evident in younger subjects. There was a gradual increase in the early 90's, followed by much more pronounced increases, occurring first in 1996/97, then in 2001/02. It should be mentioned that in England, just in the year 2007, there were more than 50.000 cases of *C. difficile* infections, 20% of them in young patients.

There are no comprehensive data on the Italian situation. However, Epidemiological Observatory of the Lombardia Region study, on the spread of hospital infections

reported, in the early months of 2008, an increase of 60% of patients compared to the same period in 2007.

Available below there is the 2008 distribution of *Clostridium difficile* ribotypes in Europe (according to Martijn P. Bauer, Daan W. Notermans, Birgit H. B. van Benthem, Jon S. Brazier, Mark H. Wilcox, Maja Rupnik, Dominique L. Monnet, Jaap T. van Dissel, Ed J. Kuijper, for the ECDIS Study Group. *Clostridium difficile* infection in Europe: a hospital-based survey Lancet 2011; 377: 63–73. November 16, 2010).

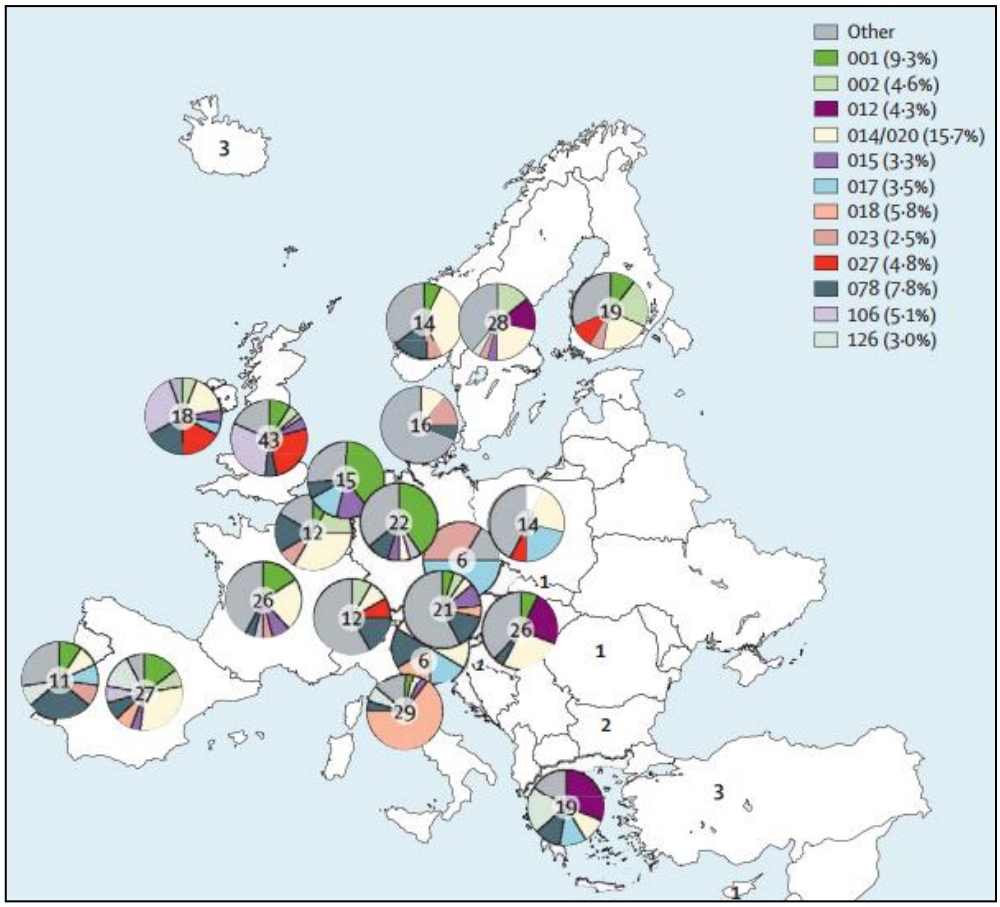



**Chart 16 Geographical distribution of *Clostridium difficile* PCR ribotypes in European countries with more than fi ve typable isolates, November, 2008.**

The increasing incidence and severity of human *Clostridium difficile* infection (CDI) has been attributed to the emergence of the hypervirulent strains polymerase chain reaction (PCR) ribotypes 027 and 078. A research published in 2010 (Clinical infectious diseases, Volume 50 Issue 1 January 1, 2010) reported that these hypervirulent strains, that are highly resistant to macrolides, are spreading in Italy.

### 4.10.1. Method

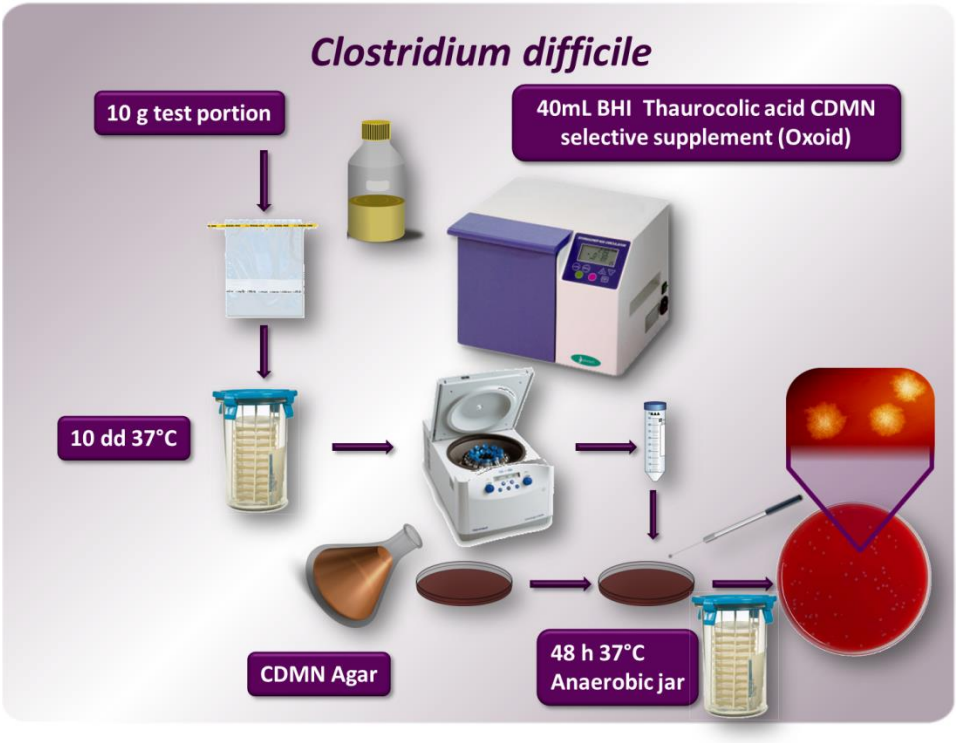



**Image 10 Flow chart of microbiological technique for *C.difficile* detection.**

According to *Clostridium difficile* isolation protocol, an aliquot of 10 g of meat and 40 mL of BHI (Oxoid), supplemented with sodium taurocholate (Sigma) and the supplement (Oxoid) SR0173 containing norfloxacin, and moxalactam, must be added to a Stomacher bag.

Homogenized Stomacher bags are incubated at 37 ° C for 10 days in anaerobic jar.

On the tenth day the sample undergoes an indirect method of determination: 2 mL of the suspension are removed from the envelope and poured into a falcon containing 2 mL of 96% ethanol in order to eliminate vegetative forms, leaving only spores. After kept at 20°C for 50 minutes, the falcon is placed in a centrifuge at 3800 rpm for 10 minutes: the precipitate is than streaked on CDMN Agar (Oxoid), a selective medium whose composition differs from that of the enrichment broth because of the replacement of taurocholate with agar. In addition, the agar preparation requests the adding of a 5% rate of defibrinated horse blood and the Oxoid supplement SR0173.

SR0173 Supplement contains moxalactam, a broad spectrum antibiotic, effective especially against Gram-negative and norfloxacin, a broad spectrum antibiotic directed

against Gram-positive and some Gram-negative. The resulting plates are incubated at 37°C for 48 hours in anaerobiosis conditions. The colonies appear grayish, rhizoids and the characteristic dung horse smell is recognizable.

In case of identification of a typical colony, it is necessary to proceed in order to identify *C. difficile* using an immunoassay test. Latex particles are coated with IgG antibodes specific for *Clostridium difficile* cell wall antigens. When mixed on a reaction card with selective or enrichment broth containing the organism, or with a suspension of *Clostridium difficile* colonies from solid media, the latex particles agglutinate in large visible clumps within 2 minutes.

A further identification method consists on realizing a Gram coloration to identify the typical structure of the *Clostridium*; using a green malachite colorant it is achievable to evidence spores.

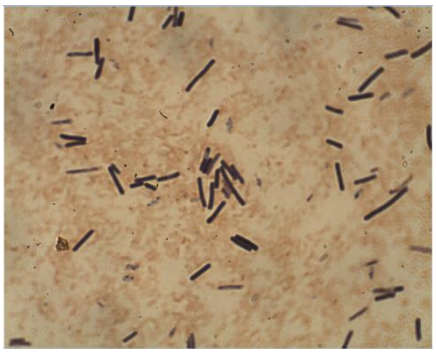


**Image 11 *C.difficile* Gram coloration.**

## 4.11. *Clostridium perfringens*

It is a Gram-positive anaerobic and spore-forming rod, ubiquitous from decaying plant to marine sediment, intestinal tract of humans and other vertebrates, insects and soil. Virtually every soil sample examined, with the exception of the sands of the Sahara, contains *C. perfringens*.

*C. perfringens* is usually responsible for benign infections, as part of the normal flora.

*C. perfringens* infections show tissue necrosis, bacteremia, cholecystitis and emphysema gas gangrene, also called Myonecrosis. The toxin involved in gas gangrene is as α-toxin, which produces alterations in the cell membrane, by interfering with its normal functions. The gas Gangrene is a process that begins with the entry of spores in a wound poorly

oxygenated generally lacerocontuse; these, once germinated, if producing specific toxins (α and ϑ), will cause the degeneration of the adjacent soft tissue with relative production of gas. In blood agar plates, the *C. perfringens* grows anaerobically and produce β-hemolysis; colonies are flat, wrinkled, translucent with jagged edges.

### 4.11.1. Method

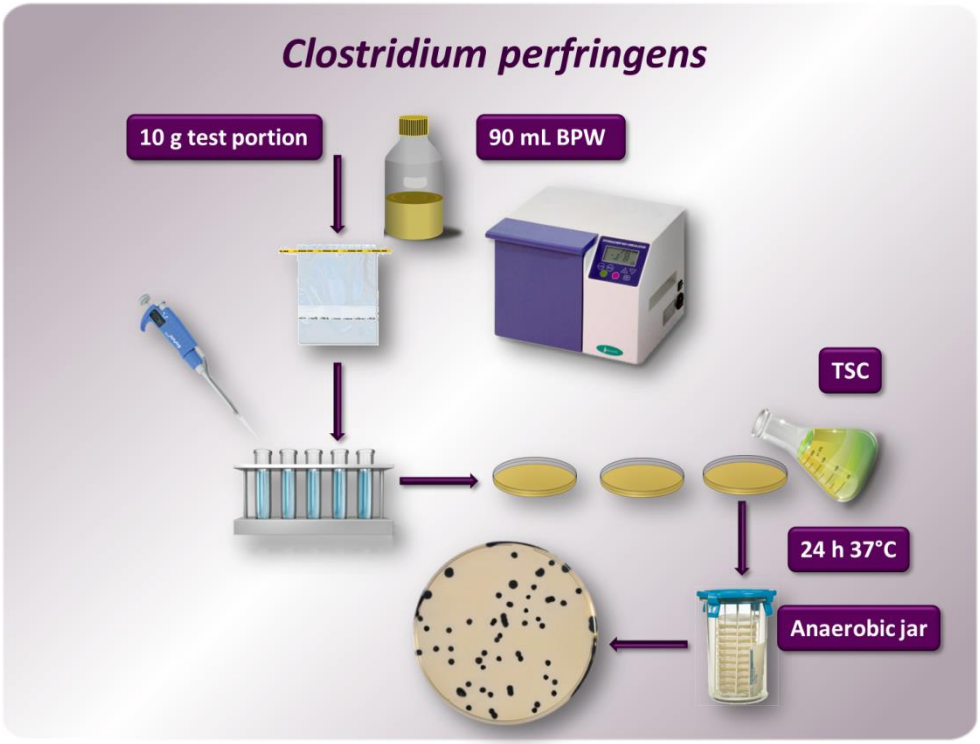



**Image 12 Flow chart of microbiological technique for *C.perfringens* detection.**

To determine the presence of *C. perfringens,* an aliquot of 10 g of meat and 90 mL of Buffered Peptone Water (Oxoid) are added to a Stomacher bag. After homogenization with Stomacher, 1 mL from the sample is included in TSC Agar Base (Oxoid), using the pour plate method. Plates are incubated at 37 ° C for 20 hours in anaerobic jar.

TSC Agar Base is a selective medium consisting of tryptone, peptone soy, yeast extract, iron ammonium citrate, sodium metabisulfite anhydrous agar, D-cycloserine Antimicrobic Supplement, D-cycloserine 4-MUP supplement, 4-metilumbelliferilphosphate.

Selective medium, with the precautions of asepsis, is supplemented with Egg Yolk Emulsion (Oxoid).

Typical colonies of *C. perfringens* are black, convex, surrounded by a halo of egg clarification that appears opaque.

A confirmation test consists on realizing two streaks on blood agar plates; once conducted the microbiological step, one plate is incubated in aerobic condition at 37° C and another one in anaerobic jar to same condition of temperature. After incubated, the plate presenting *C. perfringens* must only be the one put in anaerobic conditions and has to present a β-haemolysis halo around colonies.


**Image 13 *C.perfringens* β Haemolysis on Blood agar plate on the left (anaerobic conditions); no grow on the right (aerobic conditions).**

An additional test to reveal the identity of the presumptive *C. perfringens* is based on the use of Api 20 test (Biomerieux); it presents 20 microtubes containing dehydrated substrates. These tests are inoculated with a bacterial suspension which reconstitutes the media.

During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The reactions are read according to the reference table and the identification is obtained by referring to using the identification software.

## 4.12. *Escherichia coli* O157: H7

The strain most frequently involved in food-borne diseases is *E. coli* O157:H7, EHEC belonging to the group.

*E. coli* O157:H7 was first identified in 1976 and described as a human pathogen in 1982 in the United States despite the serotyping methods used were available for a long time. More severe diseases, that causes the hemolytic uremic syndrome (from verocytotoxin), have been described since the 1950 in Switzerland. The microorganism is a carrier of phages capable of directing the synthesis of molecules of the group of shiga-toxins, which are activated in particular conditions, for example after exposure to sublethal doses of certain antimicrobials. In addition to the gut of infected animals (mainly cattle), the microorganism easily tends to persist in the external environment, even in the aquatic environment.

The pathogenicity of *E. coli* O157:H7, which represents the prototype of the group of EHEC, is due to a multifactorial mechanism not yet fully understood. However, at least two critical factors essential in the pathogenesis of clinical manifestations have been widely described: the ability to adhere to intestinal mucosal cells with a characteristic mechanism called "attachement and effacement" (A/E), that involves the adhesion of the bacterium to the enterocyte (phase A), by a protein, encoded by the EAE gene and the consequent destruction of microvilli (step E). Only strains that possess such capabilities would be able to give rise to bloody diarrhea.

The production of a potent toxin, known as verocytotoxin (VT), acts at the level of the colon, causing abdominal cramps, nausea, vomiting and bloody diarrhea. The VT can spread into the bloodstream by binding through glycolipids receptors, the cell membranes of target organs, especially the kidneys, giving rise to Haemolytic Uraemic Syndrome.

The disease is characterized by acute renal failure and Microangiopathic Hemolytic Anemia and Thrombocytopenia, which may result in the loss of renal function and give Purpura Thrombotic Thrombocytopenic, a form of Haemolytic Uraemic Syndrome accompanied by fever and neurological symptoms observed more frequently in adults than in children.

The strong virulence of *E. coli* verotoxigenic, demonstrated by the low infectious dose (estimated to be less than 50 cells), resulted in the inclusion of Zoonoses to be monitored in the field of human and veterinary use in the European Union Member States.

About verotoxigenic groups, it is worth to remember the recent episode including a large *E. coli* O104:H4 outbreak in Germany which later spread to the Scandinavian countries. E. coli O104:H4 represented the origin of the phenomenon of infection with the involvement of one hundred subjects with a mortality rate of approximately 20% for hemolytic uremic syndrome.

### 4.12.1. Method

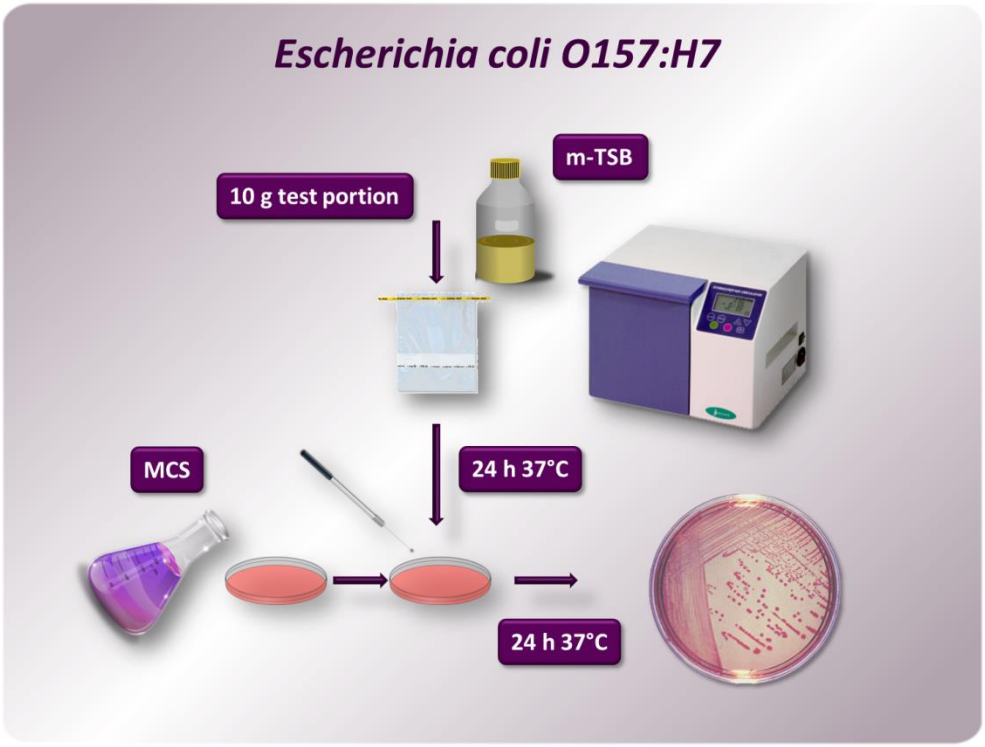



**Image 14 Flow chart of microbiological technique for *E. coli* O157H:7 detection.**

*E. coli* O157:H7 isolation protocol consists on adding 10 g of meat and 90 mL of mTSB (Modified Tryptone Soya Broth) (Oxoid) to a Stomacher bag.

mTSB enrichment broth contains a pancreatic digest of casein, digestion of soybean meal, sodium chloride, dipotassium hydrogen phosphate, glucose and bile salts. Homogenized Stomacher bags are incubated at 37 ° C for 24 hours.

After incubation, the sample is streaked to selective medium MCS (MacConkey Sorbitol Agar Oxoid) containing peptone, sorbitol, bile salts, sodium chloride, natural red, crystal violet, agar.

Plates are incubated for 24 hours at 37° C. Unlike *Escherichia coli, E. coli O157:H7* does not ferment sorbitol and hence colonies are colorless or slightly colored pink.

Additionally, suspected colonies are subjected to the Latex test (Oxoid) for presumptive identification. Latex particles are coated with an antiserum against the antigen of *E.coli O157*. When these particles are mixed with fresh colonies of *E. coli* serotype O157, the bacteria bind to the antiserum causing apparent agglutination of the latex particles.

# 5. VIRULENCE FACTORS

## 5.1. Exoenzymes expression

Biochemical investigations were performed for each isolate. The exoenzymes expression, using a culture medium base with the addition of specific substrates, human red blood cells, protease, lipase, collagen, mucin, elastin allows to highlight the activity of the strains with different substrates.

**Proteinases**

Tryptic soy agar (Oxoid) has been used by adding 1% of skim milk; the same has been carried out for the casein 1% and for collagen 1% (Burke et al., 1991; Edberg etal., 1996). A zone of clearing around the inoculums spot was regarded as a positive test (Burke et al., 1991; Edberg et al., 1996).

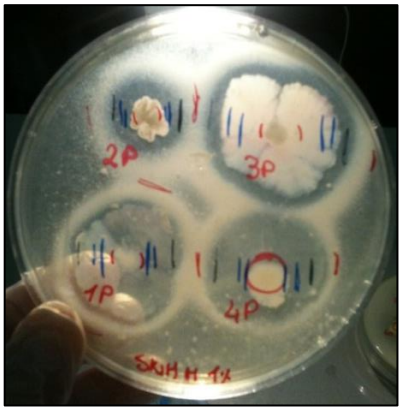



Image 15 Skim milk halo.


Image 16 Caseine halo.

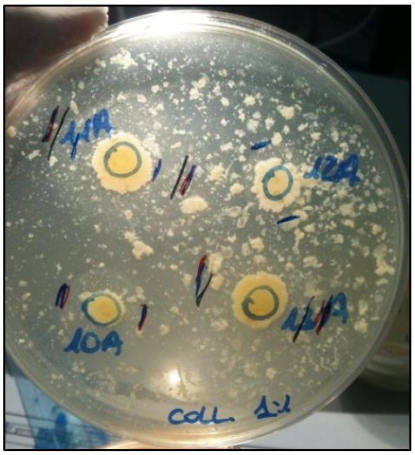



Image 17 Collagen halo.

**DNase**

DNase agar (Difco Laboratories) was employed as the substrate (Janda and Bottone, 1981; Edberg et al., 1996). Halo or a zone of clearing around the bacterial colonies indicate a positive test (Edberg et al., 1996).

**Elastase**

Elastin powder from bovine neck ligament (Sigma) was prepared as a 1% suspension in Tryptic soy agar (Oxoid) (Sbarra et al., 1960), (Janda and Bottone, 1981), (Edberg et al., 1996). Positive tests were observed by the clearing of the opaque medium around the inoculum spot (Sbarra et al., 1960; Edberg et al., 1996).

**Lecithinase**

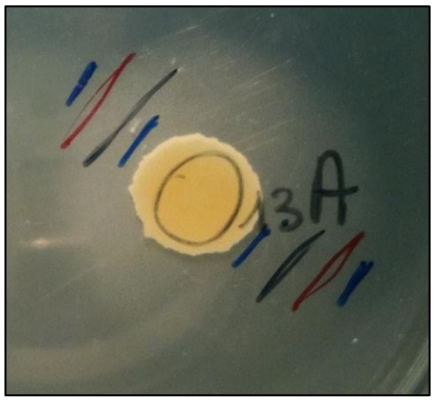


Image 18 Egg Yolk halo.

Lecithinase was determined by utilising a 2% solution egg yolk in Tryptic soy agar (Oxoid) (Edberg et al., 1996). A white precipitate around or beneath the inoculum spot was taken as a positive test (Edberg et al., 1996).

**Haemolysis**

Hemolytic assays were performed by measuring the zone of hemolysis around colonies on a blood-agar plate (Oxoid) (Imzilm et al. 1996).

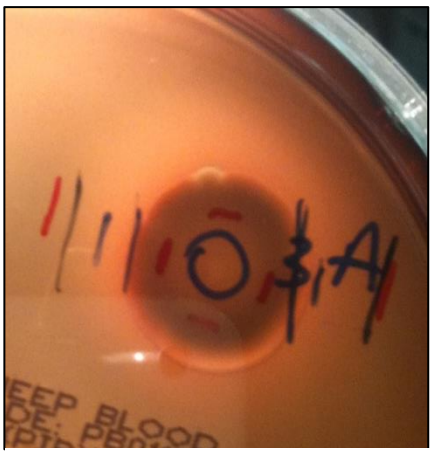


Image 19 Hemolysis.

**Collagenase**

Endopeptidase activities were assayed with Tryptic soy agar (Oxoid) to 0,1% of collagen type I (Sigma).

**Mucinase**

This activity were assayed with Tryptic soy agar (Oxoid) to 1% Mucin II (Sigma) and 1% Mucin III (Sigma).

Image 20 Collagen type I halo.

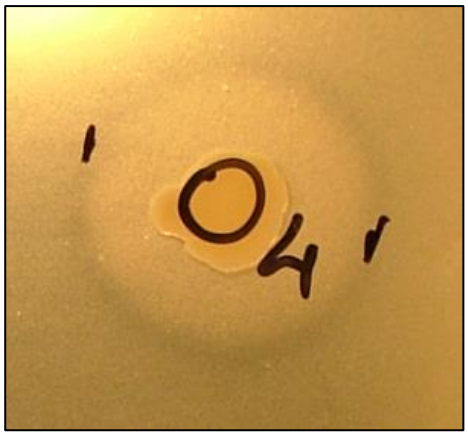


Image 21 Mucin halo.

**Amilase**

This activity was assayed with Tryptic soy agar (Oxoid) to 0,5% soluble starch (Sigma).

**Chitinase**

This activity were assayed with Tryptic soy agar (Oxoid) to 0,5% Chitin (Sigma).

Image 22 Chitin halo.

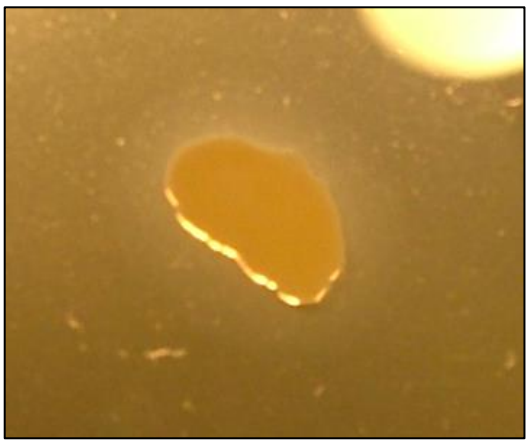


Image 23 Amilase halo.

Positive tests were observed by the clearing of the opaque medium around the inoculum spot after incubation at 37° C. Halos were measured for seven consecutive days (Sbarra et al., 1960; Edberg et al.,1996); to be able to reveal the presence on the seventh day, the plates were treated with appropriate solutions in order to verify the possible halo. For plates containing Mucin, 1% $CaCl_2$ solution was used; the highlighting of the protease halos require the use of a saturated solution of $(NH_4)_2SO_4$; for amylase, Lugol's solution was used.

## 5.2. Biofilm

Biofilm consists of microorganisms and polymeric substances, usually exopolysaccharides, produced by the microorganisms. The biofilm, once formed, protects the microorganisms living in it by providing an ecological niche.

In the matrix of the biofilm, non-cellular materials can also be found: mineral crystals, corrosion particles, clay particles depending on the environment in which it is generated.

In biofilms, besides bacteria generated by high adhesion and producing exocellular polymers, there are also several other organisms: Algae, Mold, Fungi and Protozoa.

Biofilms can grow on a wide variety of surfaces, including living tissues, medical devices, industrial or potable water pipes. The scanning electron microscopy has shown that many pathogens and spoilage of foods are able to adhere and accumulate as biofilms on surfaces of stainless steel, aluminum, glass, rubber, teflon and nylon, which are materials commonly used in the food industry.

It has been shown that the adsorption of certain types of proteins to surfaces plays an important role in the processes of microbial adherence: for example, the presence of casein and ß-lactoglobulin inhibits the adhesion of *Listeria monocytogenes* and *Salmonella Typhimurium* to stainless steel and to the rubber, while other milk proteins are able to promote the attachment of some microorganisms.

In a path of water it is estimated that approximately 99% bacteria in the biofilm is established, with a constant exchange with environment, regulated by very complex mechanisms, which involves the flow velocity of water in various points, proliferation,

competition among species and other mechanisms equally variable and difficult to predict. This compaction of the biofilm, leading to unpredictable releases of portions of material of different size, clearly explains why the biological tests conducted regularly brought conflicting results, with fluctuating bacterial counts and often without relationship to sanitation interventions.

Assessing biofilm formation of a microorganism can make a projection of the same formation on host tissues or on inanimate surfaces: the biofilm contributes to bacteria pathogenicity, inhibits the effectiveness of antibiotic therapy, protects against immune defense mechanisms, facilitates bacterial communication and contributes to the expression of other virulence factors; this property was assessed following the Stepanovich et al. (2004) protocol, mentioned below:

Day 1:

- Prepare a broth culture of the strain in question in tubes containing 5 mL of Tryptic Soy Broth (TSB, Oxoid) with 1% NaCl and incubated under stirring for 16-18 hours at 37 ° C.

Day 2:

- Diluted the broth culture 1:200 in TSB 1% NaCl, in particular, take 25µL of culture broth and place in test tubes containing 5 mL of the broth; transferred 200 µl of dilution in the wells of a microtiter plate in sterile polystyrene 96-well plates (Nunclon, Nunc, Roskilde, Denmark); insert a negative control by transferring 200 µl of TSB in a sterile cockpit; incubate the plate at 37 °C for 24 ± 2 hours.

Day 3:

- After incubation, aspirate the culture medium from each well; wash the wells with phosphate buffered saline (PBS, Oxoid) three times; add 200 µL 96% ethanol to fix the bacteria that may be immobilized on the walls of the wells and leave for 10 minutes; aspirate the ethanol and dry the plate; after added to the wells 200 µL of the dye Crystal Violet 2% (w / v), leave for 5 minutes;

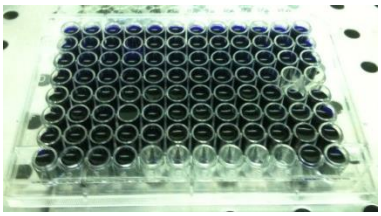


**Image 24 Multiwell plate.**

wash the wells 3 times with distilled water and air dry; solubilize the dye by adding 200 µL 96% ethanol to each well; read the absorbance of each well by microplate at a wavelength 570 nm. The absorbance measured in the tubes gives us a value that expresses the ability of bacteria to develop microbial biofilms, since it is directly proportional to the biofilm formation.

## 5.3. Hydrophobicity

To investigate the degree of hydrophobicity, given the nature of biological membranes, it is important to understand how the bacteria are able to overcome the phospholipid bilayer, thus emphasizing their most or less marked virulence; the hydrophobicity was assayed through the use of the protocol Rosenberg et al., 1980.

Day 1:

- prepare a broth culture of the strain of interest in test tubes containing 5 mL of TSB and incubate for 24 hours at 37° C;

Day 2:

- centrifuge tubes containing the bacterial cultures for 10 minutes at 3800 rpm with subsequent removal of the supernatant; wash the pellet with 5 mL of saline solution by means of successive centrifuges. Repeat for 3 times; add 4 mL of 50 mM PBS (pH 7.4) +0,15 M HCl and vortex; transferring 1,5 mL of the solution made in cuvettes of glass and make the reading of the spectrophotometer at a wavelength 660 nm; transfer the remaining 2,5 mL into glass tubes with a conical base, like falcon, add 1 mL of xylene (2,5: 1 v/v) and wait 20 minutes leaving at

room temperature; vortex the tubes for 2 minutes and let for 20 minutes to allow the hydrocarbon to solubilize the hydrophobic portion of the crop; gently pick, with a Pasteur pipette, the aqueous phase visible on the bottom under the layer of xylene, put it in a glass cuvette measuring absorbance at 660 nm comparing to a white represented by phosphate buffered saline (PBS).

The decrease in absorbance of the aqueous portion following the addition of xylene has provided the value of hydrophobicity of the bacterial cells (H) expressed in %.
Results were calculated to according the following formula:

$$100-[100\ x(At/Ao)]=H$$

where:

At = absorbance of the aqueous phase after the addition of xylene;

Ao = absorbance of the aqueous phase before the addition of xylene.

Values converted to percentage and distributed in ranges (low, medium, high) were ranked as follows: isolates with % hydrophobicity > 70% were classified as highly hydrophobic and those with a hydrophobicity index < 30% were classified as low range hydrophobic while those with a hydrophobicity index between 30 and 70% like on the average hydrophobicity (Denise de Oliveira Scoaris, Jean Colacite).

## 5.4. Antibiotic resistance: MRSA

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a type of staph bacteria that is resistant to certain antibiotics called beta-lactams. These antibiotics include methicillin and other more common antibiotics such as oxacillin, penicillin and amoxicillin.
Within the monitoring, S. *aureus* strains have been

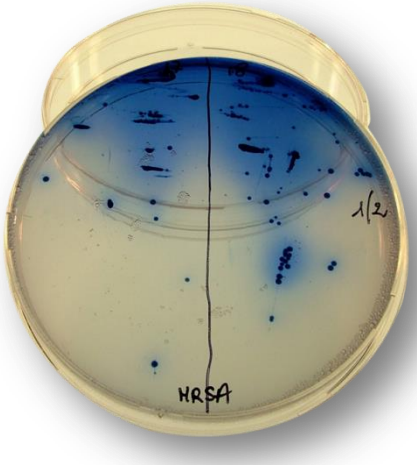



**Image 25 MRSA on ORSAB.**

isolated from various food matrices and subsequently the development of resistance to methicillin has been verified.

For this purpose Oxacillin resistance screening agar base (Oxoid ORSAB) has been employed; the antibiotics contained in the ORSAB Selective supplement (Oxoid) are oxacillin 2 mg/L, which has the purpose of inhibiting the methicillin-sensitive *Staphylococcus aureus* (MSSA) and polymyxin B, for the suppression of other bacteria able to grow (ex. *Proteus spp.*).

## 5.5. PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing

PCR-ribotyping is a system based on the sequencing of a polymorphic region of DNA that is located between 16S and 23S ribosomal RNA subunits. This method generated bands of high and close molecular masses, thus the patterns were difficult to read on agarose gel electrophoresis. The innovation in the method consisted on using a primer closer to the intergenic space to improve the reading of the electrophoresis runs. The PCR generates bands of easy separation by electrophoresis on agarose gel. Each of the 10 serogroups and 11 subgroups of serogroup A produced a different model and therefore it is possible to discriminate the type of *Clostridium difficile*.

Strains of *C. difficile* were grown in an anaerobic atmosphere of plates made with brain heart infusion agar supplemented with 5% defibrinated horse blood, taurocholate 0.1%, 10 mg/mL cefoxitin and 10 mg/mL cycloserine.

The DNA was extracted using a Chelex resin base DNA extraction commercial kit (InstaGene Matrix 1, Bio-Rad, France). Three major *C. difficile* colonies were inoculated to a final volume of 200 µL.

Amplification reactions were carried out in 100 µL volumes containing 10 mM Tris-HCl (pH 8,8), 50 mM KCl, 1,5 mM $MgCl_2$, 200 µL of each dXTP, (Pharmacia), 100 pmol of each primer, 2,5 units of Taq polymerase (Pharmacia) and 10 µL of DNA extract.

Amplifications were carried out in the thermal cycler (Perkin Elmer Cetus 480) for 1 cycle of 6 min at 94° C for denaturation, followed by 35 cycles (1 min at 94° C, 1 min at 56° C

and 1 minute at 72° C) and a final extension of 7 min at 72° C. Amplification products were fractionated by electrophoresis through standard 1,5% agarose (Eurobio) for 16 hours at 50 V in Tris-borate-EDTA buffer (Eurobio), with a distance of 35 cm between electrodes and analyzed on a UV table after ethidium bromide staining. Isolated groups were cut and in preweighed tubes. DNA was extracted using the kit GFX1 (Pharmacia Biotech, France).

DNA sequencing was performed on an Applied Biosystems 373A DNA sequencer with the DNA sequencing kit (Applied Biosystems). Sequences were then analyzed and aligned on the Sequence Navigator 1.0.1 software (Applied Biosystems).

# 6. RESULTS

Foodborne and waterborne bacterial pathogens constitute a major cause of mortality in developing countries and cause significant morbidity in developed nations. Some countries carry a disproportionately heavy burden of these infectious diseases due to inadequate resources to provide sanitation and hygienic facilities, and safe water. The most important bacterial pathogens transmitted through contaminated water and food include species or strains of *Salmonella spp., Escherichia coli, Staphylococcus* and *Campylobacter* spp..

The huge amount of data has been processed and transposed in graphs to efficiently handle the results. The individually discussed graphs were grouped by type of sampled matrix and results for individual microbial species investigated were also shown within each matrix. Each graph is annotated individually in order to describe collected results.

## 6.1. Matrix: Poultry meat

### 6.1.1. Chicken

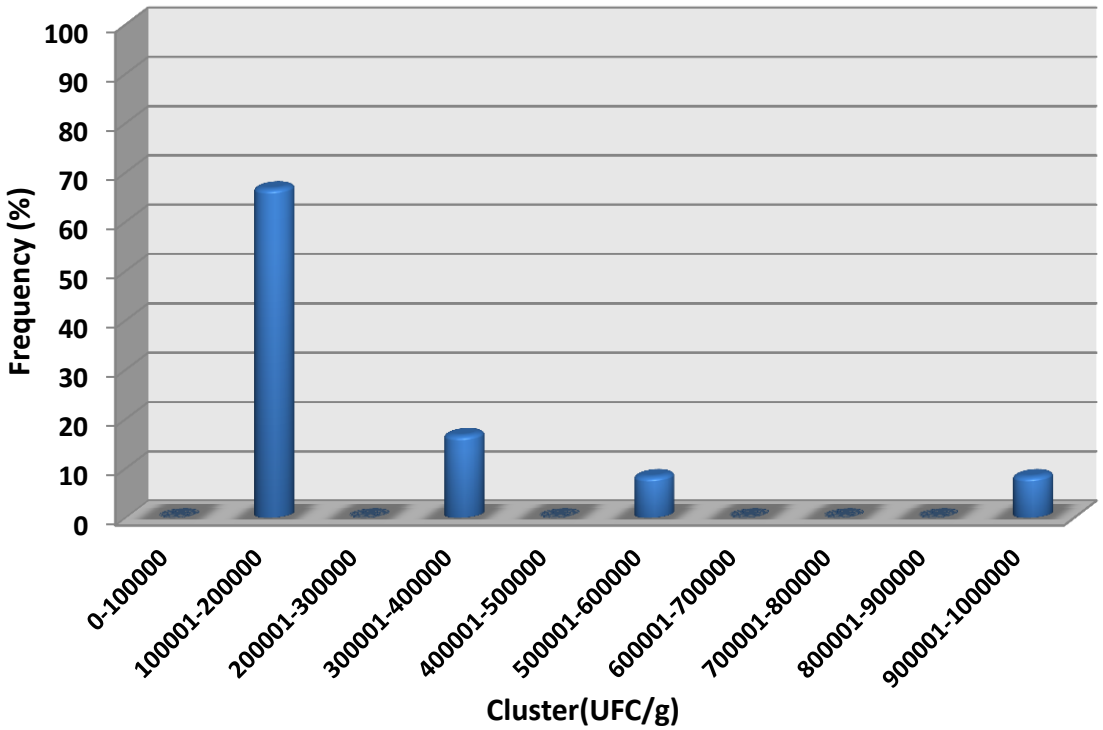



**Chart 17 Chicken matrix: Mesophilic frequency distribution.**

The distribution of frequencies of mesophilic microorganisms regarding chicken matrix highlights a normal pattern with evidence of high percentages for low microbial loads and very high microbial loads for a low confirmation percentage.

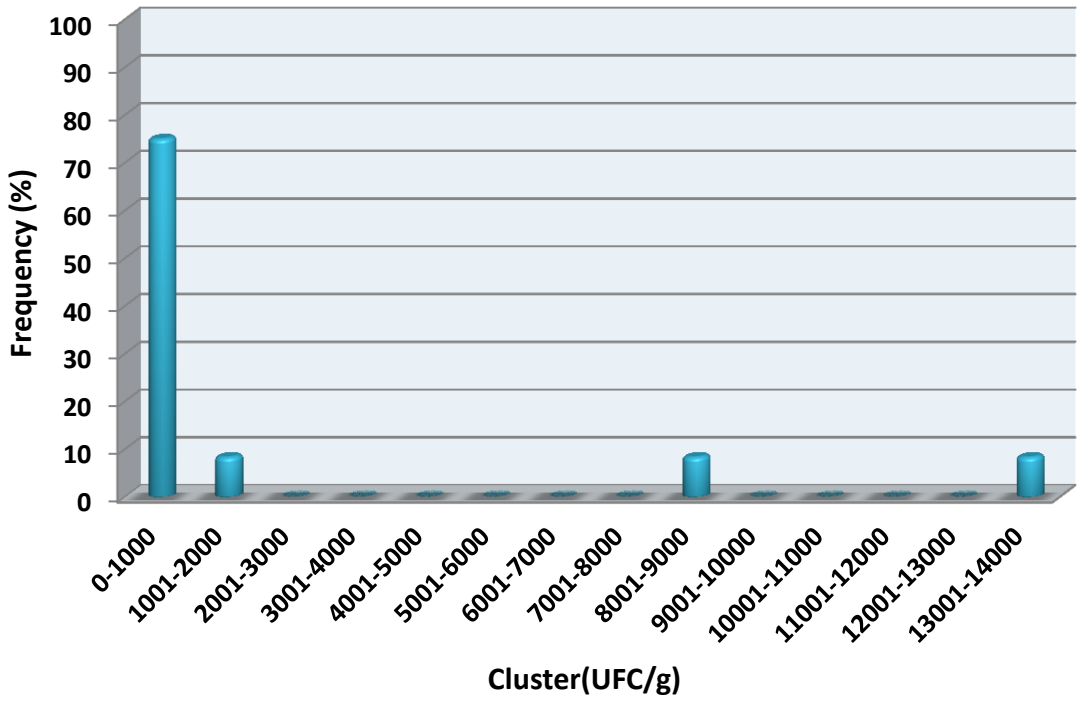



**Chart 18 Chicken matrix: *Escherichia coli* frequency distribution.**

*E. coli* frequency distribution focuses an evident non-compliance with current legislation, which states 500 CFU/g limit. The analyzed samples levels resulted beyond the limits of the law with a very high cluster within 1000 CFU /g, two peaks with higher microbial loads though the very low confirmation rates.

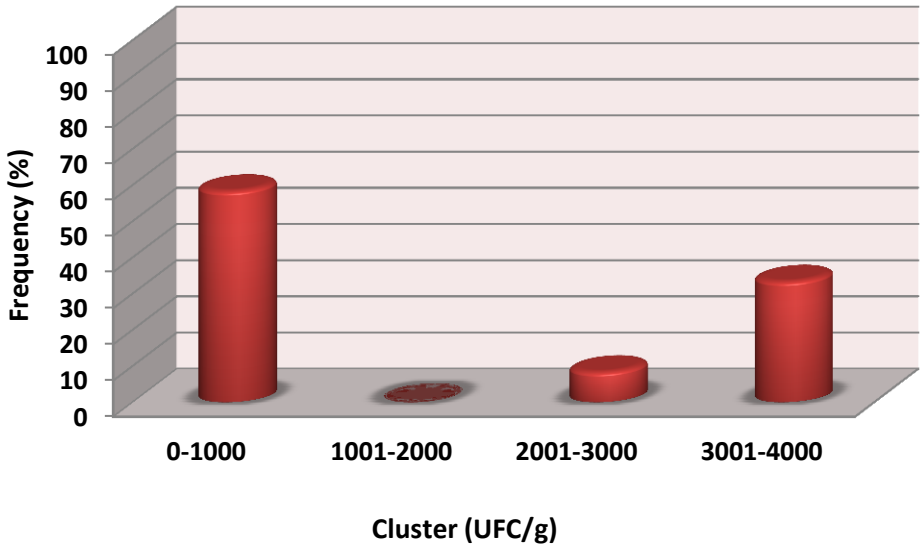



**Chart 19 Chicken matrix: *Aeromonas spp.* frequency distribution.**

The samples analyzed always presented high levels of contamination by *Aeromonas spp.* ; the chart indicates the presence of two populations of data which stand within values of 1000 CFU/g and another population of data in the cluster over the 3000 CFU/g. This brings out a significant observation of this pathogen. This trend clearly emerges as abnormal if compared to the expected decreasing frequency distribution.

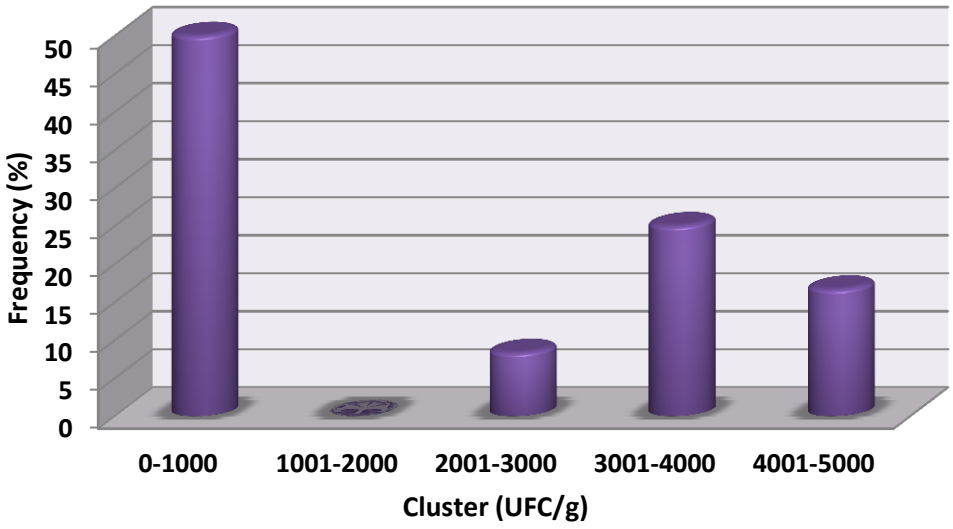



**Chart 20 Chicken matrix: *Enterococcus spp.* frequency distribution.**

Concerning *Enterococcus spp.,* the population data orientate around two peaks, one of 1000 CFU/g and the other with very high values: even in this case the reported trend is abnormal, since a standard data distribution suggests low frequencies at high values of microbial load.

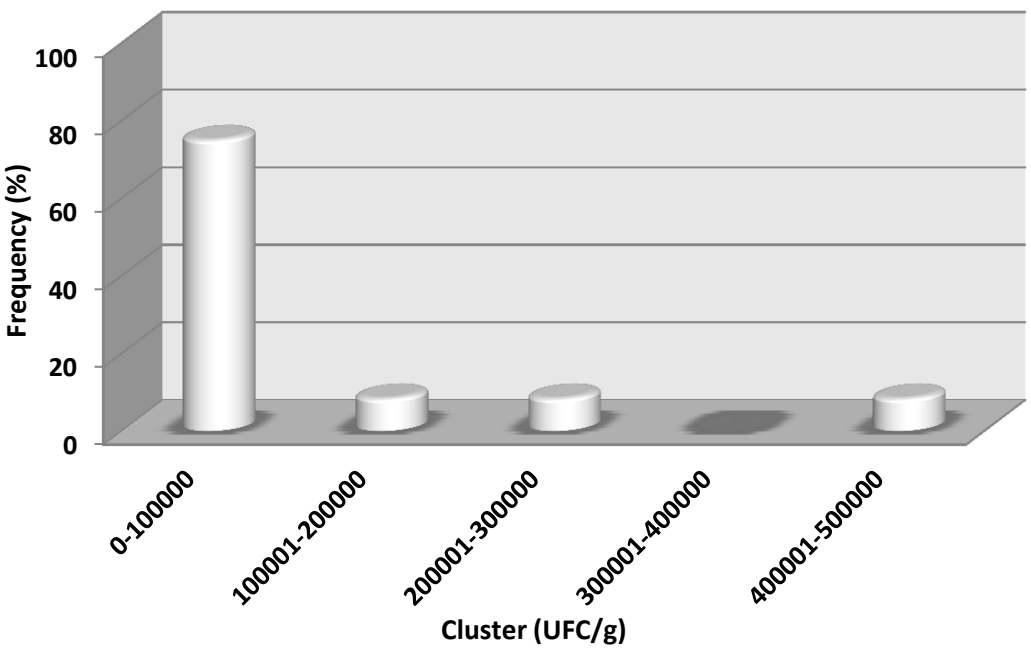



**Chart 21 Chicken matrix: *Staphylococcus spp.* frequency distribution.**

From the observation of the frequency distribution for *Staphylococcus spp.*, a normal single-mode pattern emerges: the data mostly correspond to the first cluster, being comprised into medium-high microbial loads range.

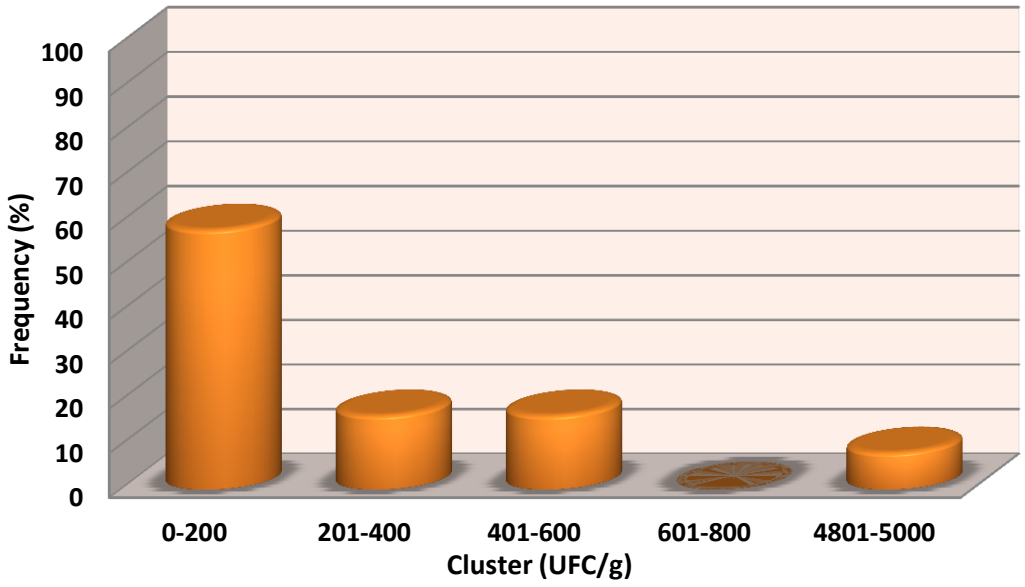



**Chart 22 Chicken matrix: *Staphylococcus aureus* frequency distribution.**

Most of the data stood on low microbial loads and the trend of the population data results logical, with high values of microbial load for few samples.

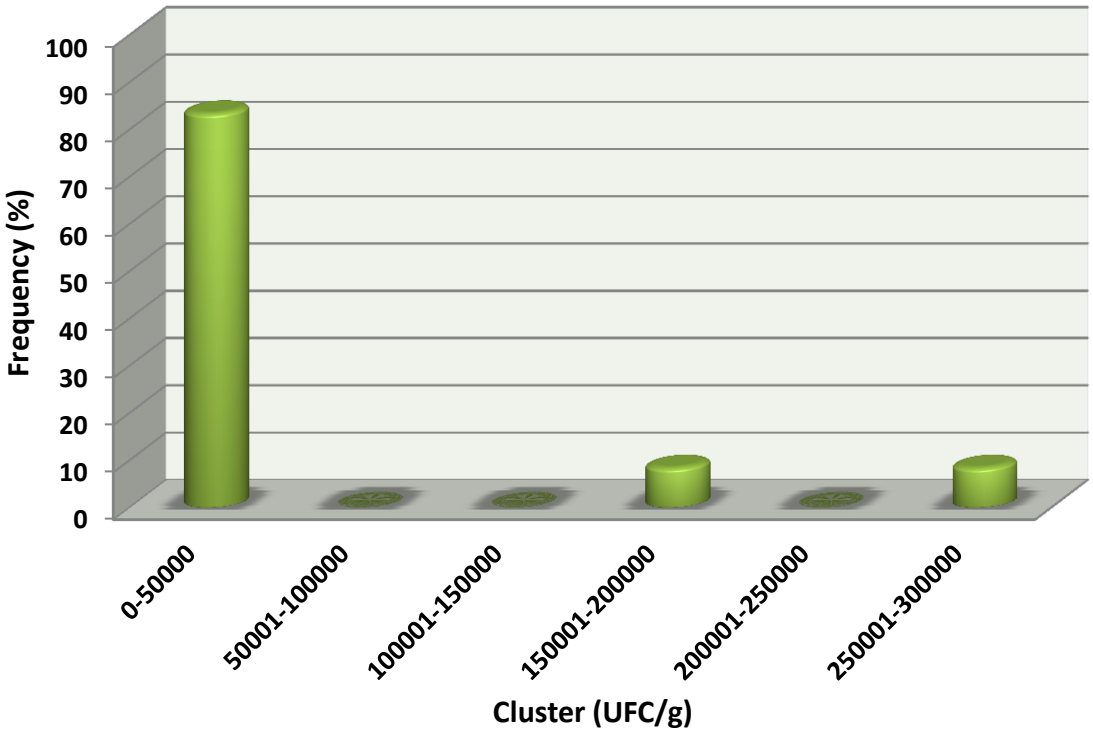



**Chart 23 Chicken matrix: *Listeria spp.* frequency distribution.**

The values for bacteria of the Genus *Listeria spp.* were ranked at high level but the detected data are concentrated almost entirely in the first cluster; *Listeria monocytogenes* evaluation was not easy due to the highly variable concentration of *Listeria spp.*, that sometimes did not allow easy identification of the opaque halo typical of *L. monocytogenes*. The sequencing of some suspect colonies of *L. monocytogenes* did not provide positive responses and therefore this pathogenic microorganism was never isolated.

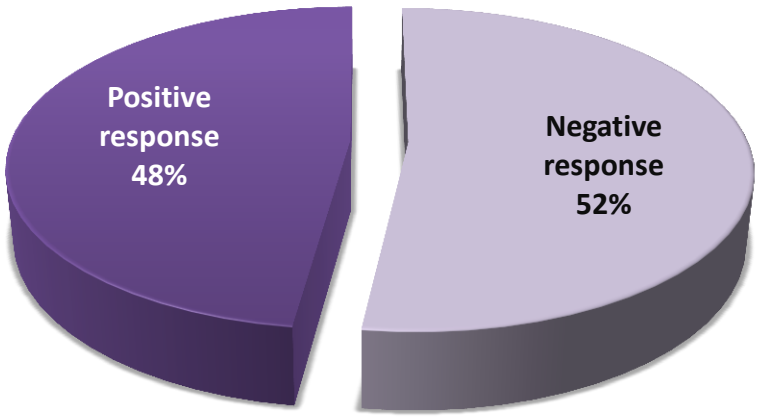



**Chart 24 *Salmonella spp.* in chicken matrices.**

The determination of *Salmonella spp.* consists on a survey of qualitative type: for this reason it was not possible to generate frequency distributions but pie charts; thus it is clear that in the samples of chicken there are very high percentages of positive values, around 48%. This data is alarming and underlines the lack of useful interventions aimed at limiting *Salmonella spp.* presence in chicken meat.

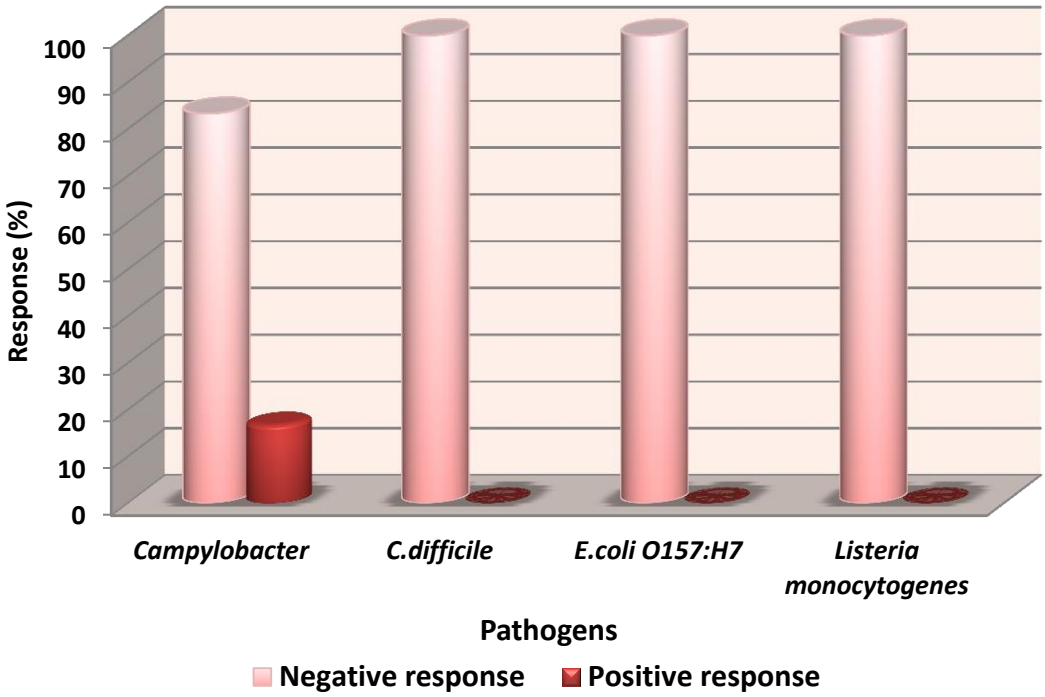



**Chart 25 Chicken matrix: *Campylobacter spp.*, *Clostridium difficile*, *E.coli* O157:H7, *Listeria monocytogenes.***

In particular, the research focused on investigations of emerging Pathogens through qualitative methods, bringing a presence/absence response. In chicken matrix, only emerging pathogen *Campylobacter spp.* was found: the most defined specie was, with a 99% percentage, *C.coli*.

Other pathogens were not isolated.

### 6.1.2. Turkey

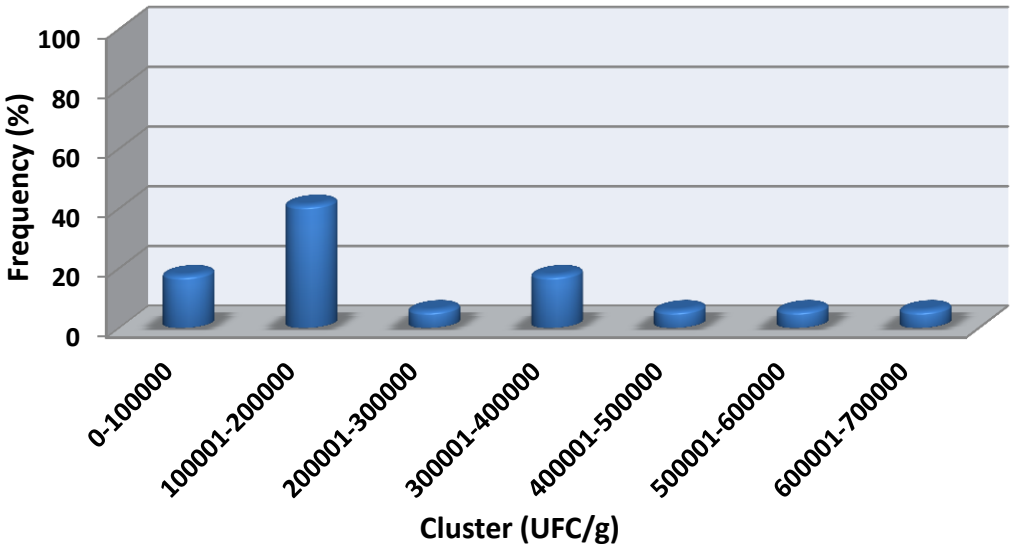



**Chart 26 Turkey matrix: Mesophilic frequency distribution.**

In turkey matrix the frequency distribution of mesophilic shows a trend that can be defined as a single-mode and levels of microbial contamination fulfill the limits of the law.

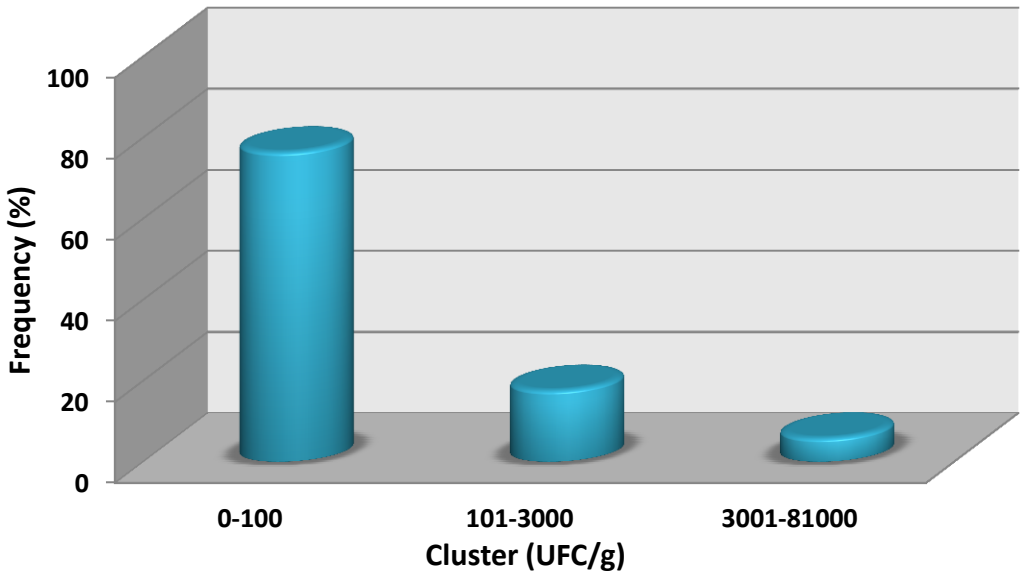



**Chart 27 Turkey matrix: *Escherichia coli* frequency distribution.**

The detection of *Escherichia coli* in turkey meat evidences a single-mode trend with values almost entirely amounting to law defined limits. Only a low percentage of samples count high microbial loads.

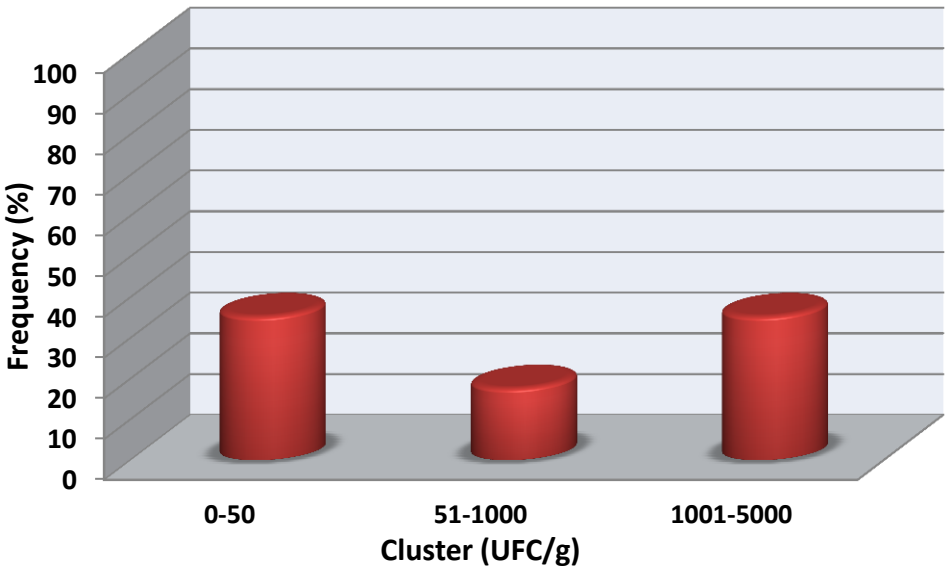



**Chart 28 Turkey matrix: *Aeromonas spp.* frequency distribution.**

The research for Aeromonas spp. is not required by laws but a lot of positive samples for the Genus have been notified. Values were mostly belonged to medium-high loads range. This trend clearly emerges as abnormal if compared to the expected decreasing frequency distribution.

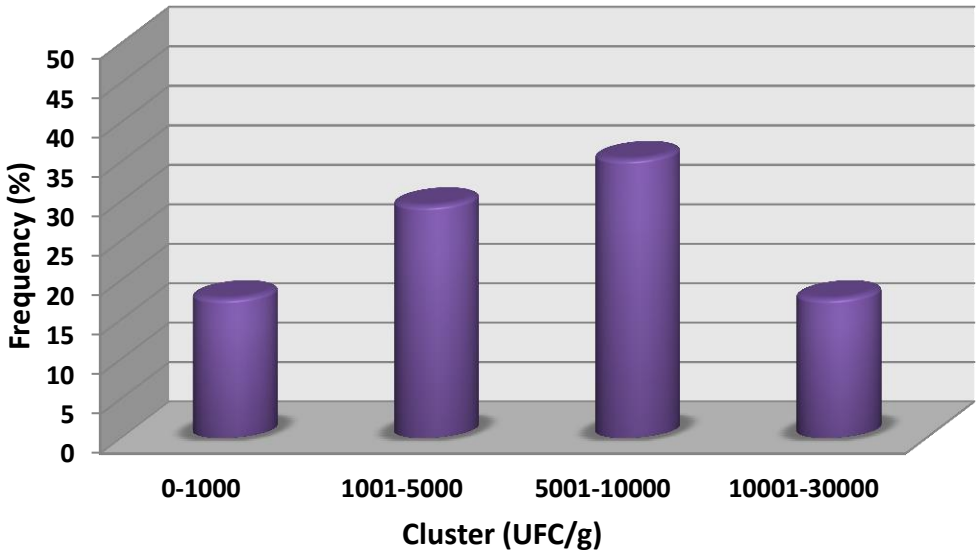



**Chart 29 Turkey matrix: *Enterococcus spp.* frequency distribution.**

*Enterococcus spp.* in turkey meat shows a single-mode trend with a peak in the cluster ranged from 5000 to 10000 CFU/g, higher microbial loads samples were less frequently detected. The trend reflects the abnormal frequency distribution already reported for *Aeromonas spp.*.

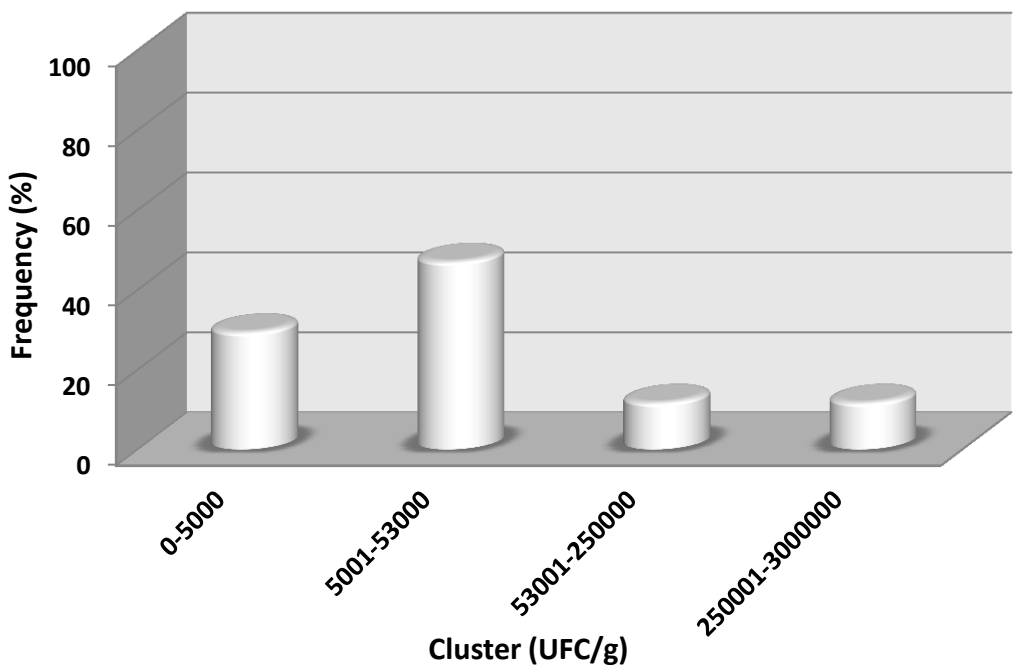



**Chart 30 Turkey matrix: *Staphylococcus spp.* frequency distribution.**

The trend of *Staphylococcus spp.* underlines a unic mode and the data are mostly gathered into lower microbial loads group.

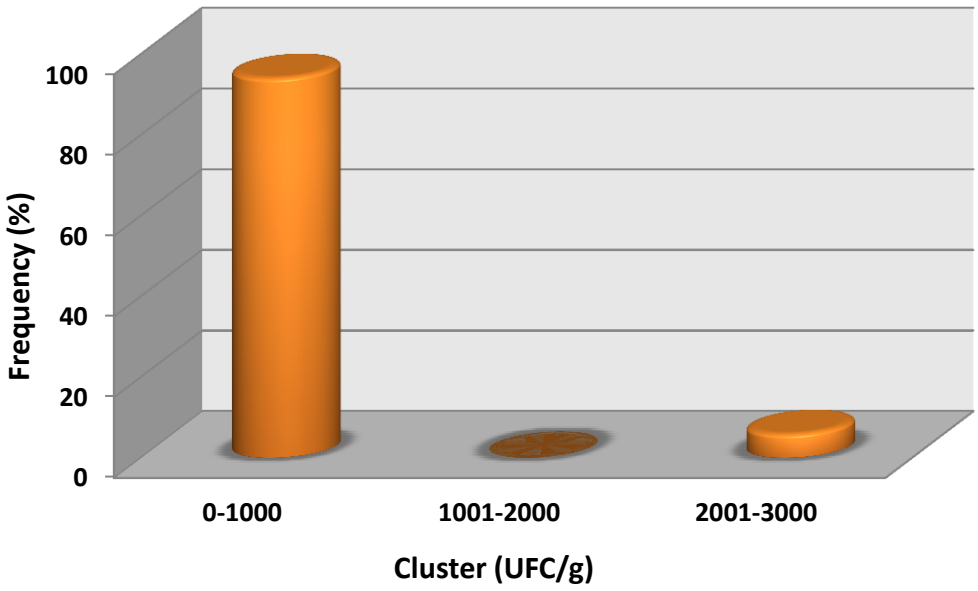



**Chart 31 Turkey matrix: *Staphylococcus aureus* frequency distribution.**

The presence of *Staphylococcus aureus* is restricted to a limited number of samples: many zero values belong to the first cluster and a slight peak presents very high values of microbial loads.

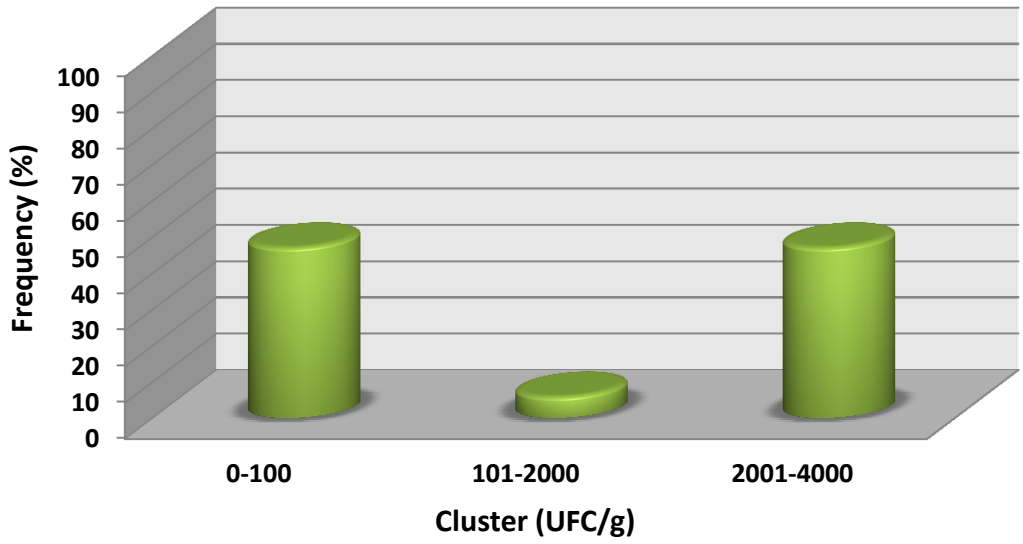



**Chart 32 Turkey matrix: *Listeria spp.* frequency distribution.**

The results for *Listeria spp.* see the data lining up in two groups: one with microbial load up to 100 CFU/g and the other with higher values.

The difficulty isolating these microorganisms consists on being able to work with dilutions sufficient to a practical interpretation of the data, because the samples show a high variability in these investigations. The resulting trend does not fit the expected decreasing frequency distribution.

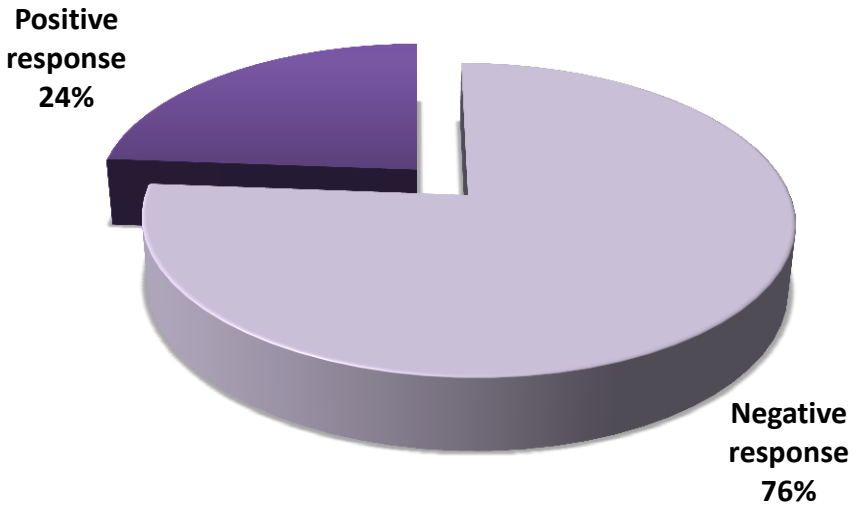



**Chart 33 Turkey matrix: *Salmonella spp.*.**

Almost one quarter of the population of turkey samples emerged positive for *Salmonella spp.*, being qualitative analysis only approachable with a pie chart.

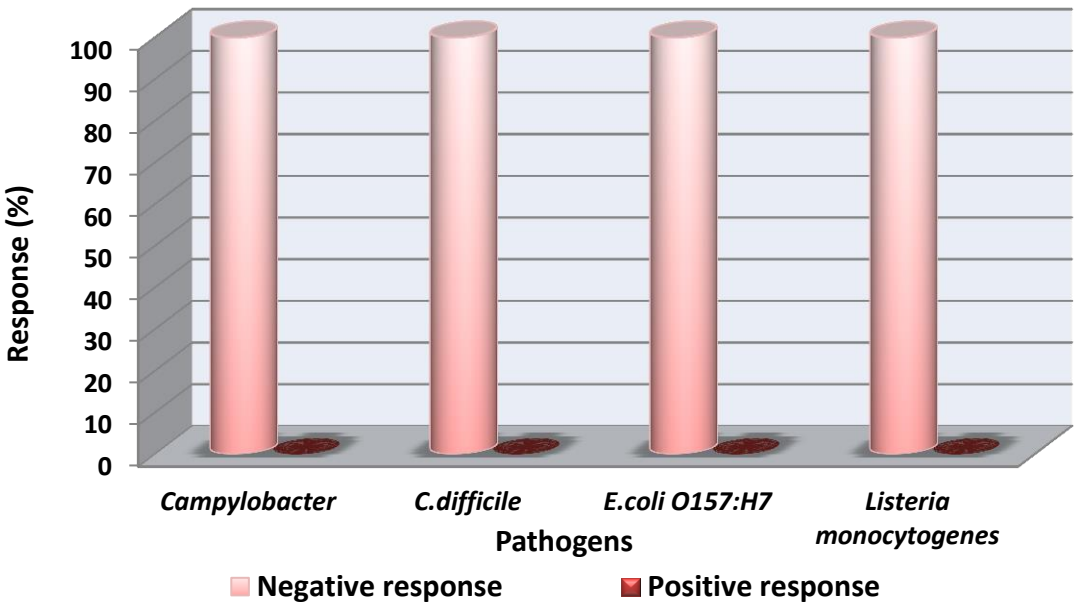



**Chart 34 Turkey matrix: *Campylobacter spp.*, *Clostridium difficile*, *E.coli* O157H:7, *Listeria monocytogenes*.**

Regarding turkey matrix, no emerging Pathogens were isolated.

## 6.2. Matrix: Beef

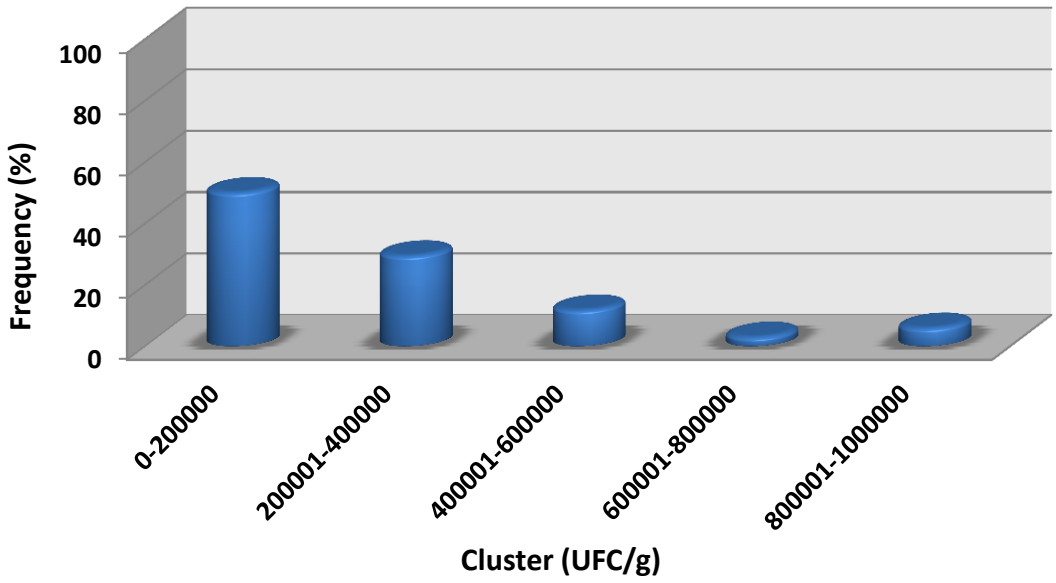



**Chart 35 Beef matrix: Mesophilic frequency distribution.**

The distribution of the data related to the mesophilic microorganisms in beef recalls an ideal trend, with low microbial loads for most of the samples and high microbial loads for a fewer onces. The values state within the limits of regulations.

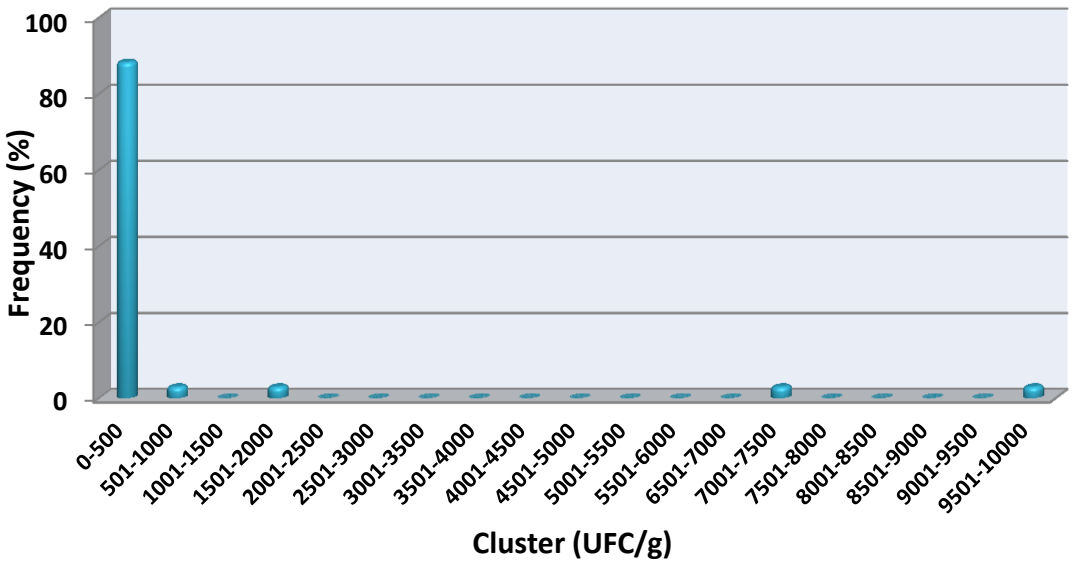



**Chart 36 Beef matrix: *Escherichia coli* frequency distribution.**

The analyzed beef samples have given almost all results within the legal limits for contamination indicator *Escherichia coli*.

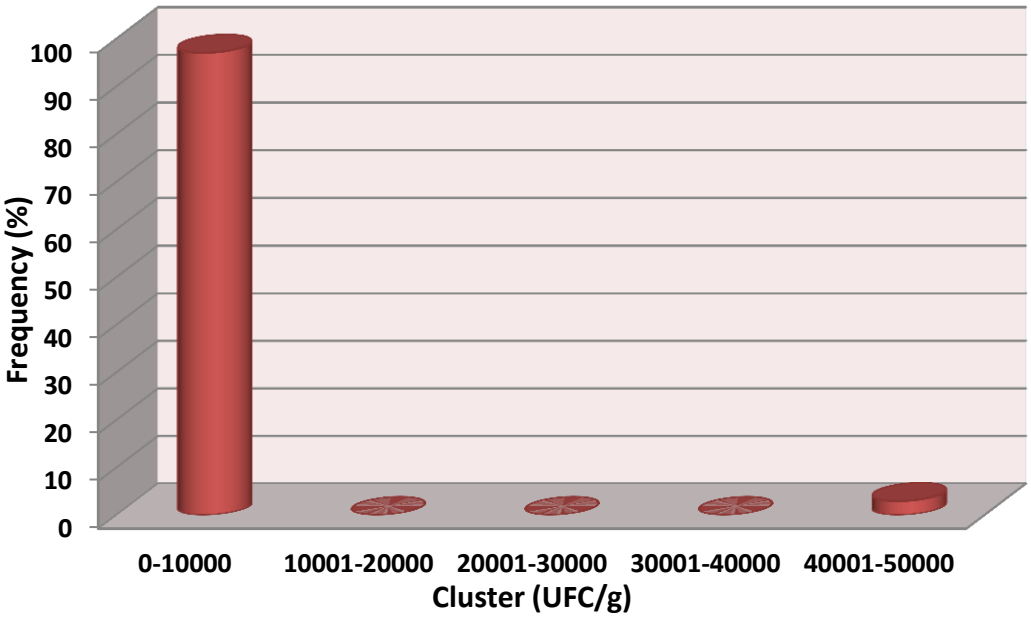



**Chart 37 Beef matrix: *Aeromonas spp.* frequency distribution.**

Analyzing the determination of *Aeromonas spp.* in beef samples, almost the totality of the data were encountered in the first cluster.

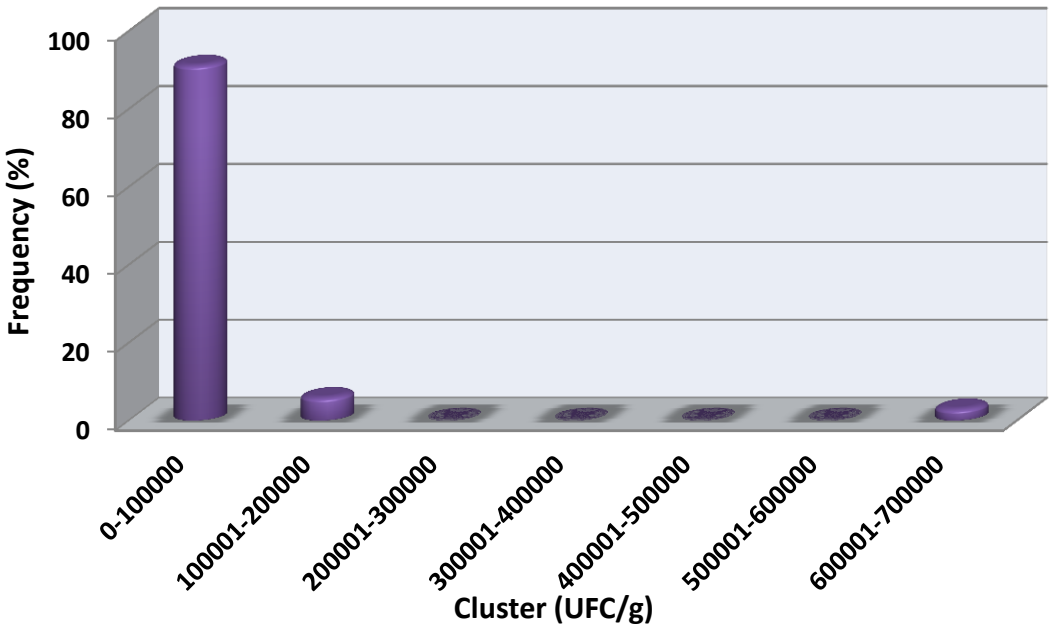



**Chart 38 Beef matrix: *Enterococcus spp.* frequency distribution.**

The microbial loads of *Enterococcus spp.* are distributed in a logical flow: in particular most of the samples for the indicator of fecal contamination showed low load levels.

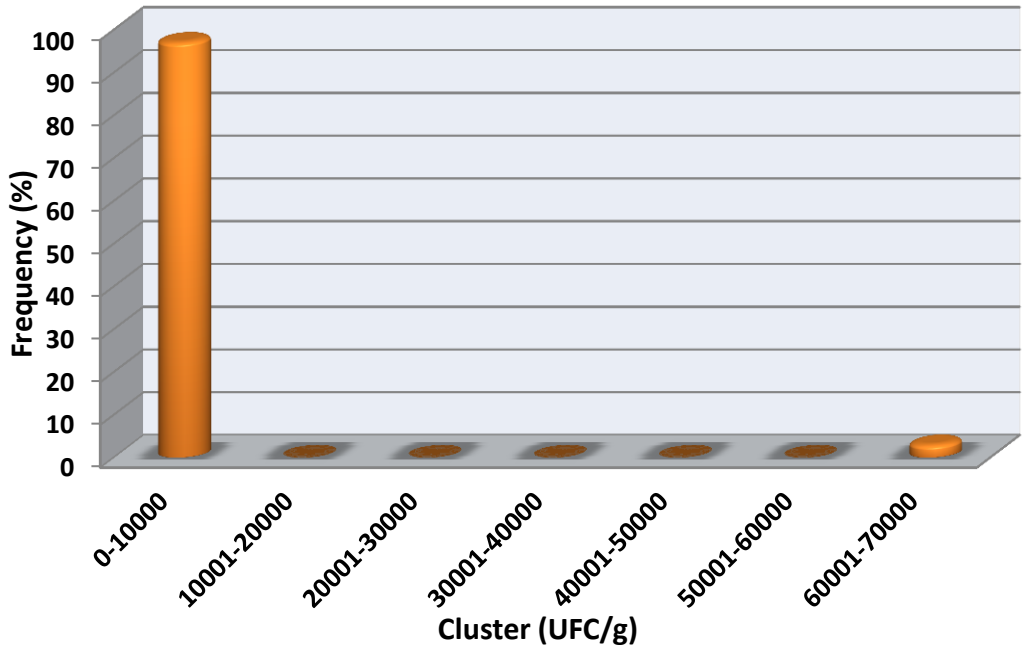



**Chart 39 Beef matrix: *Staphylococcus aureus* frequency distribution.**

Given the choice of clusters for the frequency distribution, it is necessary to emphasize that most of the data relating *S. aureus* weighed values equal to zero.

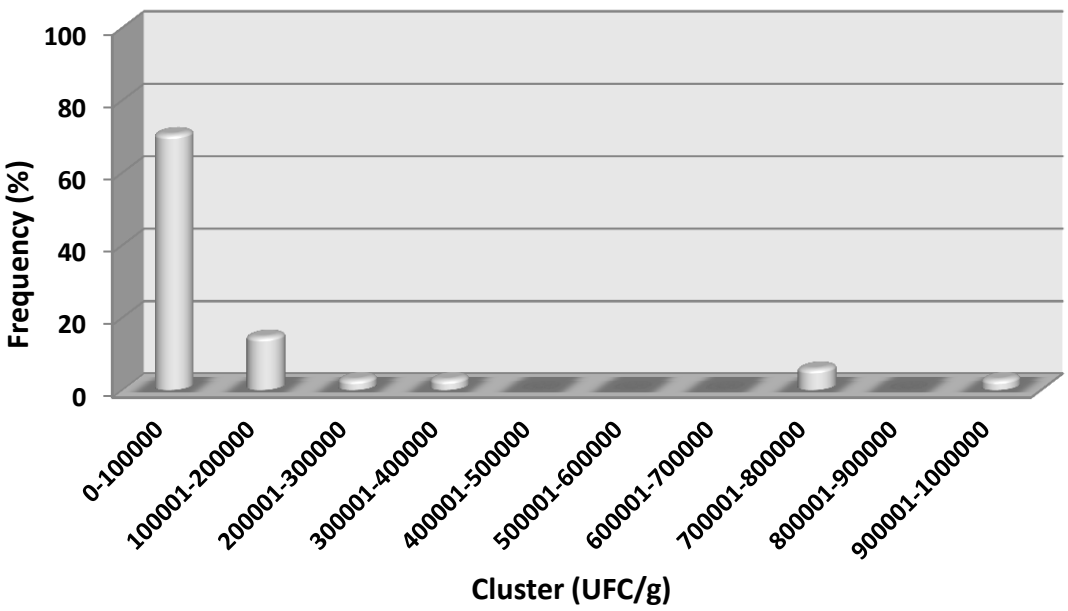



**Chart 40 Beef matrix: *Staphylococcus spp.* frequency distribution.**

*Staphylococcus spp.* distribution shows a single-mode trend, with a significant peak situated in the first cluster, gradually decreasing towards higher microbial loads.

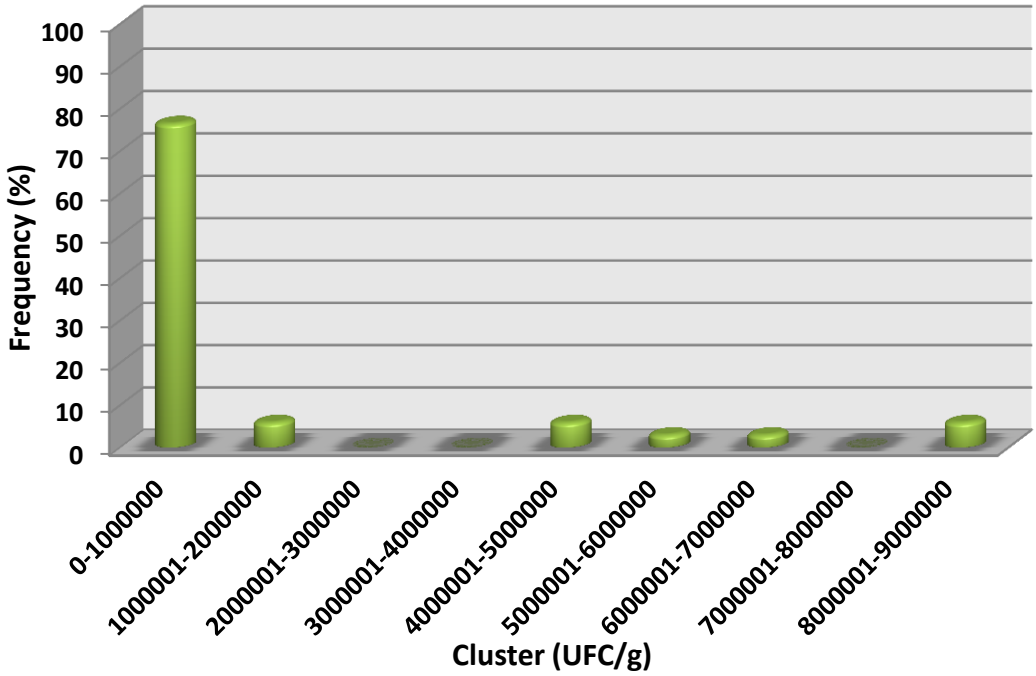



**Chart 41 Beef matrix: *Listeria spp.* frequency distribution.**

The trend in determination of *Listeria spp.* is typical of a normal pattern; the high variability of responses related to the various samples did not allow to use a universal

protocol considering the number of dilutions to realize. For this reason, the identification of potential *L. monocytogenes* colonies was not easy.

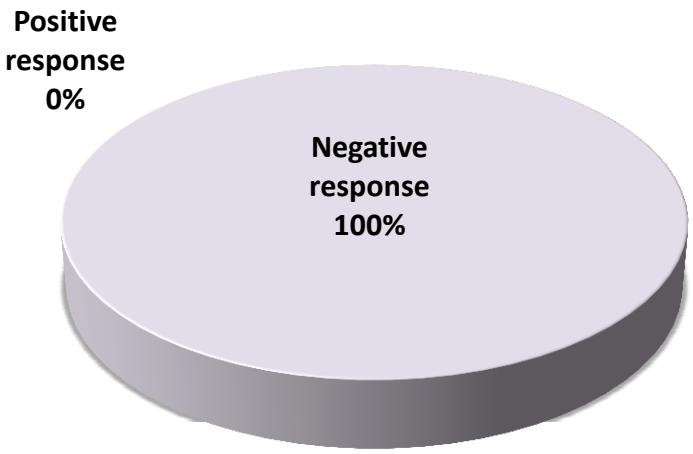



**Chart 42 Beef matrix: *Salmonella spp.*.**

The results for the detection of *Salmonella spp.* in beef were negative.

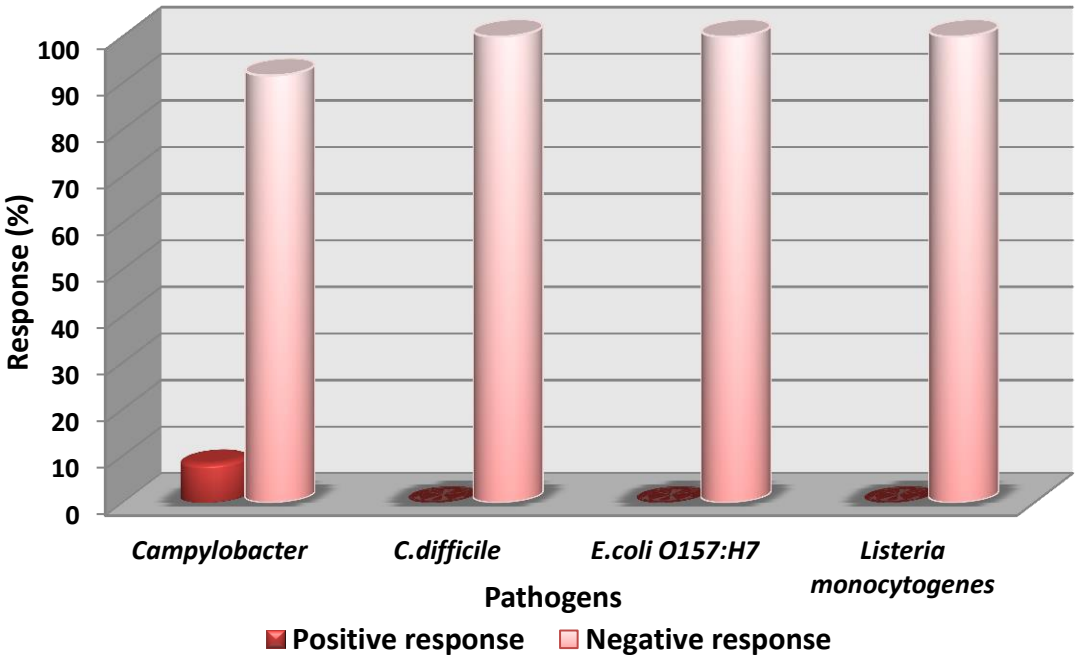



**Chart 43 Beef matrix: *Campylobacter spp.*, *Clostridium difficile*, *E.coli* O157H:7, *Listeria monocytogenes*.**

The research for Pathogenic microorganisms in beef only set off the isolation of *Campylobacter spp.* germ.

## 6.3. Vegetables

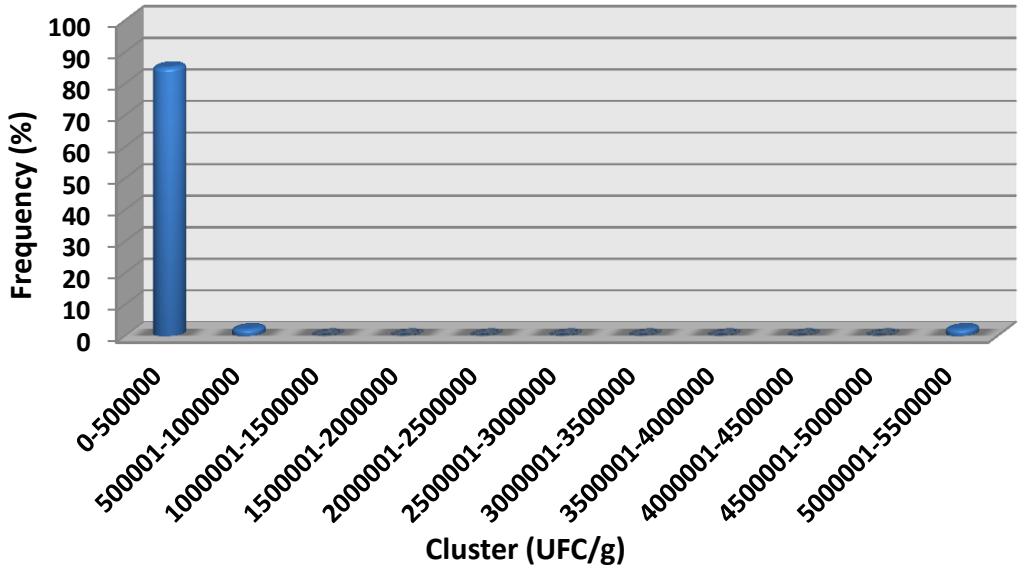



**Chart 44 Vegetables matrix: Mesophilic frequency distribution.**

Vegetable matrix (which includes "I gamma" and "IV gamma" products) data distribution for Mesophilic microorganisms states a concentration of values in the first cluster with low levels of contamination. A small peak for high microbial loads is evident from the observation of "I gamma" products.

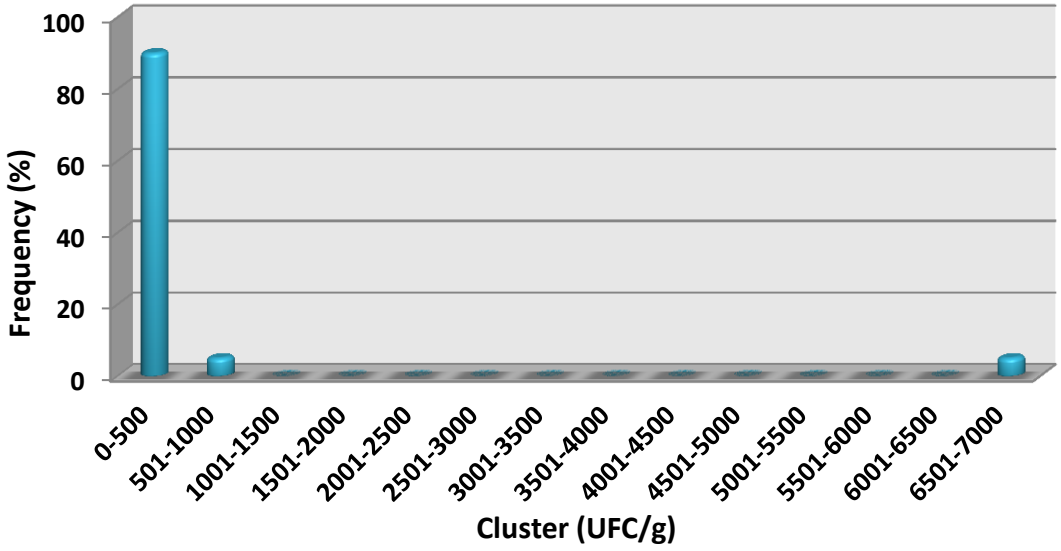



**Chart 45 Vegetables matrix: *Escherichia coli* frequency distribution.**

The microbial loads for the analyte *Escheria coli* are collocated within the second cluster, 1000 CFU/g, which represents the limit imposed by Regulation CE N° 2073 of 2005; it is clear, in addition, a high microbial loads lower peak.

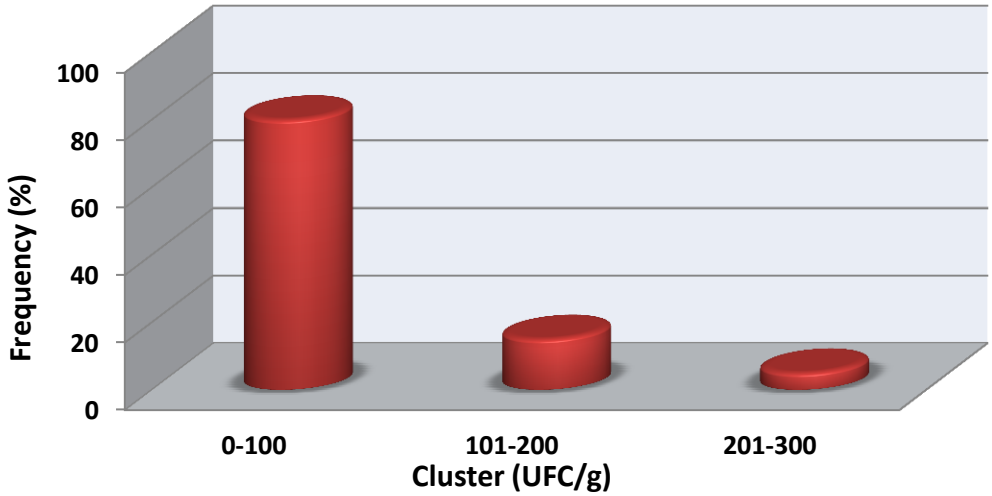



**Chart 46 Vegetables matrix: *Aeromonas spp.* frequency distribution.**

The trend concerning *Aeromonas spp.* microbial loads results as logical and, according to a normal distribution, percentages decrease as much as microbial loads increase.

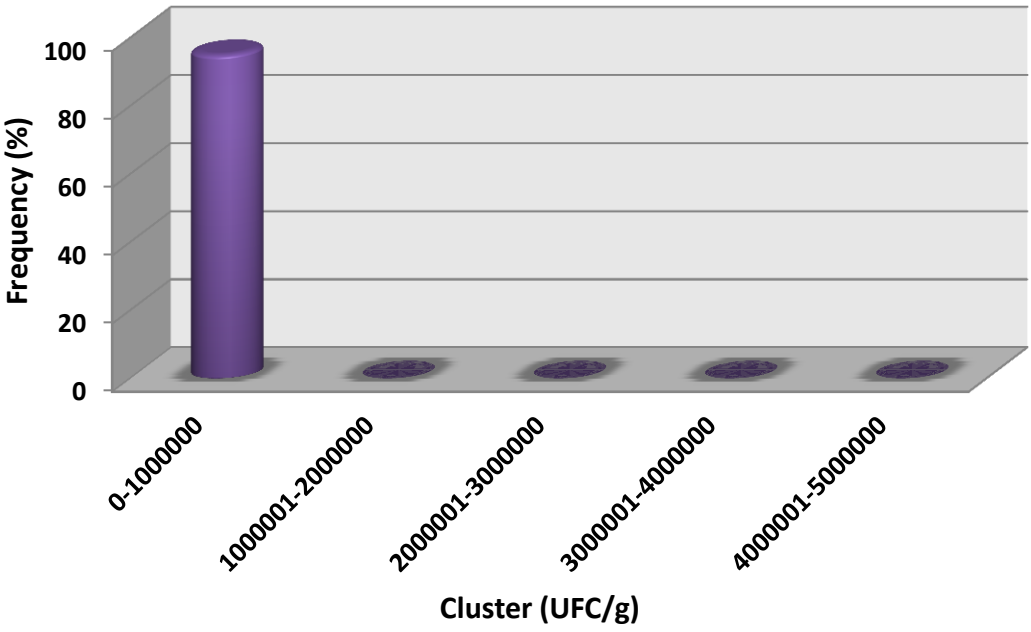



**Chart 47 Vegetables matrix: *Enterococcus spp.* frequency distribution.**

A behavior similar to Pathogens previously described is available for the indicator of contamination *Enterococcus spp.*.

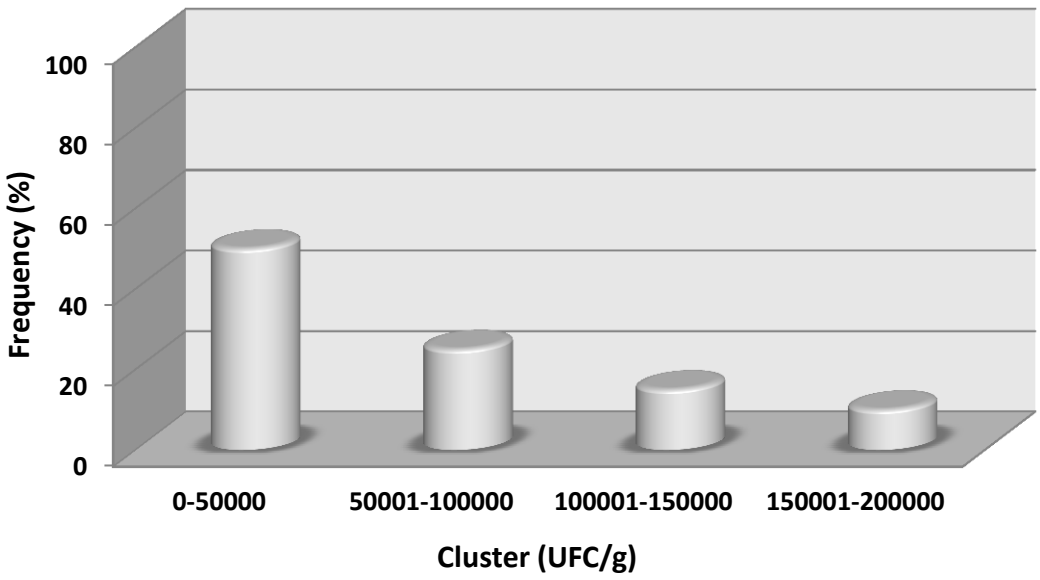



**Chart 48 Vegetables matrix: *Staphylococcus spp.*frequency distribution.**

The trend of the analyte *Staphylococcus spp.* suggests a gradual decrease compared to increasing microbial loads.

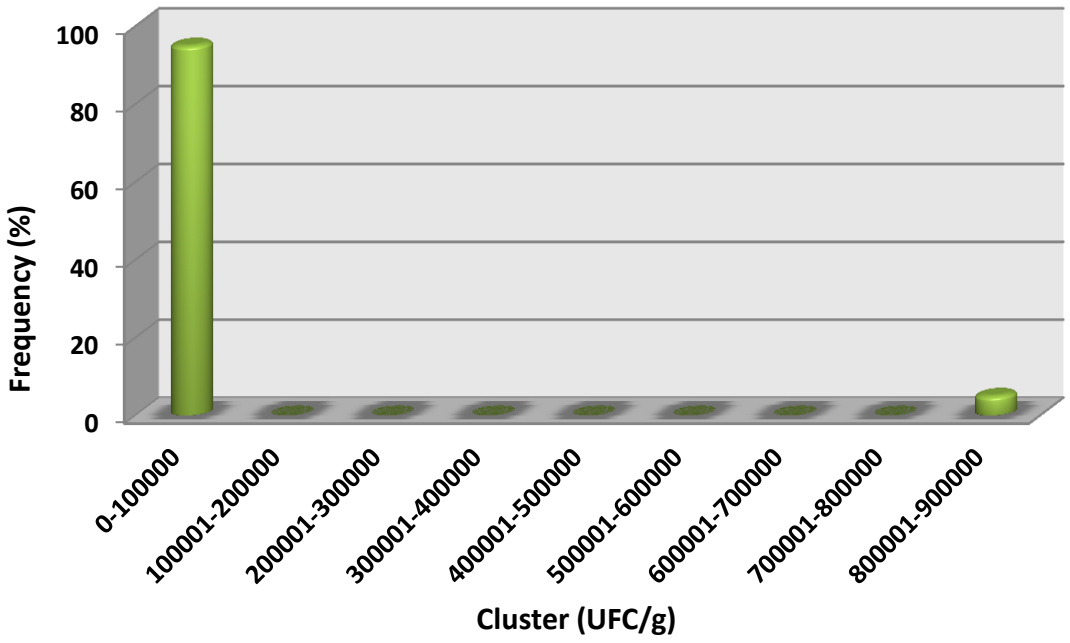



**Chart 49 Vegetables matrix: *Listeria spp.* frequency distribution.**

*Listeria spp.* trend evidences a high frequency distribution within the first cluster (low microbial loads) and a spike, referred to high loads, with a low rate of response.

Samples of vegetables never gave positive results for *Salmonella spp.*.

Alarming instead is the data regarding Pathogens on vegetables samples; the presence of *Clostridium difficile*, highly virulent bacterium responsible for pseudomembranous Colitis, was detected.

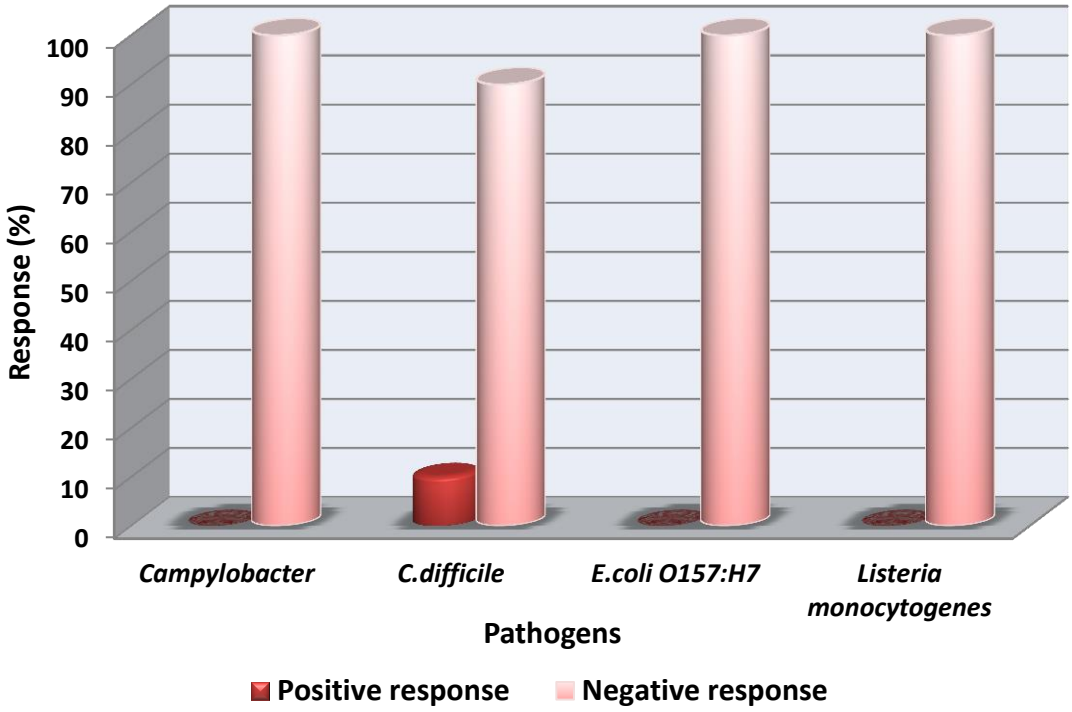


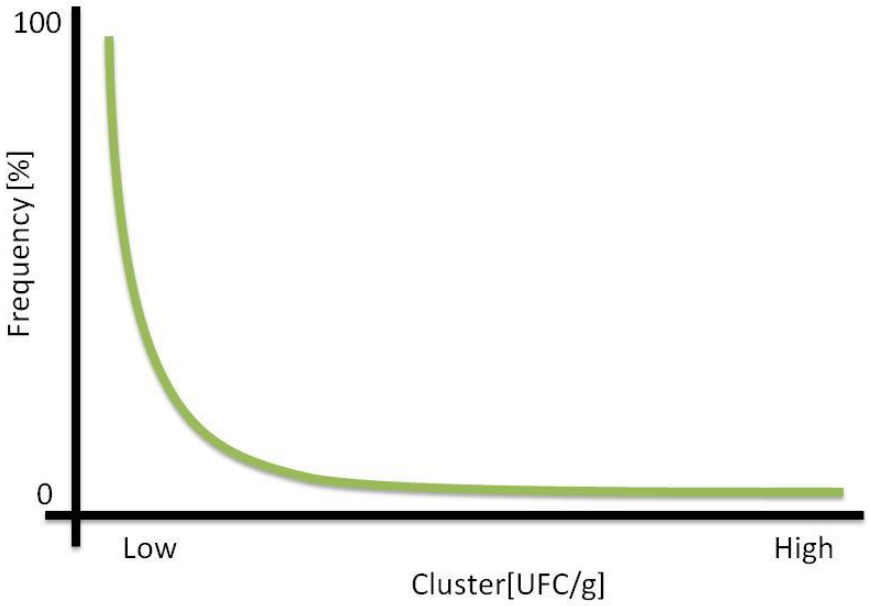

**Chart 50 Vegetables matrix: *Campylobacter spp.*, *Clostridium difficile*, *E.coli* O157H:7, *Listeria monocytogenes*.**

Most of the information obtained on microbial loads suggests compliance with the limits imposed by current legislation regarding food.

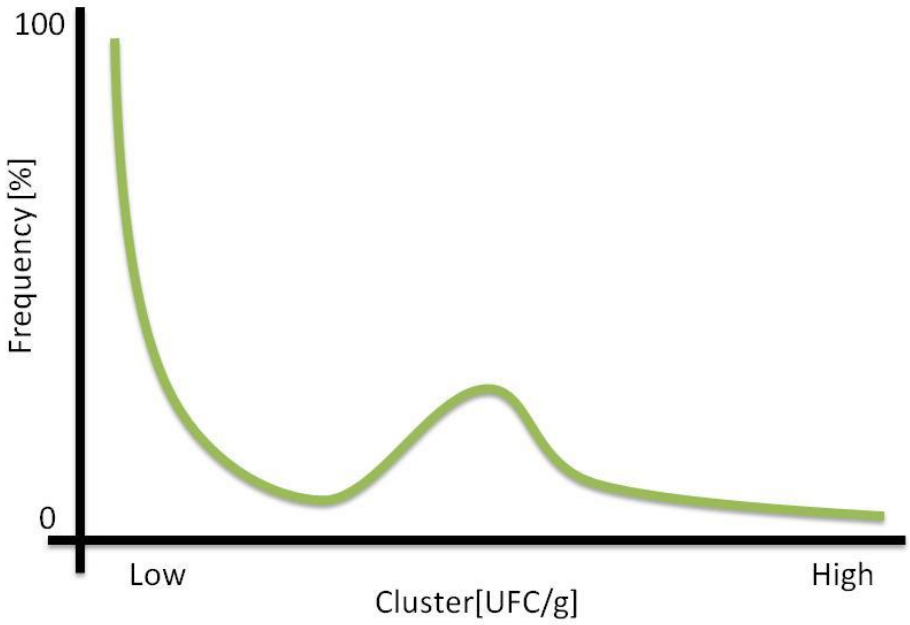
Available quantitative data were organized in frequency distributions. Through the observation of the results, the majority of samples were ranked in low microbial loads range and it was less likely to register greater microbial loads.

An ideal trend of frequency distribution sees a rapid decline towards high microbial loads.

An ideal curve should look like the one following shown:




Abnormal distribution with multimode trend as shown below is an indication of an anomaly that can be due to various factors.




It can be hypothesized that the possible causes are:

- batches of matrix sampled at different levels of contamination

- incurance or sporadic and systematic errors in the individual matrices production process.

However, these abnormalities may represent evidence of a potential hazard and may therefore allow the programming of a more thorough investigation on the production chains.

In this work, different results have been evidenced: some similar to the ideal performances and some others far from ideal.

Trends approaching the ideals are provided below*:*

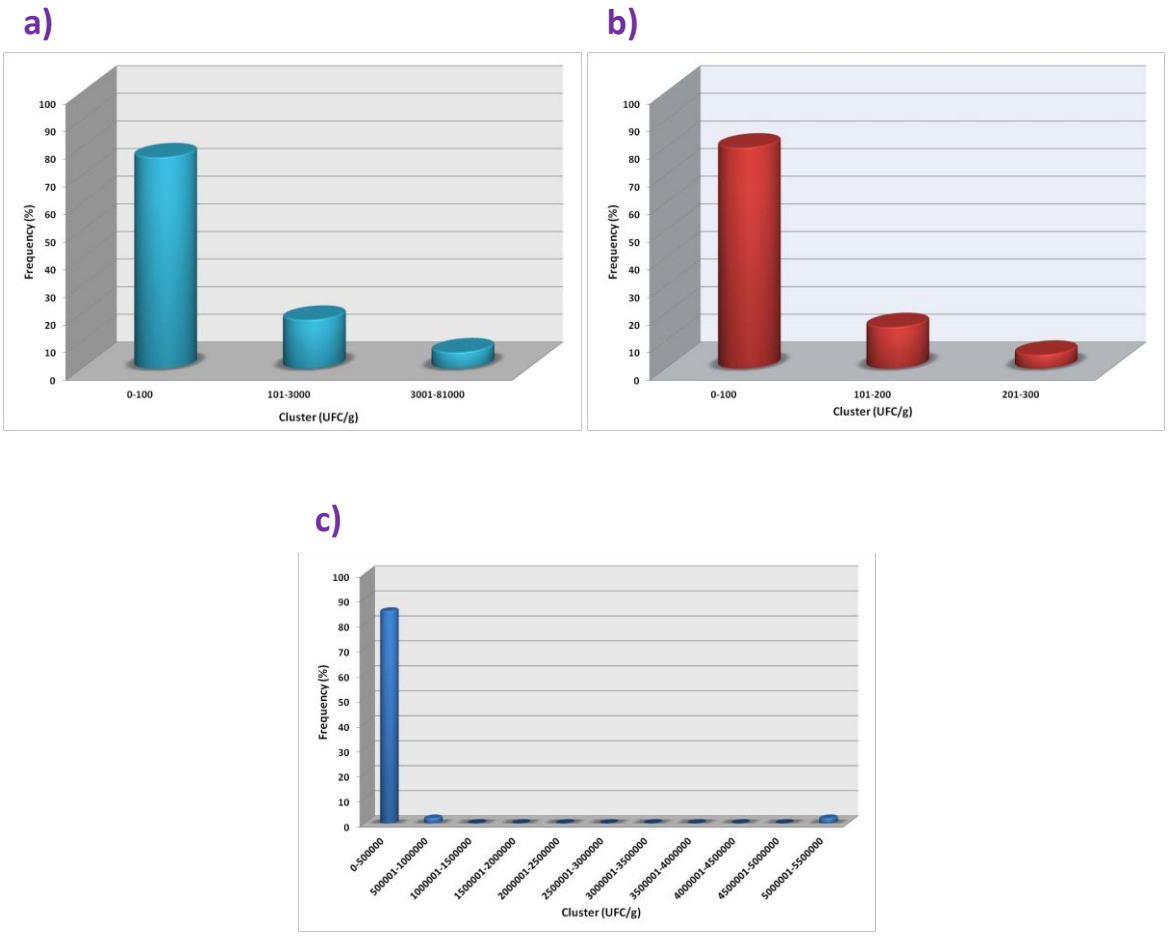
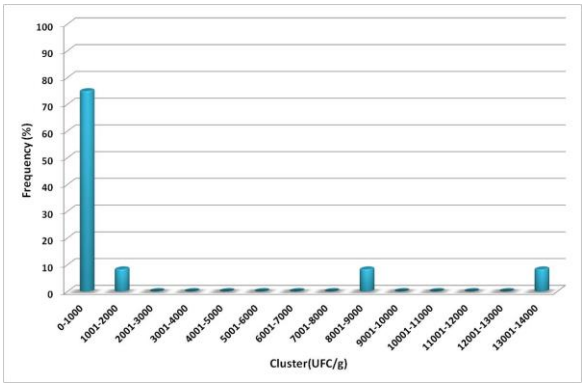


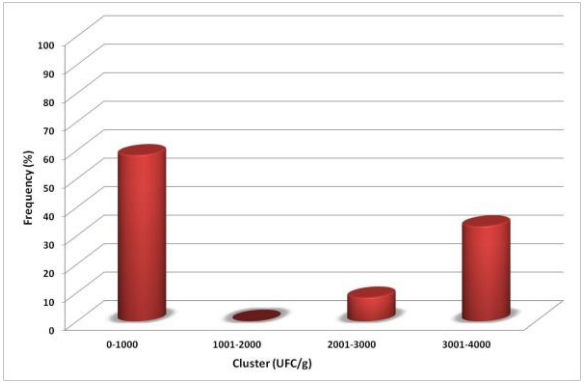

**Image 26 Single mode trands: a) *E.coli* in turkey; b) *Aeromonas spp.* in vegetables; c) TBC in vegetables.**

Following, examples of trends far from ideal:

a)




b)




c)

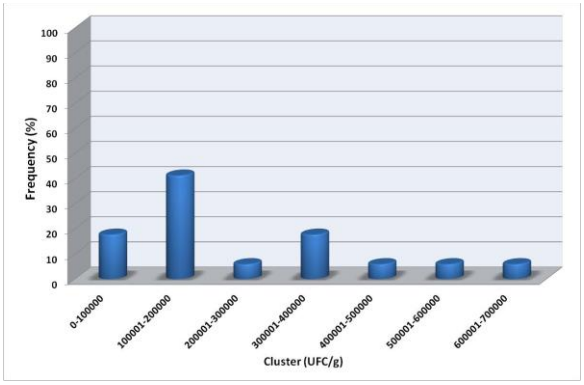



**Image 27 Multimodal trends: a) *E.coli* in chicken; b) *Aeromonas spp.* in chicken; c) TBC in turkey.**

Systematically checking all calculated distributions, both data close to the ideal trends and those showing evident abnormalities were compared.

The overview is described in the following figure:

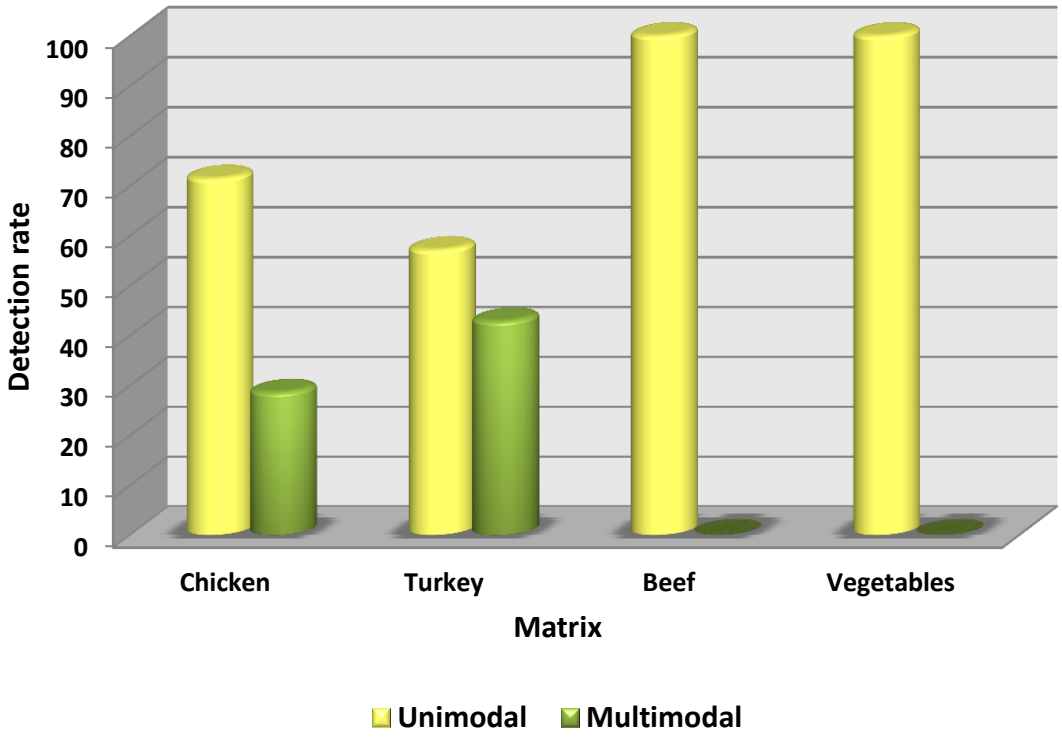



**Chart 51 Frequencies trends for all matrices.**

It is quite clear that beef and vegetables matrices did not indicate any abnormality. Instead, poultry meats – and, in particular, turkey meat - putting the investigated foods in order of potential risk, are those presenting higher risk than other matrices.

This approach allows a more detailed study on the supply chain of food production to better know the preventive strategies that will treat the stages of production of poultry products, improving the quality of the finished product.

## 6.4. Studies on isolated microbial species

The monitoring may have a greater value when the virulence factors of isolated bacterial species can be investigated: these contribute to the characterization of the risk of food contamination. In our study several microbial species were isolated and the most significant results are following described.

### 6.4.1. *Salmonella spp.*

All results of laboratory tests performed on isolates of *Salmella spp.* are tabulated below:

| Code | matrix | Serotype |
|---|---|---|
| **1S** | turkey | *Salmonella Agona* |
| **2S** | chicken | *Salmonella enterica enterica* with one ciliary phase |
| **3S** | chicken | *Salmonella Infantis* |
| **4S** | chicken | *Salmonella Thompson* |
| **5S** | chicken | *Salmonella Infantis* |
| **6S** | chicken | *Salmonella Give* |
| **7S** | chicken | *Salmonella Paratyphi B* |
| **8S** | chicken | *Salmonella Give* |
| **9S** | turkey | *Salmonella Typhimurium* |
| **10S** | turkey | *Salmonella Newport* |
| **11S** | chicken | *Salmonella Infantis* |
| **12S** | chicken | *Salmonella Infantis* |
| **13S** | pork | *Salmonella Edinburgh* |
| **14S** | chicken | *Salmonella Bovismorbificans* |
| **15S** | chicken | *Salmonella Newport* |
| **16S** | turkey | *Samonella Paratyphy B* |
| **17S** | turkey | *Salmonella Paratyphy B* |
| **18S** | chicken | not detected |
| **19S** | pork | not detected |
| **20S** | turkey | not detected |

**Table 30 *Salmonella spp.* detected serotypes from food matrices.**

| | AMCL | AMP | ANA | CAT | CAZ | CIP | CTX | ENR | GN | KF | KAN | STR | SUL | SXT | TET | CT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1S** | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| **2S** | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| **3S** | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| **4S** | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| **5S** | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| **6S** | S | S | S | S | S | S | S | S | S | S | S | S | R | S | S | S |
| **7S** | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **8S** | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| **9S** | S | R | S | S | S | S | S | S | S | S | S | R | R | S | R | S |
| **10S** | S | R | S | S | S | S | S | S | S | S | S | S | R | R | R | S |
| **11S** | S | S | R | S | S | S | S | S | S | S | R | S I | R | R | R | S |
| **12S** | S | S | R | S | S | S | S | S | S | S | R | R I | R | R | R | S |
| **13S** | S | S | S | R | S | S | S | S | S | S | S | R | S | S | R | S |
| **14S** | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| **15S** | S | R | R | S | S | S | S | R | S | S | S | S | R | R | R | S |
| **16S** | S | R | S | S | S | S | S | S | S | S I | S | S I | S | R | R | S |
| **17S** | S | R | S I | S | S | S | S | S | S | R | S | R | S | S | R | S |
| **18S** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **19S** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **20S** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

**Table 31 Antibiogram for isolates of *Salmonella spp.*.**

Grouping the data on the isolation of strains of *Salmonella spp.* considering investigated matrix, it is evident that the highest detection of *Salmonella spp.* has occurred in poultry meat and, with a fewer rate, in turkey. Relatively limited is instead the results concerning pork and beef.

This observation already allows to associate the transmission risk of *Salmonella spp.* to food matrix poultry and confirms that EFSA aim of eradicating *Salmonella* problem in poultry chicken, in particular, is far from solved.

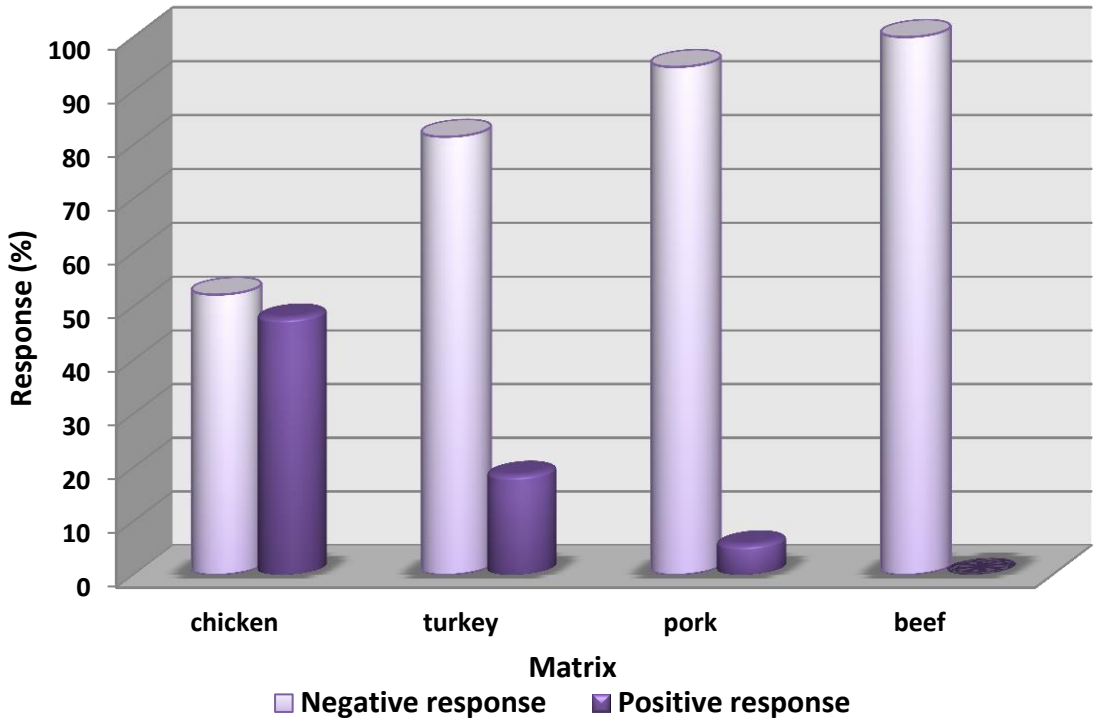



**Chart 52 Positivity percentage of *Salmonella spp.* in the matrices investigated.**

Data show that different serotypes of *Salmonella* have a high variability in the attitude to generate biofilm: identical strains present different attitudes in the formation of biofilms. This feature constitutes a virulence factor since a great power of adhesion can promote tissue invasion and pathogenic action as well as a greater permanence in the food.

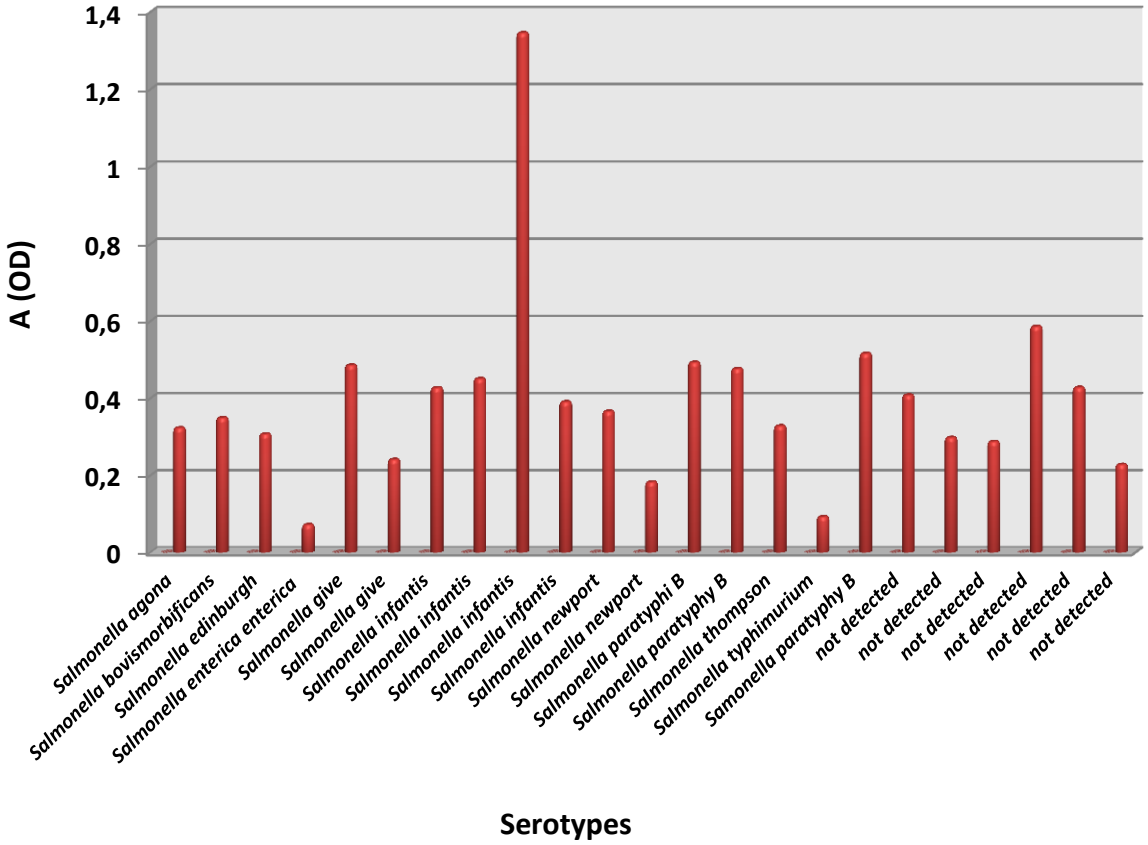



**Chart 53 Biofilm results for *Salmonella spp.* serotypes.**

The ability to produce biofilm was remarkable for a strain of *S. Infantis,* while the other strains of the same serotype possess a more limited ability to generate biofilm. The other species isolated, *S. Enterica* sub. *Enterica*, *S.Give, S.Newport* and *S. Tiphymurium* exhibited a restricted ability of generating biofilm. Latter species' attitude were intermediate.

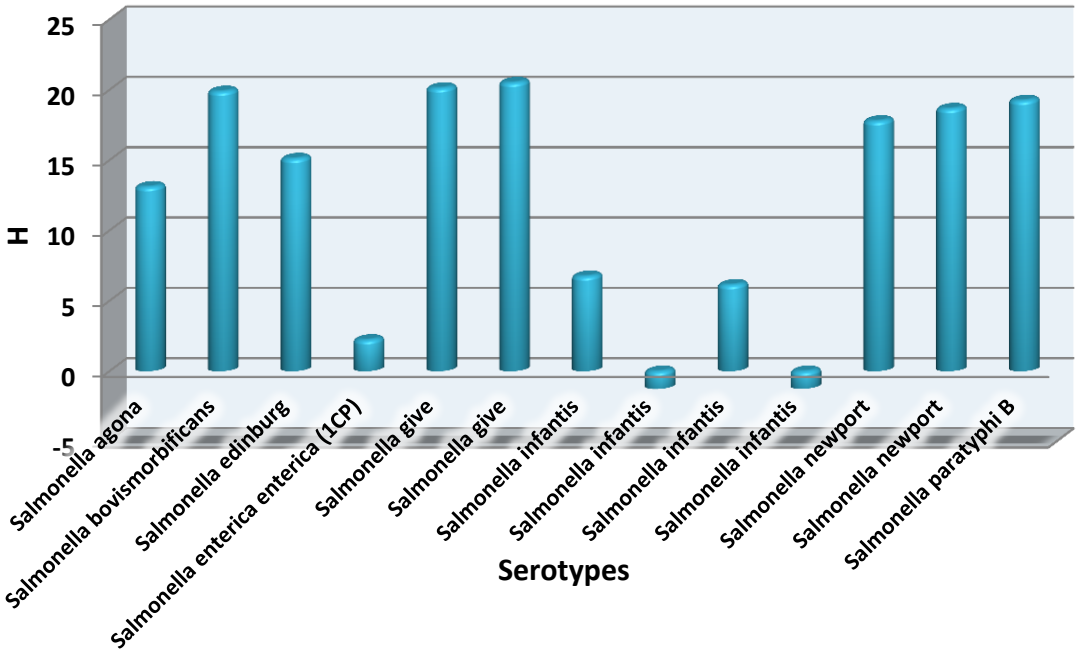



**Chart 54 Hydrophobicity levels for *Salmonella spp.* serotypes.**

Hydrophobicity is a well known virulence factor, which reveals the possibility of joining the biological membranes. From investigated strains, only *S. Enterica sub. enterica* and two strains of *S.Infantis* reported low or zero hydrophobicity levels. *S. give, S. bovismorbificans S. paratiphy* and *S. newport* hydrophobicity evidenced, instead a more significant degree.

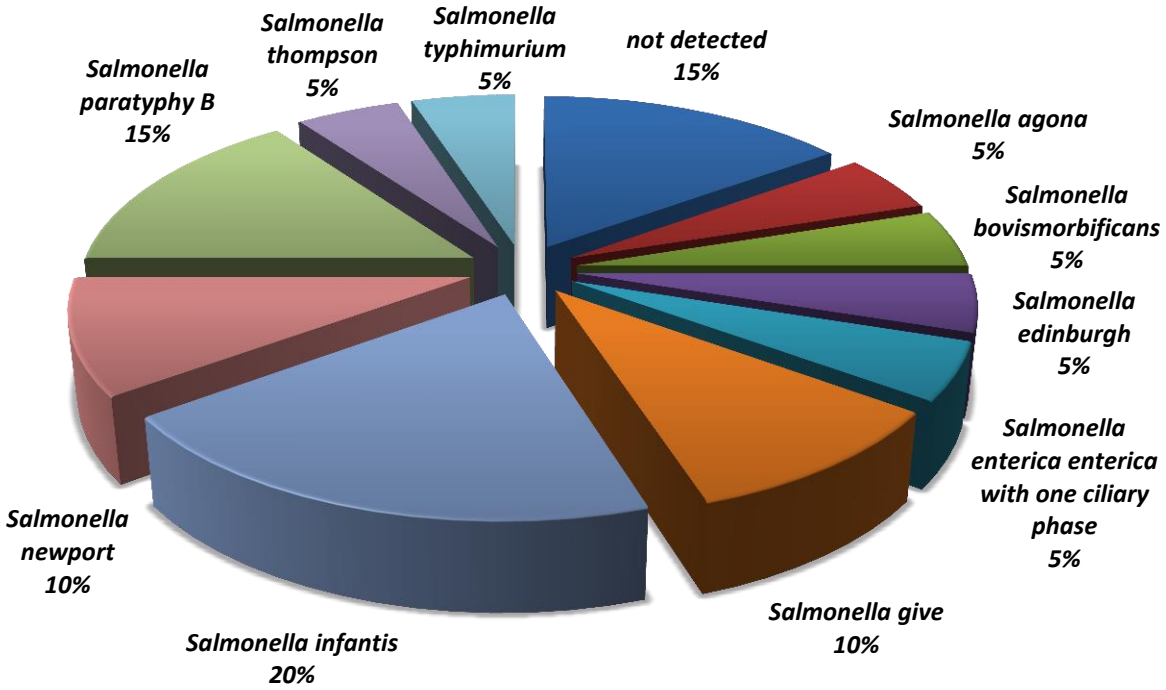



**Chart 55 Response rate of *Salmonella spp.* serotypes.**

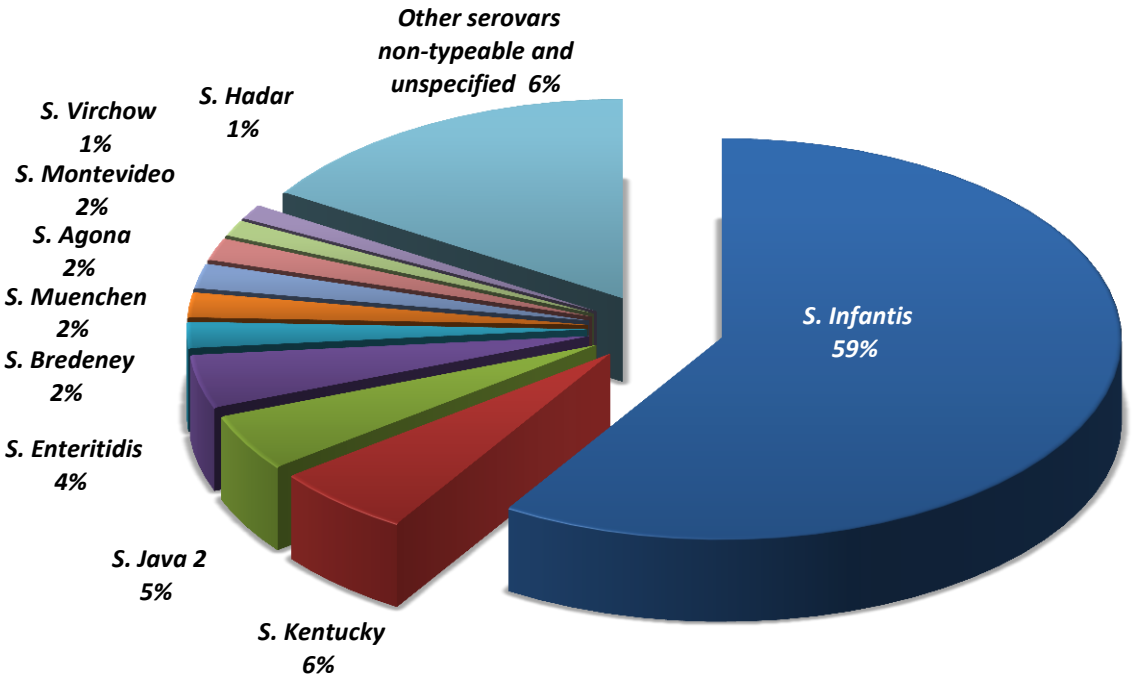



**Chart 56 Distribution of the 10 most common *Salmonella* serovars in broiler meat, 2010 The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 20101 European Food Safety Authority.**

As indicated above, our data differ from the EFSA's. Even though *Salmonella Infantis* represents the dominant specie, the response is limited from that declared by EFSA. Some species typed in our study are not even counted in the European report. It's important, in the future, to create a mapping of the isolates based on the origin of meat and production chains to better understand the distribution areas of each Salmonella *spp.* serotype.

It is recalled that international programs in veterinary medicine were intended to eradicate *Salmonella spp.* from farms and poultry supply chains. Our results show that this goal has not been reached.

### 6.4.2. *Staphylococcus aureus*

| | | MRSA |
|---|---|---|
| **1A** | turkey | - |
| **2A** | beef | - |
| **3A** | beef | + |
| **4A** | vegetables | + |
| **6A** | beef | - |
| **7A** | other | - |
| **8A** | chicken | - |
| **9A** | chicken | + |
| **10A** | other | - |
| **11A** | chicken | + |

| | | MRSA |
|---|---|---|
| **12A** | beef | + |
| **13A** | beef | + |
| **14A** | other | + |
| **15A** | turkey | - |
| **16A** | vegetables | - |
| **17A** | other | + |
| **18A** | chicken | - |
| **20A** | turkey | + |
| **21A** | other | - |

**Table 32 *Staphylococcus aureus* isolated and MRSA strains.**

Also for the isolation of *Staphylococcus aureus* in chicken it was experienced the highest frequency of positive response, that confirms the potential major risk for food derived from poultry meat than other products.

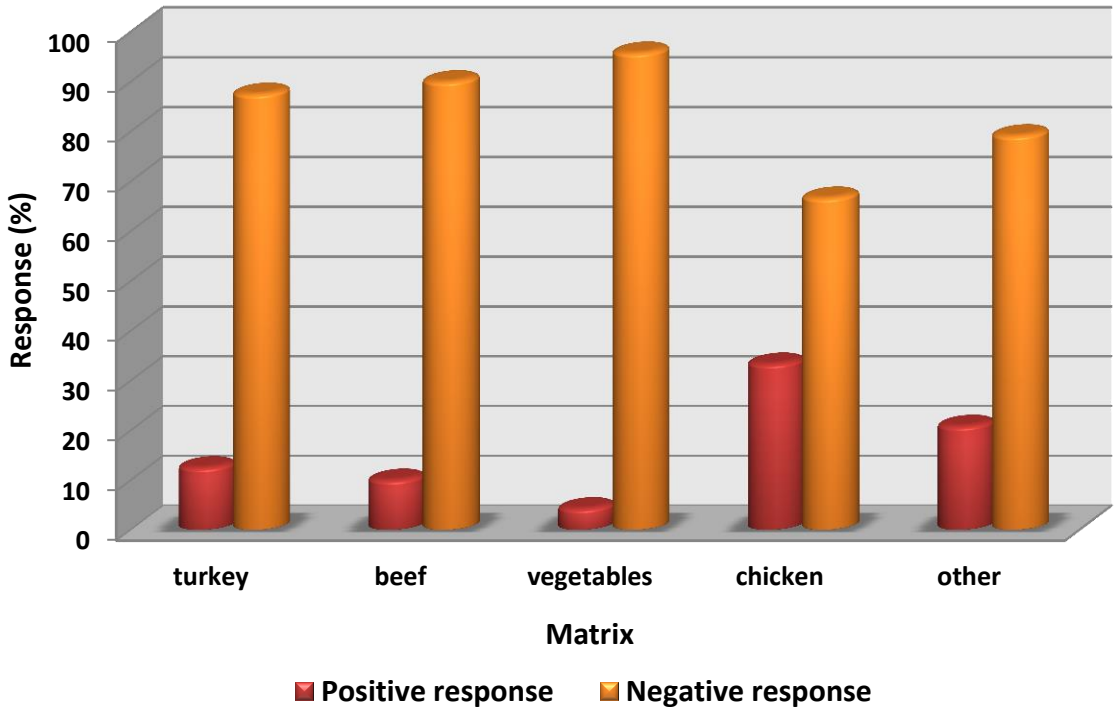



**Chart 57 Positivity percentage of *Staphylococcus aureus* in the matrices investigated.**

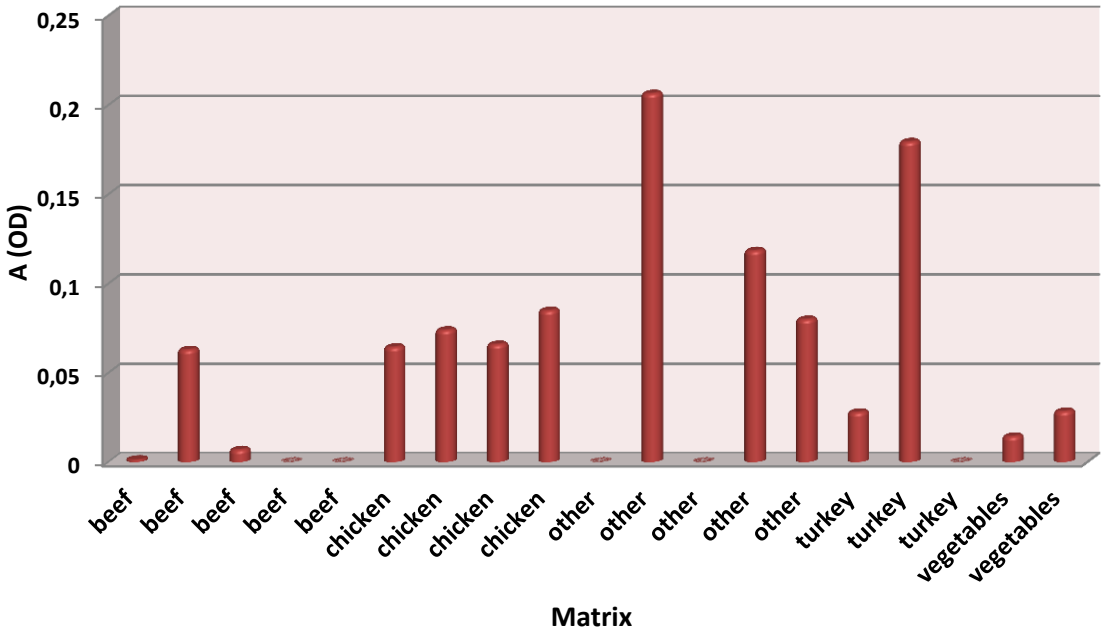



Chart 58 Biofilm values for *S.aureus* strains.

*S. aureus* ability of forming biofilms in different matrices has been proven somehow variable. The isolates from chicken meat and other matrices evidenced high ability to form biofilms.

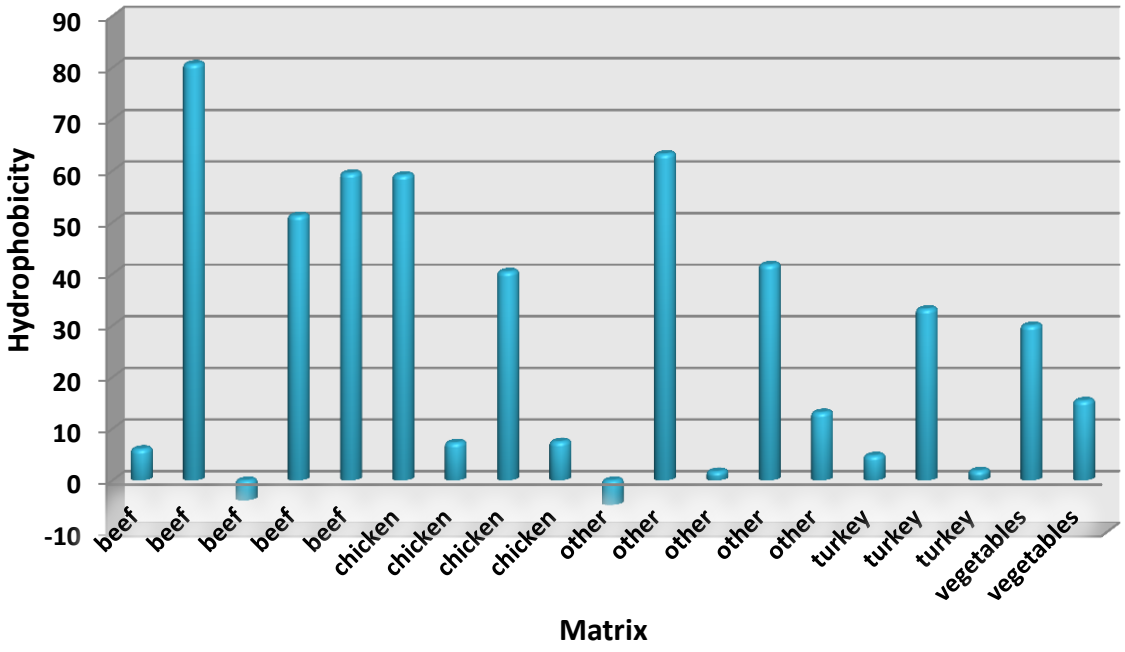



Chart 59 Hydrophobicity values for *S.aureus* strains.

Also the obtained hydrophobicity results were very different and heterogeneous. Different hydrophobicity levels can not be related to different matrices.

### 6.4.3. *Clostridium perfringens*

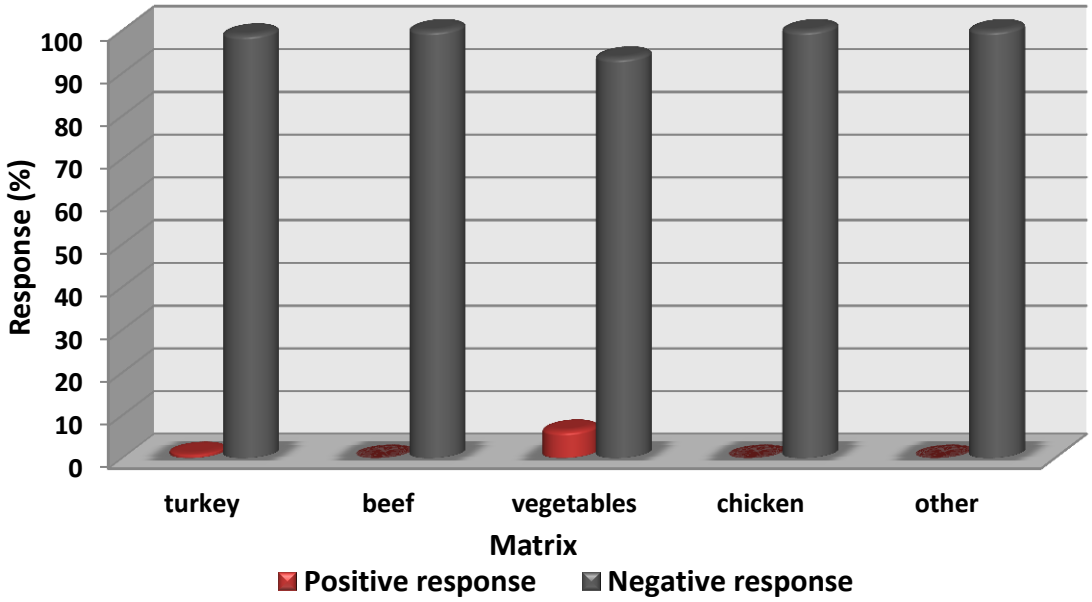



**Chart 60 Positivity percentage of *Clostridium perfringens* in the matrices investigated.**

Risk associated with the isolation of *Clostridium perfringens,* as expected, is more probable in vegetables and negligible or zero in other food matrices.

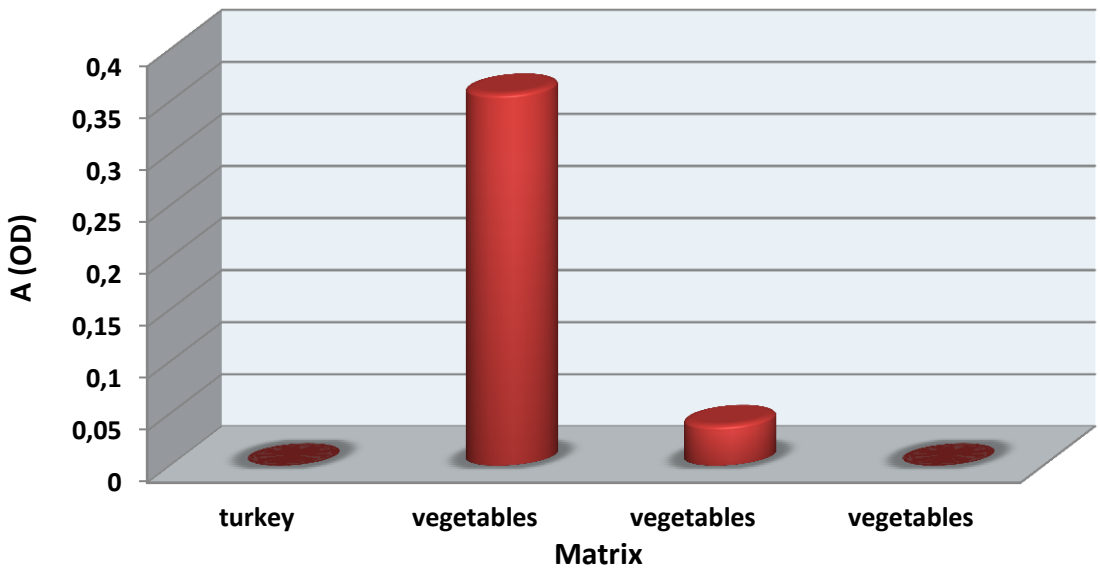



**Chart 61 Biofilm values for *C.perfringens* strains.**

*C. perfringens* isolates have however brought out a considerable variability in the attitude to form biofilms: even in the same matrix, in fact, a strain with high attitude and a strain with a limited ability to generate biofim have been isolated.

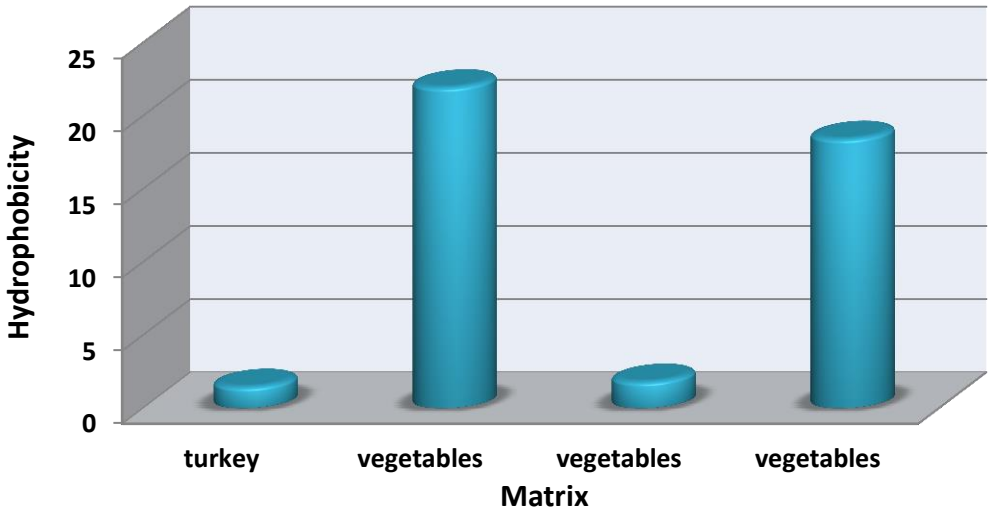



Chart 62 Hydrophobicity levels for *C. perfringens* strains.

Analyzing the test responses, hydrophobicity levels on the same matrix have underlined a considerable variability. Results concerning turkey isolates reported a low hydrophobicity.

### 6.4.4. *Campylobacter spp.*

*Campylobacter spp.* was only isolated from a few samples of chicken and beef. This confirms the potential risk of the chicken meat and the most commonly encountered species in this matrix.

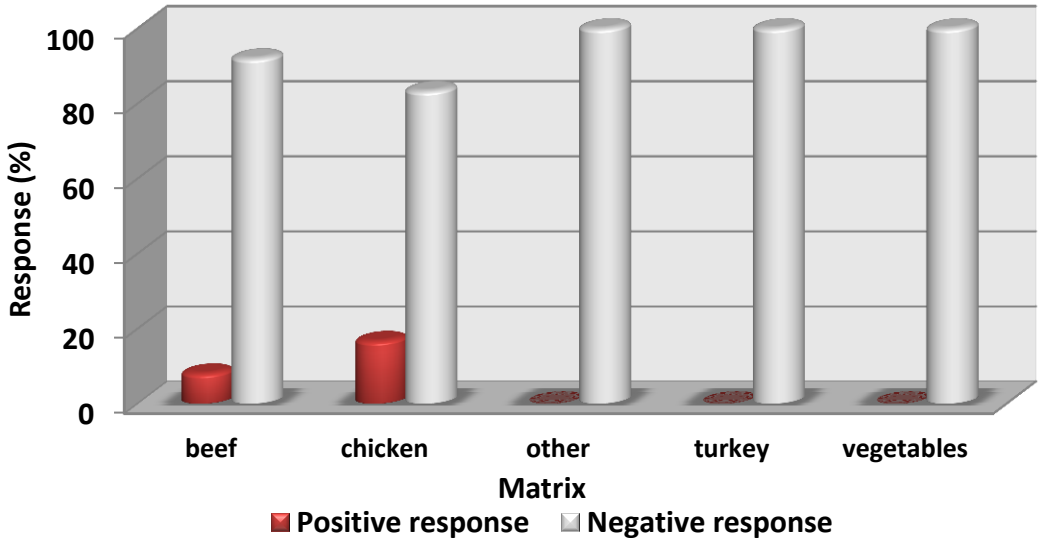



Chart 63 Positivity percentage of *Campylobacter spp.* in the matrices investigated.

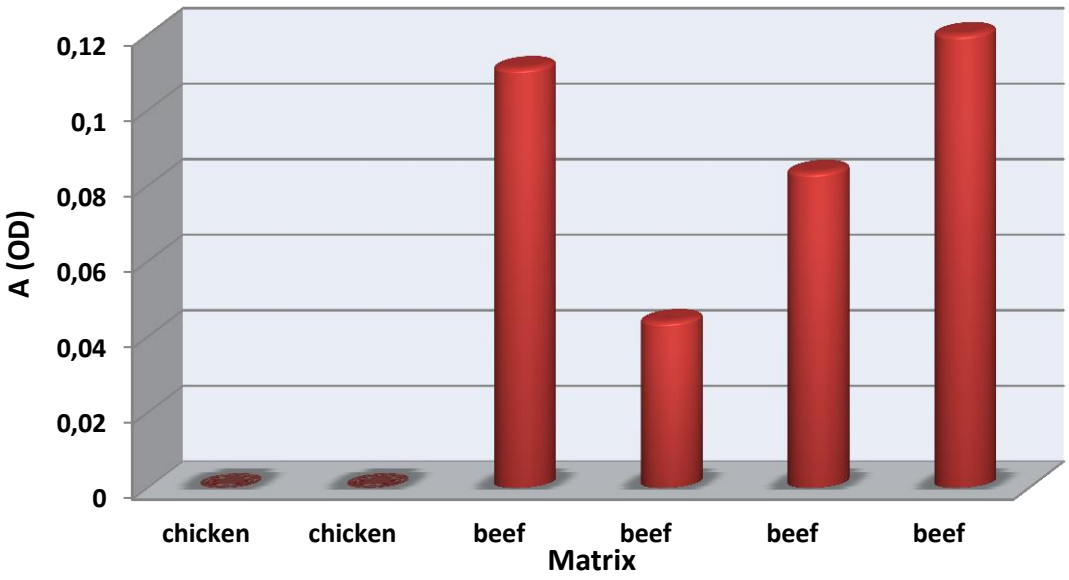



**Chart 64 Biofilm data for *Campylobacter spp.* strains.**

The strains isolated from beef probed high ability to form biofilms although with a considerable variability in the results.

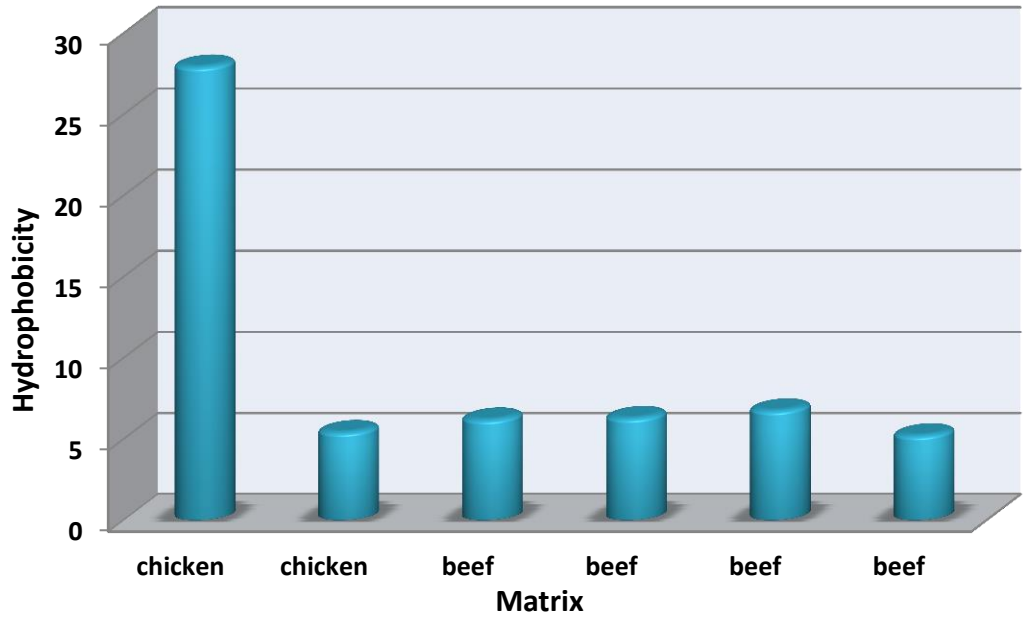



**Chart 65 Hydrophobicity levels for *Campylobacter spp.* strains.**

Only one strain of *S. aureus* isolated from chicken meat has shown a considerable hydrophobicity.

### 6.4.5. *Clostridium difficile*

*Clostridium difficile* evaluation in food matrices constitutes a recently acquired scientific problem. In our study, the isolates were found exclusively in the vegetable matrix: "I gamma" products.

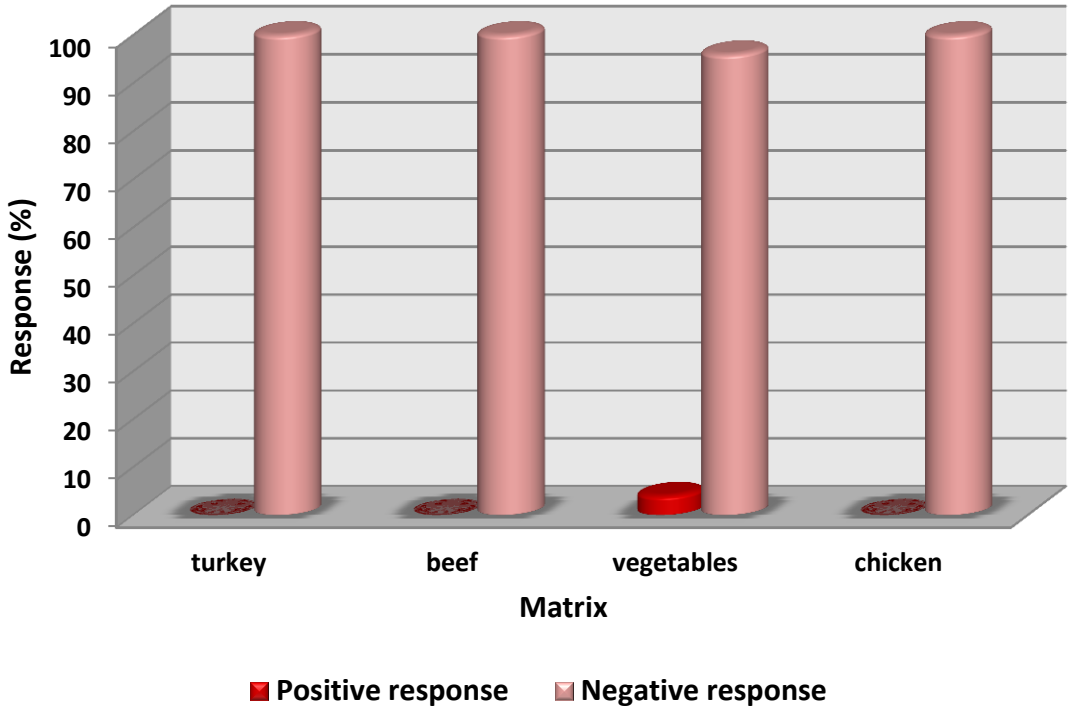



**Chart 66 Positivity percentage of *C. difficile* in the matrices investigated.**

## 6.5. Hydrophobicity and biofilm formation

### 6.5.1. Statistical evaluation

The parameters of hydrophobicity and biofilm formation for *Salmonella spp.* and *S.aureus* strains revealed considerable variability in the analysis of the Student's t that showed no significant differences. A further approach has involved the use of correlation test to support the conclusions of Student's t test. The study of the correlation between the

trends of the data acquired on the same parameters has not verified significant relationships.

The results of hydrofobicity and biofilm for *S.aureus* reported no significant differences between data; Student's t test stated a value of 0,000338 that does not fit the reference value due to the processed data (P = 0,05%).

The same conclusions can be described after elaboration for *Salmonella spp.* data (Student's t test value: 0,00000170; P = 0,05%).

An additional statistical evaluation consisted on realizing the Wilcoxon-Mann-Whitney Ranks Sum Test for large samples.

The Wilcoxon Ranks Sum Test has the same purpose as the t Test: to determine whether there is a difference between two groups. Specifically, it was determined whether the two sample groups could be associated.

It is a hypothesis test and concerns of two hypotheses:

- $H_0$ - Null Hypothesis states that the two sample groups could be associated, that is, there is no difference between the two populations.
- $H_1$ - Alternate Hypothesis states that the two sample groups could not be associable.

Results obtained for *Staphylococcus aureus* report a |Z| value of 0,071, which is lower than the respective Z Critical (1,960): therefore it is possible to accept the Null Hypothesis, stating that there is a 95% certainty that the two sample groups were associable and that, therefore, supporting Student's t results, Biofilm and Hydrophobicity could be related.

A different picture can be described for *Salmonella spp.*: in the specific case, $H_0$ hypothesis has to be rejected since |Z| (2,934) is greater than Z Critical (1,960) for the given $\alpha$ (0,05).

### 6.5.2. Correlation studies on biofilm and hydrophobicity

In order to manage highly different data, it was essential to convert the values to percentage, distribute them in ranges (low, medium, high) and define an eventual correlation between biofilm and hydrophobicity considering each food matrix, thus confirming statistical analysis results.

Isolates with % hydrophobicity > 70% were classified as highly hydrophobic and those with a hydrophobicity index < 30% were classified as low range hydrophobic while those with a hydrophobicity index between 30 and 70% like on the average hydrophobicity (Denise de Oliveira Scoaris, Jean Colacite).

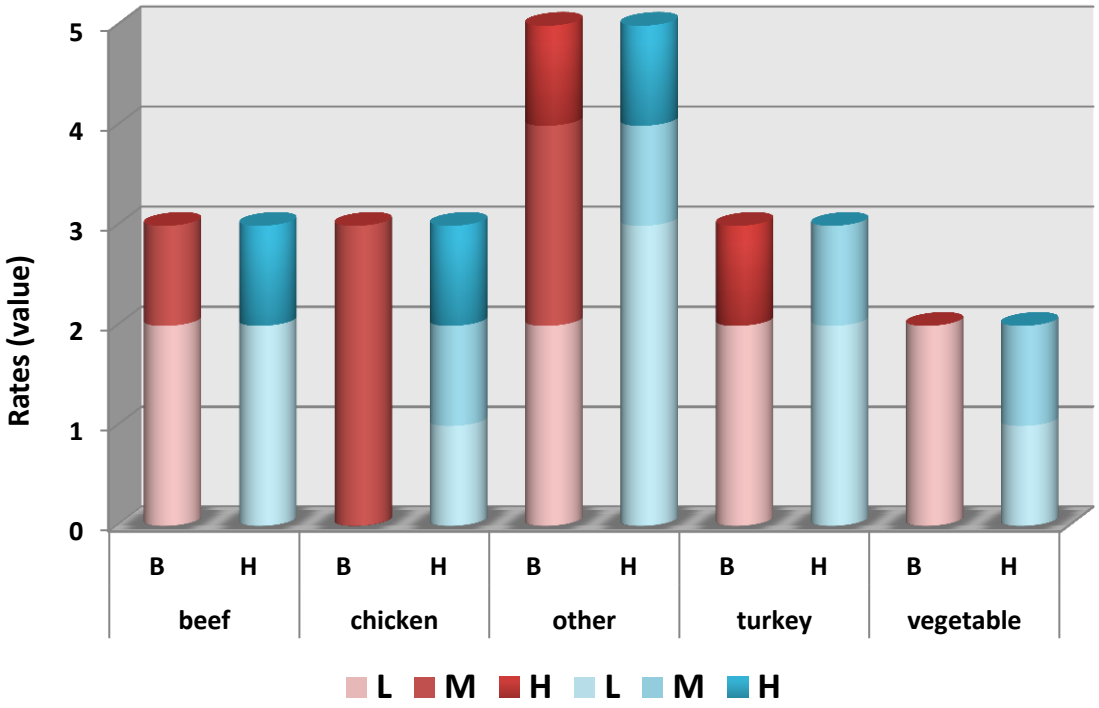



**Chart 67 *S.aureus* strains: Biofilm and Hydrophobicity levels for each matrix.**

The graph shows association between the levels of biofilm and hydrophobicity for the different matrices. The majority of *S. aureus* strains isolated from beef are ranked in low range for both parameters.

Considering the strains isolated from chicken samples, despite the fact that biofilm values are always ranked in high range, hydrophobicity once highlights a wider variability.

Data referred to *S.aureus* in turkey and vegetables reveal biofilm values mostly in the low range and low-medium hydrophobicity.

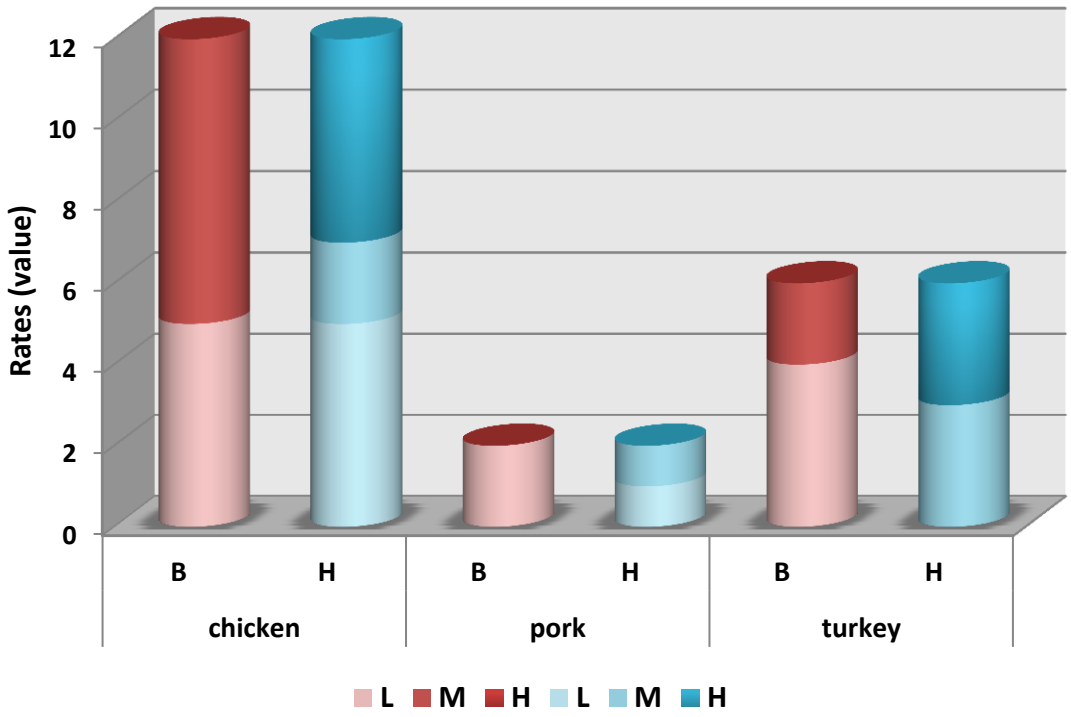



**Chart 68 *Salmonella spp.* strains: Biofilm and Hydrophobicity levels for each matrix.**

*Salmonella* strains isolated from turkey and chicken highlight either low or high biofilm rates compared to high-medium hydophobicity frequencies.

## 6.6. Virulence factors

Virulence factors are enzymes known to destroy cell components, such as nucleic acids and proteins. It is generally considered necessary to contain more than one extracellular enzyme in order for a microbe to be virulent (Edberg et al., 1996). Although no single extracellular enzyme has been proved to be the sole factor responsible for bacterial virulence, there is no doubt that such enzymes do play a role in their pathogenic process (Edberg et al., 1996).

To determine if isolated strains can be potentially pathogenic, analyses where performed to examine the production of various virulence factors by the evaluated bacteria.

As previously stated, positive tests were observed by the clearing of the opaque medium around the inoculum spot after incubation at 37° C. Halos were measured for seven consecutive days (Sbarra et al., 1960; Edberg et al., 1996); to be able to reveal the presence on the seventh day, the plates were treated with appropriate solutions in order to highlight the possible halo. For plates containing Mucin, 1% $CaCl_2$ solution was used; the highlighting of the protease halos require the use of a saturated solution of $(NH_4)_2SO_4$; for amylase, Lugol's solution was used.

In addition, an evaluation of the number of virulence factors expressed by each strain has been performed: this step of the study has been carried out basing on several researches stating a positive correlation between higher numbers of virulence factors and their potential for determining diseases. (Foodborne and Waterborne Bacterial Pathogens: Epidemiology, Evolution and Molecular Biology -Shah M. Faruque).

### 6.6.1. *Salmonella spp.*

Table 32-33 summarizes *Salmonella spp.* isolates' responses to examined virulence factors.

Against a panel of 13 enzymes, 20 strains were tested: none of the isolates produced the halo excepting for amylase results. Using Starch as a substrate, for amylase evaluation, 15% samples emerged positive to the specific virulence factor.

| | Mucin type II 1% | Mucin type III 1% | Elastine bovine neck ligament 1% | Collagen Type I 1% | Collagen Type I 0,1% | Skim milk 1% | Casein 1% |
|---|---|---|---|---|---|---|---|
| % | 0% | 0% | 0% | 0% | 0% | 0% | 0% |
| 1S | - | - | - | - | - | - | - |
| 2S | - | - | - | - | - | - | - |
| 3S | - | - | - | - | - | - | - |
| 4S | - | - | - | - | - | - | - |
| 5S | - | - | - | - | - | - | - |
| 6S | - | - | - | - | - | - | - |
| 7S | - | - | - | - | - | - | - |
| 8S | - | - | - | - | - | - | - |
| 9S | - | - | - | - | - | - | - |
| 10S | - | - | - | - | - | - | - |
| 11S | - | - | - | - | - | - | - |
| 12S | - | - | - | - | - | - | - |
| 13S | - | - | - | - | - | - | - |
| 14S | - | - | - | - | - | - | - |
| 15S | - | - | - | - | - | - | - |
| 16S | - | - | - | - | - | - | - |
| 17S | - | - | - | - | - | - | - |
| 18S | - | - | - | - | - | - | - |
| 19S | - | - | - | - | - | - | - |
| 20S | - | - | - | - | - | - | - |

**Table 33 Virulence factor: response metabolic profile.**

| | Gelatine 1% | Soluble chitin 0,5% | DNasi | Erythrocytes | Starch 0,5% | Egg Yolk emulsion 2% |
|---|---|---|---|---|---|---|
| % | 0% | 0% | 0% | 0% | 15% | 0% |
| 1S | - | - | - | - | - | - |
| 2S | - | - | - | - | - | - |
| 3S | - | - | - | - | - | - |
| 4S | - | - | - | - | - | - |
| 5S | - | - | - | - | - | - |
| 6S | - | - | - | - | - | - |
| 7S | - | - | - | - | + | - |
| 8S | - | - | - | - | + | - |
| 9S | - | - | - | - | - | - |
| 10S | - | - | - | - | - | - |
| 11S | - | - | - | - | - | - |
| 12S | - | - | - | - | - | - |
| 13S | - | - | - | - | - | - |
| 14S | - | - | - | - | - | - |
| 15S | - | - | - | - | - | - |
| 16S | - | - | - | - | - | - |
| 17S | - | - | - | - | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| **18S** | - | - | - | - | - | - |
| **19S** | - | - | - | - | - | - |
| **20S** | - | - | - | - | + | - |

**Table 34 Virulence factor: response metabolic profile.**

Strains that evidenced positivity to starch (amylase) were 7, 8, 20.

| Strains | Enzyme production |
|---|---|
| **1S** | 0 |
| **2S** | 0 |
| **3S** | 0 |
| **4S** | 0 |
| **5S** | 0 |
| **6S** | 0 |
| **7S** | 1 |
| **8S** | 1 |
| **9S** | 0 |
| **10S** | 0 |

| Strains | Enzyme production |
|---|---|
| **11S** | 0 |
| **12S** | 0 |
| **13S** | 0 |
| **14S** | 0 |
| **15S** | 0 |
| **16S** | 0 |
| **17S** | 0 |
| **18S** | 0 |
| **19S** | 0 |
| **20S** | 1 |

**Table 35 Number of enzymes producted by each strain of *Salmonella spp.*.**

### 6.6.2. *Staphylococcus aureus*

After analyzing the 21 *Staphylococcus aureus* isolates against a panel of 13 enzymes the following positive results were obtained: mucynase (with 1% mucin type II substrate) 63%, mucynase (with 1% mucin type III substrate) 63%, elastase 0%, collagenase (1% collagen type I) 74%, collagenase (0,1% collagen type I) 68%, skim milk (1% skim milk), casein (1% casein) 79%, gelatin (1% gelatin) 47%, chitinase (0,5% soluble chitin) 0%, DNase 16%, hemolytic activity (erythrocytes) 42%, amylase (0,5% starch) 32%, the activity of the lecithinase (2% egg yolk emulsion) 84%.

| | Mucin type II 1% | Mucin type III 1% | Elastine bovine neck ligament 1% | Collagen Type I 1% | Collagen Type I 0,1% | Skim milk 1% | Casein 1% |
|---|---|---|---|---|---|---|---|
| **%** | 63 | 63 | 0 | 74 | 68 | 58 | 79 |
| **1A** | + | + | - | + | + | + | + |
| **2a** | + | + | - | + | + | - | + |
| **3A** | + | + | - | + | + | + | + |
| **4A** | - | - | - | - | - | + | + |
| **6A** | + | + | - | + | + | + | + |
| **7A** | - | - | - | + | + | + | + |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **8A** | + | + | - | + | + | + | + |
| **9A** | - | + | - | - | - | + | + |
| **10A** | - | + | - | + | + | - | + |
| **11A** | + | + | - | + | + | + | + |
| **12A** | + | + | - | + | + | - | + |
| **13A** | + | + | - | + | + | + | + |
| **14A** | - | - | - | - | - | - | - |
| **15A** | + | - | - | - | - | - | - |
| **16A** | - | - | - | + | - | - | - |
| **17A** | + | - | - | - | - | - | - |
| **18A** | - | - | - | + | + | - | + |
| **20A** | + | + | - | + | + | + | + |
| **21A** | + | + | - | + | + | + | + |

**Table 36 Virulence factor: response metabolic profile.**

| | Gelatine 1% | Soluble chitin 0,5% | DNasi | Erythrocytes | Starch 0,5% | Egg Yolk emulsion 2% |
|---|---|---|---|---|---|---|
| **%** | 47 | 0 | 16 | 42 | 32 | 84 |
| **1A** | + | - | - | - | - | + |
| **2a** | - | - | + | + | + | + |
| **3A** | + | - | - | + | - | + |
| **4A** | + | - | - | + | - | + |
| **6A** | + | - | + | - | + | + |
| **7A** | - | - | - | - | + | + |
| **8A** | - | - | - | - | + | + |
| **9A** | - | - | - | - | - | + |
| **10A** | - | - | - | + | - | + |
| **11A** | - | - | - | + | - | + |
| **12A** | + | - | - | - | - | + |
| **13A** | + | - | - | - | + | + |
| **14A** | - | - | - | - | - | - |
| **15A** | - | - | - | - | - | - |
| **16A** | - | - | - | - | - | - |
| **17A** | - | - | - | + | - | + |
| **18A** | + | - | - | - | - | + |
| **20A** | + | - | + | + | + | + |
| **21A** | + | - | - | + | - | + |

**Table 37 Virulence factor: response metabolic profile.**

In respect to *Salmonella spp.* results, *S. aureus* strains showed a more variable spectrum of positivity to virulence factors evaluated: strains 20, 6, 2, 3, 13, 21 present a higher

number of virulence factors expressed; a lower number were registered for strains 15, 16, 17; only one strain (14) resulted negative to all tests.

| Strains | Enzyme production |
|---|---|
| 1A | 8 |
| 2a | 9 |
| 3A | 9 |
| 4A | 5 |
| 6A | 10 |
| 7A | 6 |
| 8A | 8 |
| 9A | 4 |
| 10A | 6 |
| 11A | 8 |

| Strains | Enzyme production |
|---|---|
| 12A | 7 |
| 13A | 9 |
| 14A | 0 |
| 15A | 1 |
| 16A | 1 |
| 17A | 3 |
| 18A | 5 |
| 20A | 11 |
| 21A | 9 |

**Table 38 Number of enzymes producted by each strain of *S.aureus.***

A further investigation has been carried out by comparing the mean numbers of enzymes expressed by *S. aureus* strains isolated from different matrices. In particular, strains isolated from beef and turkey evidence a higher number of positivities to enzymes evaluated. It can be presumed that, even referring to previous studies on different microorganisms (The role of pigs in the epidemiology of *Yersinia enterocolitIca* foodborne Infection- S.Barbieri, S.Bonardi- University of Parma, 2007) the most pathogenic strains were isolated from beef and turkey matrices.

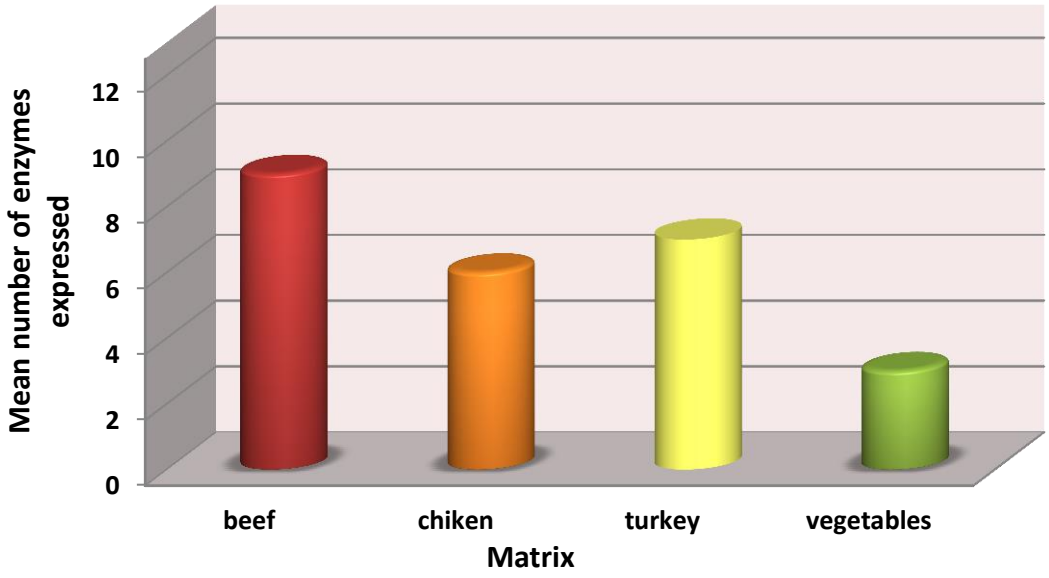



**Chart 69 Mean number of enzymes expressed by matrix.**

### 6.6.3. *Clostriudium perfringens*

The same enzymatic activity evaluation was performed for *Clostridium perfringens* (4 isolates); in this case, data evidenced the following positivity rates: mucynase (with 1% mucin type II and 1% mucin type III substrate) 0 %, elastase 0%, collagenase (1% collagen type I) 25%, collagenase (0,1% collagen type I) 0%, skim milk (1% skim milk) 100% , casein (1% casein) 100%, gelatin (1% gelatin) 100%, chitinase (0,5% soluble chitin) 0%, DNase 0%, hemolytic activity (erythrocytes) 100%, amylase (0,5% starch) 0%, the activity of the lecithinase (2% egg yolk emulsion) 100%.

| | Mucin type II 1% | Mucin type III 1% | Elastine bovine neck ligament 1% | Collagen Type I 1% | Collagen Type I 0,1% | Skim milk 1% |
|---|---|---|---|---|---|---|
| % | 0% | 0% | 0% | 25% | 0% | 100% |
| vegetables | - | - | - | - | - | + |
| vegetables | - | - | - | + | - | + |
| turkey | - | - | - | - | - | + |
| vegetables | - | - | - | - | - | + |

**Table 39 Virulence factor: response metabolic profile.**

| | Casein 1% | Gelatine 1% | Soluble chitin 0,5% | DNasi | Erythrocytes | Starch 0,5% | Egg Yolk emulsion 2% |
|---|---|---|---|---|---|---|---|
| % | 100% | 100% | 0% | 0% | 100% | 0% | 100% |
| vegetables | + | + | - | - | + | - | + |
| vegetables | + | + | - | - | + | - | + |
| turkey | + | + | - | - | + | - | + |
| vegetables | + | + | - | - | + | - | + |

**Table 40 Virulence factor: response metabolic profile.**

*Clostridium perfringens* strains results described a tendentially similar trend in respect to analyzed enzymes: worth to evidence that positivities refer to the same virulence factors (casein, gelatin, erythrocytes, egg yolk).

| Strains | Enzyme production |
|---|---|
| 1P | 5 |
| 2P | 6 |
| 3P | 5 |
| 4P | 5 |

**Table 41 Number of enzymes producted by each strain of *C.perfringens*.**

### 6.6.4. *Campylobacter spp.*

The six *Campylobacter spp.* isolates results, against the 13 enzymes panel, reported what follows: mucynase (with 1% mucin type II and 1% mucin type III substrate) 0 %, elastase 0%, collagenase (1% collagen type I) 0%, collagenase (0,1% collagen type I) 0%, skim milk (1% skim milk) 67%, casein (1% casein) 0%, gelatin (1% gelatin) 0%, chitinase (0,5% soluble chitin) 0%, DNase 0%, hemolytic activity (erythrocytes) 100%, amylase (0,5% starch) 100%, the activity of the lecithinase (2% egg yolk emulsion) 0%.

| | Mucin type II 1% | Mucin type III 1% | Elastine bovine neck ligament 1% | Collagen Type I 1% | Collagen Type I 0,1% | Skim milk 1% |
|---|---|---|---|---|---|---|
| % | 0% | 0% | 0% | 0% | 0% | 67% |
| chicken | - | - | - | - | - | - |
| chicken | - | - | - | - | - | + |
| beef | - | - | - | - | - | - |
| beef | - | - | - | - | - | + |
| beef | - | - | - | - | - | + |
| beef | - | - | - | - | - | + |

**Table 42 Virulence factor: response metabolic profile.**

| | Casein 1% | Gelatine 1% | Soluble chitin 0,5% | DNasi | Erythrocytes | Starch 0,5% | Egg Yolk emulsion 2% |
|---|---|---|---|---|---|---|---|
| % | 0% | 0% | 0% | 0% | 100% | 100% | 0% |
| chicken | - | - | - | - | + | + | - |
| chicken | - | - | - | - | + | + | - |
| beef | - | - | - | - | + | + | - |
| beef | - | - | - | - | + | + | - |
| beef | - | - | - | - | + | + | - |
| beef | - | - | - | - | + | + | - |

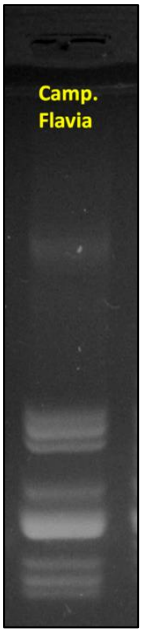
**Table 43 Virulence factor: response metabolic profile.**

The same concordance, concerning enzymes types, discussed for *C. perfringens* can be mostly evidenced for *Campylobacter spp.*: strains analyzed underline same results (from 2 to 3 positivities) for erythrocytes, starch, skim milk.

| Strains | Enzyme production |
|---|---|
| 1C | 2 |
| 2C | 3 |
| 3C | 2 |
| 4C | 3 |
| 5C | 3 |
| 6C | 3 |

**Table 44 Number of enzymes producted by each strain of *Campylobacter spp.*.**

### 6.6.5. *Clostridium difficile*




As previously stated, DNA sequencing was performed on an Applied Biosystems 373A DNA sequencer with the DNA sequencing kit (Applied Biosystems) and sequences were then analyzed and aligned on the Sequence Navigator 1.0.1 software (Applied Biosystems).

The only analyzed strain was isolated from a vegetables matrix; after processing the sample through ribotype technique it was not possible to identify the strain since it does not match any of those available in the "Parthenope" University of Naples library.

**Image 28 Evaluated *C.difficile* strain.**

The electrophoretic profiles of *C.difficile* ribotypes are following showed: third profile from left corresponds to the evaluated strain.

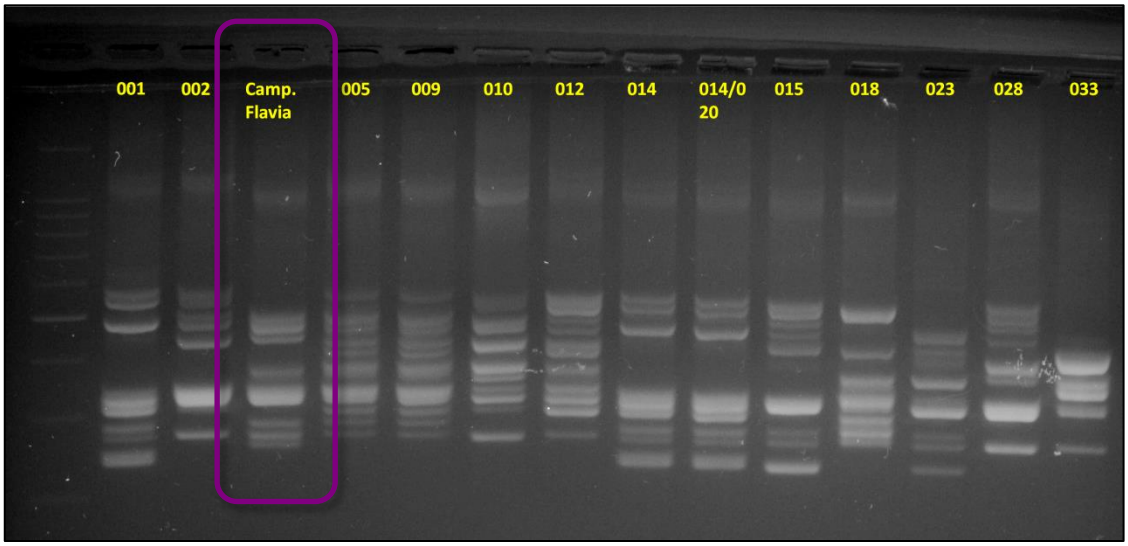



**Image 29 Electrophoretic run of *C.difficile* ribotypes.**

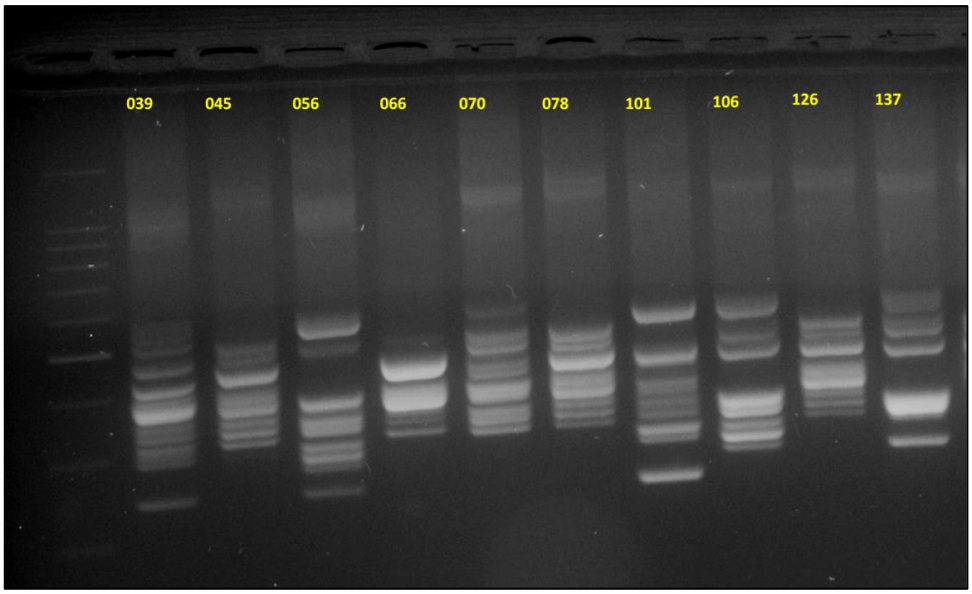



**Image 30 Electrophoretic run of *C.difficile* ribotypes.**

### 6.6.6. Considerations

Virulence factors analysis allowed the comparation of collected data to available bibliography referred to the same topic.

Half of the *S.aureus* isolated strains present at least 8 positivities over the 13 enzymes examined as virulence factors.

Therefore, it can be supposed that these strains are the most pathogenic because of the larger number of expressed enzymes, proving the positive correlation between higher numbers of virulence factors and their potential for determining diseases, as reported by previously quoted bibliography (Foodborne and Waterborne Bacterial Pathogens: Epidemiology, Evolution and Molecular Biology -Shah M. Faruque 2012).

In addition, strains isolated from beef and turkey evidence a higher number of positivities to enzymes evaluated; since previous studies on different microorganisms (S.Barbieri, S.Bonardi- University of Parma, 2007) correlate the most pathogenic strains to specific food matrices, a hypothesis can be moved: because strains with higher number of virulence factors expressed were isolated from specific matrices, it can be presumed that those represent the ideal substrate where the growth of highly pathogenic bacteria is optimal.

# 7. CONCLUSIONS

The performed study resulted pretty diversified, regarding both key points and examined matrix types. This does not mean a dispersion within the research but rather a strong point since immediately evaluates several aspects of microbial contamination.

First of all, the research on meat (chicken, turkey, beef, pork) and vegetables matrices brought out higher levels of microbial load, considered generic contamination markers: an example is provided by TBC evaluation on poultry meat, which implies the higher risk linked to production and consumption of these matrices. This result was even confirmed by the higher isolation of *Aeromonas spp.* from these matrices (in particular, chicken).

*E.coli* evaluation further supported data gathered from previous determinations; *Salmonella spp.* analysis describes a larger isolation rate in poultry meat, mostly, even in this case, in chicken and generally counting a significant isolation rate in respect to other matrices examined.

Summarizing, the majority of samples, excepting those in which *Salmonella spp.* was detected, was in compliance with current Regulations.

Noncompliancy rates resulted limited although even these confirm the higher risk linked to white meat.

The application of HACCP method, considered in particular TBC, *E.coli* and *Salmonella spp.* in order to fulfill the quality and safety standards contemplated by current regulations (Reg. CE 2073/2005), should have solved every issue related to food chains, by detecting the CCP (Critical Control Points) along the different production phases and the deriving monitoring strategies and corrective actions.

The study evidenced that, within the 30% products, the efficacy of the HACCP method does not seem to have provided positive outcomes.

This actually constitutes a problem in relation to the evaluation of products safety at the end of production chain: since, within this phase, sampling is probabilistic, the available data could even reveal not reliable results concerning noncompliancies.

On the other hand, a systematic examination of the totality of supply chains, a poser, is reacheable in longer times; next step consisted therefore on detecting a “characteristic”

that, though analyzing the product at the end of the chain (that is, the distribution phase), could be able to highlight chain's abnormalities tied to a more substantial risk.

In the specific case, disposing of TBC and *E.coli* microbial load values, this allows to evidence, through frequency distributions analysis, that the abnormal trends could indicate, even in samples conform to the limits of the Law, a production chain not adequately monitored and consequently a greater risk for consumers.

Autorities' controls dispose a considerable quantity of numeric data arbitrarily collected at the end of the production chains.

The frequency trends elaboration, performed using these results, could easily indicate, according to abnormal tendencies, more risky chains.

Having a greater quantity of data available in respect to the effective low samples numerosity of this monitoring, it could be even possible to classify the several abnormalities degrees: for example, thanks to the analysis of abnormal peaks' number and within the distribution trend and together with the comparation to an ideal curve, a risk index could have been generated.

This approach can result crucial to detect and address the control actions on each production chain in order to minimize eventual gaps within the monitoring and reduce handling expenses.

Focusing the study on frequency distributions, it appears to be a promising and cutting edge approach that deserves, in our opinion, a deeper analysis on the study itself and its application.

Some evidences acknowledged in terms of relations between different microbial virulence factors were supported by, in the first analysis, non-parametric tests (Wilcoxon-Mann-Whitney). Again, a major number of samples could have allowed other interesting correlation studies.

The research of minor pathogens or potentially pathogenic species such as *Aeromonas spp.* and *Campylobacter spp.* allowed to underline several critical points; for this reason, these represent an undeniable signal for the evaluation of a Laws upgrade able to consider these microorganisms as critical analytes as much as the traditional ones; in fact, 10% *Campylobacter spp.* was evidenced in beef matrices, 20% in chicken matrix. *Aeromonas spp.* strains were isolated from 100% chicken, 82% turkey, 74% beef, 62% vegetables matrices.

A third aspect regards the non compliance showed by some samples due to the detection of *Salmonella spp.* ; within this research 21 *Salmonella spp.* serovars were isolated: after proceeding with serovars typing, it is possible to describe a notable diversification even in respect to bibliography (EFSA-ECDC report). This point obviously worths further follow-ups considering a possible adaptive diversification of species depending on meat types and geographical areas of origin.

An additional interesting topic regards *Clostridium difficile*, a pathogen which is rarely isolated from foods in Italy, but aim of systematic studies, especially in northern Europe, both for environmental and food matrices analyses. Limits of available procedure allowed the detection of two strains which still need to undergo a genomic characterization for ribotype identification.

*Salmonella spp.* isolated serovars were additionally examined for eventual positivities to specific virulence factors together with ability to form biofilm, hydrophobicity and extracellular enzymes production: the analysis conducted suggest that potentially more virulent strains are those isolated from beef. This result seems innovative and worths further analysis.

In conclusion, putting together all the “tiles” of the research not only sets the basis for the creation of a food chains’ linked risk index, which could be more appropriate even for samples in compliance with Legislation, but also provides interesting questions which need to be answered in a systematic manner in order to guarantee a better and more adequate food chains safety management and the optimal protection of consumers' interests.

# 8. FUTURE PERSPECTIVES

The study highlighted several strong points such as the statistical-mathematical approach of frequency distributions for the evaluation of the Risk related to production chains, through the assessment of their final step: the distribution phase.

A possible weak point, instead, could be the lack of epidemiological data especially if referred to the specific consumers' area which has been subjected to study.

A research conducted this way should comprehend questionnaire to be suggested to consumers: this to reveal both the evident GI diseases, confirmed by clinical analysis, through the isolation of their main cause, surveyed by informative flows (according to D.M. 15/12/1990: informative system of infectious and diffusive diseases) and the questions about minor toxinfective episodes, presenting mild signs and symptoms (nausea, vomiting, abdominal pain, etc.), usually not notified by doctors and therefore not reported.

This approach could be an interesting starting point for future studies to verify, on mathematical basis, the possible associations and deduce more reliable risk indices and thus schematizing the method.

Focusing attention on the distribution phase products allowed to open to interesting perspectives regarding investigations on more risky chains, together with the check of the adequate application of HACCP method and an epidemiological evaluation concerning consumers.

Within the study it was indeed evidenced a discrepancy between European data provided by EFSA-ECDC and those from our study, especially considering *Salmonella* strains isolated.

For these reasons it is advisable a higher attention throughout the processing of the data elaborated by EFSA-ECDC observatory and a major consideration to the so-called "emerging" pathogens, which have been instead emphasized in this research.

In fact, only acting in an integrated manner, availing of all available data, it is possible to generate prevention strategies and risk mitigation.

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