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STUDY ON THE INACTIVATION BY HIGH PRESSURE OF PATHOGENS IN FISH PRODUCTS: POTENTIAL APPLICATIONS.

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A Alfonso, Alessandra, Luigi, Rosalia
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SUMMARY

1. INTRODUCTION

INTRODUCTION

The increased demand from consumers of naturally and minimally processed foods, associated with the fear of chemical preservatives added during the manufacture, has improved the development of new mild technologies for the processing of foods, as High Hydrostatic Pressure.

High Pressure technology offers a great potential for the preservation of fish products having natural characteristics and an optimal microbiological quality.

Between fish products, the caviar represents a fragile foodstuff with an important nutritive value, subjected to rapid spoilage and involved in problems of safety risk due to the possible contamination by pathogens, as *Staphylococcus aureus* and *Salmonella* sp..

These pathogens are two ubiquitarious micro-organisms, respectively a Gram+ and Gram- bacterium, having many different features, but both responsible of serious foodborne diseases, the most important from a point of view of epidemiology for diffusion in France and in Italy.

The foods often involved and cause of these illness are the fish products, especially in the cases where the handling and the procedures of manufacture are achieved in not proper conditions.

So *Staphylococcus aureus* and *Salmonella* sp. can contaminate the fish eggs during the steps of fabrication of the caviar and so represent a risk for the consumer.

In this thesis a study on the feasibility of High Pressure treatment on caviar was done in order to find the optimal conditions in parameters of this procedure applied to the fish eggs.

In a first moment the behaviour of the caviar under pressure was evaluated and represented the first step for the search of the optimal conditions of treatment.
Also the baroresistance of the strains used in this work, *Staphylococcus aureus* ATCC 6538 and *Salmonella* ATCC 13076, was tested in suspensions models and subsequently in caviar samples. The effects of the matrix on the sensitivity to the pressure of the pathogens was also considered.

A detailed bibliography about the technology of High Pressure, the pathogens *Staphylococcus aureus* and *Salmonella enteritidis*, the caviar, the effects of this thermodynamical parameter on the pathogens and also on fish products, is reported for each part, in order to well understand all the subjects of this study and the action of High Pressure both on micro-organisms and caviar.

The interdisciplinar work concerned a thesis in Cotutelle France-Italy between the University "Sciences et Technologies" Bordeaux 1 and the University of "FEDERICO II" of Naples.

The experiments on High Pressures in food field are well developed at the University of Bordeaux 1: "Sciences et Technologies" (France), while the microbiology of fish products is largely studied at the University of Naples (Italy).

2. BIBLIOGRAPHY SYNTHESIS

2.1 THE MICRO-ORGANISMS

2.1.1 Generalities

2.1.1.1 Bacterial morphology

The morphology of a bacterial cell refers, besides the form and the dimensions of the single cells, to the way these are arranged among them [7]. These characteristics are encoded from the genoma of the micro-organism and transmitted by generation to generation.

Great part of the bacteria has a well defined form that allows to distinguish them in 3 morphological groups: the “cocci”: with a spherical form, the “bacilli”: with stick form and the “spirilli”: with spiral form. Some micro-organisms are filamentous and others are “pleomorphus” (from the greek: multiforms).

Cocci. Concerns spherical micro-organisms whose dimension ranges from 0,5 to 1 μm of diameter. The cocci (from the greek: “grain”) are often observed in groups because of the incomplete separation of the single cells, during the reproductive asexual process; since the division plans vary in relation to the type of micro-organism, the final disposition of the cells representing a very important feature for their identification. The division through the same plan determines the formation of cells couples (“diplococci”), or cells chains (“streptococci”, from the greek: twisted); the division in two perpendicular planes leads the formation of “tetradi”; while the division in three plans produces typical aggregates or clusters (“staphylococci”, from the greek: grape).

Bacilli. Describes micro-organisms with stick-like form, whose length ranges from 1 to 10 μm.

Some bacilli are thin and long, others are short and ovoidal ("coccobacilli"). Bacilli with comma-like form are called "vibrion". The most part of bacilli is in form of single separated cells; however coupled forms can be observed ("diplobacilli") or in chains ("streptobacilli"). Instead bacilli, as the "corynebacteria", arrange in paling or angle.

Spirilli. The micro-organisms with spiral form are less common to the other types, but they include bacteria as *Treponema pallidum*, agent of the syphilis. They are composed from two very similar groups: the "spirilli", with rigid structure and the "spirochetes", with flexible structure.

2.1.1.2 Structures of the bacterial cell

All the bacteria are characterized by common features: the genetic information in the form of DNA (the "nucleoid"), the "ribosoms" and the "cytoplasmatic membrane". Almost all the bacteria show a characteristic "cellular wall". Some accessory structures can be present as the "capsule", the appendixes of surface and the cytoplasmatic inclusions [1].

2.1.1.2.1 Structures of surface

The first structure that we can observe, examining the surface of bacterial cell, is the external mucous layer, that sometimes can make a real capsule [7]. Immediately under this formation, an extremely solid structure is always present, the "cellular wall", constituted by a dense net, quite rigid. Under the cellular wall, in contact with it, another less rigid and very thinner wrap is present, the "cytoplasmatic membrane", that contains the real living substance, the "cytoplasm". Through the cellular wall many different appendixes, as the "fimbriae", can project toward the outside and permit the bacterial mobility.

2.1.1.2.1.1 The capsule

Bacteria actively expel some materials, of polisaccharidic or polipeptidic nature, that crowd to the outside of the cellular wall constituting an additional muff. When this layer adheres in

compact way to the surface of the cell and is clearly differentiable from the surrounding environment, is called capsule. Another different structure which can surround the bacterium is the "glycocalyx", constituted by loose polisaccharidic fibrille that favor the adhesion of the bacteria to the solid surfaces and also englobe them to form a special biofilm, which protects them from the antibacterials factors. The glycocalice, that for its extreme insubstantiality and weakness, cannot be observed by the techniques able to disclose the capsule. In fact, to observe it, a particular shrewdness is necessary and the employment of the electronic microscope, after a suitable coloration [2]. In the micro-organisms these external layers, in particular the capsule, have various functions:

(i) to protect the cellular wall from the action of natural antibacterials agents of various type (bacteriophages, colicins, complement, lysozime),

(ii) to favor the adhesion and therefore the colonization of tissues,

(iii) to protect the bacterium from the ingestion and consequent destruction by the phagocytes cells of the guest.

The only difference between harmless strains and pathogen strains of *Streptococcus pneumoniae* is respectively the absence or the presence of the capsule, wich allows to the pathogens ones to escape the local defenses (phagocytosis) and so inducing the pulmonary illness.

Among the other bacteria, having a capsule, it is possible to quote: *Clostridium perfringens* (agent of the gaseous gangrenes), *Bacillus anthracis* (agent of the haematic carbuncle), *Klebsiella pneumoniae* (agent of pneumonia) and *Haemophilus influenzae* (one cause of meningitis) ...[7].

Concerning the adhesion of the bacteria to the tissues of the guest, the bacterial glycocalice has an important role. However, the bacteria, having these structures, can adhere and multiply on the mucoses of the guest despite flows of liquid (urinary apparatus) or the peristalsis movements (bowel) and induce the infection. The chemical composition of the capsule is genetically

encoded for every type able to produce it. The most part of the capsules has polisaccharidic nature or in some cases polisaccharidic-protein complexes.

Bacillus anthracis has a polipeptidic capsule, constituted precisely by a polymer of the D-glutamic acid. This peculiarity of the capsule of *B.anthracis* is responsible of the typical coloration that the bacterium shows after treatment with blue of Löffler and causes the permanence and then the individualization of the capsule constituents in the tissues and in the skin of animals, dead for carbuncle, even in avanced putrefation. In fact, the hydrolases of the tissues are incapable to demolish substances that contain D-amino acids [7].

2.1.1.2.1.2 The cellular wall

The cellular wall of the bacteria is the structure that surrounding the cytoplasmatic membrane [1]. The shape of the bacterial cell is determined by the form of the cellular wall: every bacterium, losing such structure, takes spherical form. The most important function of the cellular wall is to protect physically the cell. In the greater part of the environmental conditions, this cell would be destined to lisis, following osmotic phenomenons (in some bacteria the wall withstands to an osmotic pressure of 25 atm). The particular structure of the cellular wall is responsible of its rigidity. It is composed from a peculiar macromolecule polisaccharidic-amino acidic, called "peptidoglican" [7]. Many bacteria have more numerous overlapped layers of peptidoglican and for this reason the resistance of the wall increases. The strength of the single layers is determined especially by the degree of the intermolecular bonds occurring between the single molecules of peptidoglican.

For example, in the bacteria Gram+, it occurs a lot of additional bonds among pentaglicinic chains. The cellular wall of bacteria Gram+ is constituted by 20 overlapped layers of peptidoglican, that are held united from amino acidic bridges. In this dense net, a small percentage of substances is retained ("matrix" of cellular wall) [2]. Among the substances that constitute the matrix of such bacteria there are some acidic polisaccharides, the teicoici acids

(from the greek: wall). The teicoici acids represent, in the bacteria Gram+, the most important antigenic component of surface. Also the cellular wall of the bacteria Gram- contains a layer of peptidoglican, thinner than the structure observed in the Gram+, so representing only the 10% of the whole wall structure.

The micro-organisms Gram- have also a further structure that covers the peptidoglican layer. This additional external layer, also known with the term of "pseudocapsule", is constituted by a double phospholipidic layer where proteins, lipoproteins and lipopolisaccharides are present [7]. The lipopolisaccharides (LPS) of the external layer have important functions both concerning the antigenic characteristic (somatic antigens or antigens O) and the toxic properties that such components show (endotoxins). The LPSs represent around 40% of the total surface of the bacteria Gram-. It is possible to destroy the cellular wall by treatment with lisozime. This enzyme is present in the tears, in the saliva, in other organic liquids and in the albumen of egg. It is an enzyme able to hydrolise the peptidoglican. After the alteration of the cellular wall, the water of the surrounding environment goes into bacterial cell, that swells and finally explodes.

The bacteria Gram+, after the treatment with lisozime, lose completely the cellular wall; the resultant cellular forms are denominated "protoplastes" [7].

The bacteria Gram-, without peptidoglican, maintain instead, also the muff of lipopolisaccharides, so they are more resistant to the osmotic lysis; such forms, denominated "spheroplastes", do not maintain the original form but take a spherical one [7]. The "protoplastes" and the "spheroplastes" are derived from a chemical treatment that deprives the bacteria of the cellular wall. However, there are in nature bacteria normally deprived of the wall [1]. The most representative group is constituted by members of the genus Mycoplasma. Micoplasmi are pleomorphi due to the lack of a wall. Another group of bacteria, deprived of cellular wall, is represented by the forms L (from the initial the Listers Institute in London where they were described in 1935). These micro-organisms, derive from bacteria Gram+ or Gram-, that naturally have lost -partly or totally- the ability to produce the peptidoglicanic portion of the

wall. This event generally occurs in the organism guest. Forms of this type can also be obtained treating the bacteria with penicillin or with lisozime. Then, if such chemical products are eliminated, the forms L can return in the bacterial forms with wall, from which they are derived (reversing forms L), or they can continue to reply without wall (stable forms L) [7].

2.1.1.2.1.3 The cytoplasmatic membrane

To survive, every cell - both procariote and eucariote - has to remain delimited towards the external environment. This protection is realized by the cellular membrane or citoplasmatic membrane [7]. The alteration or destruction of such barrier causes the spillage of the citoplasmatic material and consequently death of the cell. The citoplasmatic membrane of the bacteria, having a thickness of about 8 nm, shows double structure, very similar to that present in the eucariotic cells. The proteins extend through the whole phospholipidic layer and therefore they are exposed on both the surfaces (internal and external) of the citoplasmatic membrane. The membrane can have many functions (that in the eucariotis are developed by specialized internal structures) as:

1) the transport of molecules to the inside and the outside of the cell,
2) the secretion of extracellular enzymes,
3) the respiration and the photosynthesis,
4) the regulation of the reproduction,
5) the synthesis of the cellular wall.

2.1.1.2.1.4 Bacterial appendixes

Through the cellular wall, numerous and different appendixes can project outside and influence the mobility of the bacterium (“fimbria”, “axial filaments”) or its possibility to adhere to guest cells. For every bacterium the fimbria can be single or multiple and their location is so constant in every microbial type that can be used to taxonomic use [7].

2.1.1.2.2 Intracitoplasmatic structures

All the bacteria have a region where the cromosomial material has thickened, denominated "nucleoid". The rest of citoplasm contains a high number of ribosoms. In some micro-organisms we can also observe big citoplasmatic inclusions [7]. Further the cytoplasm is constituted by water, enzymes and small molecules.

2.1.1.2.2.1 The plasmides

The bacteria can take in their cytoplasm small portions of extracromosomial genetic material, circular, called "plasmide". These structures can autonomously reply and remain in the bacterial cell for numerous generations [7]. Although the plasmides are not essential for the bacterial growth; they are able however to maintain information that allow the cell to become resistant to the antibiotics (factor R, resistance), to produce toxins, to produce adhesive appendixes, essential for the colonization and therefore for the pathogenicity of the bacterium, or to produce sexual appendixes necessary to the bacterial genetic recombination (factor F, fertility).

2.1.1.2.2.2 The bacterial spore

In some types of bacilli Gram+, it is often observed, or inside of the cell or free in the culture medium, a particular structure: the "spore". It has special structural characters and an elevated resistance toward the sterilizing action of many chemical and physical agents and toward the aging [6]. The bacteria, "spore-forming", can transform into such sleeping structures, that do not show apparent metabolism (neither they grow neither, they reproduce). Inside a single vegetative bacterial cell the spore arises through a process known as "sporulation" [7]. When the nutritional conditions of the environment become unfavorable. During this process the greater part of the water is eliminated, so this fact justifies the particular resistance of the spores to the

heat, because the proteins in dry state are less sensitive to the thermal inactivation. Such resistance has subsequently increased from the presence of dipicolinic acid, a characteristic component of spore wall, that stabilizes the spore proteins [7]. Despite the spore is metabolically inactive; it is able to quickly answer to possible changes of the external environment. If environmental conditions become again favorable to the micro-organism from which the spore is produced, the rapid transformation in the vegetative form can be observed. This process defined as "germination"[7] is characterized by the assumption of water, the elimination of the dipicolinic acid and the synthesis of RNA, proteins, DNA and the disintegration of the external protective structures. Obviously these forms have lost all the characteristics of resistance of the spore.

The spore-forming germs have a stick-like morphology and they belong to the genus Bacillus and Clostridium. Among the spore-forming pathogens there are: *C.tetani*, *C.perfringens*, *C.botulinum*, *B.anthracis*,*B.cereus* [7].

2.1.2 Human safety risks linked to consumption of fish products

2.1.2.1 Introduction

Besides the endogenous microbial flora, which is the most important cause of the organoleptic properties deterioration, fish products can essentially lodge a telluric flora, due to fishing zones polluted by waste waters, and also due to the their contamination during preparation, processing, transport, storage and distribution steps. The incidence of foodborne illnesses is not negligible: in 1992 USA 24,779,020 cases of foodborne illness occured, with a mortality of 0.0645%. Some cases were referred to the contamination of raw mussels by *Samonella* sp. (16%), of the fish products by *Vibrio* sp. (33- 46%) and by virus Norwalk (30%) [5].

"Foodborne illness" is a disease which occurs after consumption of foods containing a pathogen, a toxic substance or a toxin from bacterial origin.

There are three main types of food illness from microbial origin:

1. Food poisonings

They arise after consumption of a food which contains a toxin, result of a microbial development in the food. The bacterium can be inactivated, but the toxin remains [4; 6].

Essential features:

a) the bacterium must to be present in the food;

b) the food has to allow the bacterial growth;

c) the bacterium has to reach high growth rates;

d) during the growth it occurs development of the toxin in the food;

e) the food is ingested;

f) the guest must to be sensitive to the action of the toxin.

Examples of poisoning: botulism, staphylococcal poisoning, *B.cereus* enteritis.

2. Foodborne infections

They occur when the food contains pathogens that colonize the bowel of the guest, where they develop and cause lesions of tissues. In general it is not necessary that the bacterium grows in the food, but in this case the probability of infection can increase [4; 5].

Essential features:

a) the bacterium must to be present in the food;

b) the food has to allow the growth, that can occur or not;

c) the food must to be consumed from the guest;

d) the bacterium must to be able to adhere to the mucous tissues, to colonize the bowel and grow;

e) the guest must to be susceptible to the pathogenic action of the micro-organism.

Examples of infection are: the salmonellosis, the shigellosis, the listeriosis, the yersiniosis, the enteritis by *V.parahaemolyticus*, the septicaemia by *V.vulnificus* and the enteritis by

Campylobacter sp [5]. The microbial charge, able to cause the food infection, can be very low (<1 cell/g food).

3. Foodborne Toxic-Infections

It is a combination of the precedent forms [5]. The pathogen has to reach very high rates in the food, so it is assumed by the guest and it continues its development in the bowel, it produces the toxin which causes the symptoms.

Essential features:

a) the bacterium must to be present in the food;

b) the food has to allow the growth of the bacterium;

c) the bacterium has to reach elevated charges (> 10^5 cfu/g);

d) the food must to be consumed;

e) the bacterium has to develop in the bowel and there to release the toxin;

f) the guest must to be susceptible to the toxin.

Typical examples of toxic-infections are: enteritis by *Cl.perfringens*, *V.cholerae* and by enterotoxic strains of *E.coli* [5].

Another type of classification of the foodborne diseases is the following:

1. Foodborne Salmonellosis: for the differences in serotypes and the complexity of the epidemiological cycle (sources, ways of diffusion) they constitute a single group [4].

2. Foodborne diseases consequent to the assumption of foods manipulated in unappropriated ways concerning the temperature (of cooking and/or storage) and the times (of cooking and/or of cooling and storage); they are called "time-temperature-abuse foods". The poisoning from *S.aureus*, *V.parahaemolyticus* and the scombroid fish poisoning are some examples [4]. It is scarce the role of the healthy carriers.

3. Foodborne diseases from foods contaminated for contact oral-faecal are: the shigellosis, the cholera, the enterotoxic illness by *E.coli*, the infectious hepatitis type A and enteritis by virus of Norwalk, listeriosis [5]. The habitat of pathogens is waters polluted by waste waters and from residual organic from the intestinal content of the terrestrial animals. Anyway the foods that derive from this biosphere are potential vehicles of illness. In this epidemiological cycle the "healthy carriers" (also human) assume a final role.

4. Botulism caused by canned foods thermal treated by improper ways, or caused by marinate fish or fermented by non correct ways [5]. Among the bacterial toxins we can distinguish enterotoxins and cytotoxins. The enterotoxins have proteinic nature and are quite thermally stable (for example, the emetic toxin of *B.cereus* withstands to temperature of 129°C for over 7 min). Mechanism of action: they interact with the equilibrium of enterocitis membrane, particularly in the production of AMP-cyclic. This effect unbalances the cellular mechanism of pomp Na^+-K^+, with secretion of Na^+ in the intestinal cavity and following loss of water, profuse diarrhea is achieved, clearly watery (*Salmonella* sp., *Vibrio* sp., *B.cereus*, *Cl.perfringens*). Instead the citotoxins can cause the necrosis of the enterocitis or other types of cells. Mechanism of action: they cause necrosis of enterocitis, so inducing the exfoliation of the mucous tissues with necrosis, hemorrhages and haematic diarrhea, sometimes very serious (*Campylobacter jejuni* and *E.coli O157:H7* provoke “diarrhea all blood”) [3].

2.1.2.2 *Salmonella* sp., *S.aureus*, *E.coli* O157:H7, *Shigella* sp.

Salmonella sp. can be present in waters polluted in proximity of the coast by waste waters. The fish-products, which can result contaminated by the pathogen, are:

1) edible molluscs, because of the presence of *Salmonella* in the polluted waters;

2) manufactured products, for scarce hygiene of the workmanship.

Some cases of food infection have been declared [5]:

- in Germany in 1990-91 an episode caused from *S.enteritidis* present in raw prawns;

- in Italy in 1989 an episode of salmonellosis from *S.enteritidis* concerned 96 people that had consumed a pate of cernia.

The risk of contamination of fish-products by *Staphylococcus aureus*, Gram+ bacterium, is represented by the workmanship and handling of the products. These bacteria are salt-tollerant so all the cured preparations and fish-based preserves are subjected to the risk of contamination of *S.aureus* [5].

E.coli O157:H7 is a Gram- bacillus (family of *Enterobacteriaceae*) is present in the intestinal content of the animals. The food contamination can occur during the manipulation of the manufactured products. Its prevention is based on the cooking, on the preservation of the foods at temperatures < +4°C, as well as on the hygiene of the workmanship and the staff [4].

Shigella sp. is a coccibacillus Gram- belonging to the family of the *Enterobacteriaceae*. *S.dysenteriae* has an elevated pathogenicity: it is calculated that 10-100 cfu/g food are enough to provoke illness [1]. The principal reservoir is the man. The germ is usually transmitted to the man by contaminated food, but the contagion can also occur between humans. The foods are contaminated by direct handling or by the contact with polluted water, and the bugs are probable

vectors. The foods subjected to risk of contamination are the fish-products, the vegetables, the milk and dairy-products.

2.1.2.3 *C.botulinum*

Within *C.botulinum* spp. there are:

- proteolitic types: A, B, F that form very resistant spores;
- no proteolitic types: E, some B types and F.

Most part of the episodes of botulism, occured in Europe (for example in Germany, France, Italy), were caused by no proteolitic types B of *C.botulinum*, developed in meat and meat-products, and especially canned vegetables. In USA it is more frequently detected the type A from vegetables-based products, preserved in cans, while in countries, traditionally used to eat raw fish (as Japan, Canada, Scandinave) is *Clostridium botulinum* type E [6].

The manufactured fish-products are subjected to risk for *C.botulinum* type E and F and less for the B type [3]. The no proteolitic types are particularly dangerous because their growth in the foods cannot be warned by the appearance of abnormal odors and tastes, and it is sufficient a storage at non optimal fridge temperature (+4/7°C) to allow the growth of the micro-organism and toxin production [6]. It is evident that some risks derive from the foods blandly pasteurized, or that follow treatments of cold smoking, and often derive from manufactured foods in order to prolong the shelf-life, vacuum or in modified atmosphere packed [4]. To allow the development of the botulism some coincidences are necessary:

- the food is contaminated from spores or from vegetative cells;
- the manufacture of the product is inadequate to inactivate the spores of *C.botulinum* or the product is contaminated after the processing;
- the food has to allow the production of the toxin when it is preserved at temperatures >3.3°C, with Aw >0.95 and under anaerobic conditions.

The toxins of *Clostridium botulinum* are the most dangerous poisonings present in nature. For toxin A the lethal oral dose for the man is 0.1-1.0 μg. The toxins are very sensitive to heat and are completely inactivated by brief heating (85°C/5 min) [6].

The main features distinguishing the proteolitic types from no proteolitic types are described in Table I.

Table I. Differences between proteolitic and no proteolitic types of *C.botulinum*

	Proteolitic types	**No proteolic types**
pH minimum	4.5	4.5
Aw minimum	0.95	0.97
temperature minimum for the toxin production	10°C	3.3°C

Kramer J.& Cantoni C., Alimenti – Microbiologia e Igiene, Ed. OEMF spa, 1994.

The spores of *C.botulinum* are present in soil, in the muds of river, lakes and coastal waters, in the gills and in the intestinal content of shellfish and molluscs, in the intestinal content of fish and animals . Therefore it is a natural contaminant of the fish-products.

For prevention [5; 6] it is possible to achieve an effective action against *C.botulinum* by:

- in the case of storage of the foods at temperatures > +10°C (by treatment at 121°C for 3 minutes);
- in the case of storage of foods at temperatures < +10°C (by treatment at 90°C for 5 minutes);
- by acidification: pH <4.5;
- if only the parameter A_w is used: to decrease A_w values < 0.95;
- by addition of nitrites additives.

2.1.2.4 *Vibrio* sp. and *Aeromonas* sp.

Within the Vibrionaceae, from fish products, especially molluscs, they have been isolated: *V.cholerae* O1, *V.cholerae* biotype El-Tor, *V.parahaemolyticus*, *V.vulnificus*, *V.mimicus*, *V.hollisae*, *V.fluvialis* [3]. They are naturally present in the superficial waters, both sweet and brackish, and the gastroenteric content of animals. *Vibrio* spp. are gram negative germs, aerobs, anaerobs facultatives, salt-tollerants [6]. The symptoms of illness caused by *Vibrio* spp. are different according to the strain, and precisely: *V.cholerae, V.fluvialis, V.parahaemoyticus* cause a serious enteritis.

V.vulnificus causes septicaemic forms, also by direct contact with polluted waters, and by contaminated molluscs and other aquatic products. From an epidemiological point of view, pandemias of cholera, caused by *V.cholerae* biotype El Tor, were much serious. They occured in 1961 in Indonesia and in 1973 in Italy (Naples) [5]. In 1991 the pandemia interested countries of South America as Perù, Ecuador and Colombia, with 340000 declared cases and over 3600 dead people [5]. The incriminated foods were: raw or little cooked molluscs, marinated fish, cured fish, dried fish, inadequately cooked shellfish, squids and cuttlefish (consumed much time after the cooking), rice (cooked and cooled at room temperature), various vegetables left at room temperature, waters etc. The man is the traditional reservoir-diffuser of *V.cholerae* O1. The shellfish are important vehicles of *V.cholerae*, because the pathogens can adhere to the chitine of the carapace and use it as substratum.

Psicrotrophic bacteria (family Vibrionaceae), *Aeromonas* spp. have an aquatic habitat and are isolated by fish-products, meats etc. Pathogens for the man are: *A.hydrophila* and *A.sobria* [3].

2.1.2.5 *Listeria monocytogenes*

Gram+, facultative anaerobs, mobile, not spore-formers, these bacteria are psycrophilic, in fact they can grow at 2.5°C (up to 44°C, with optimum range 30-37°C). The optimum pH values for their growth are 5.0-9.0. *L.monocytogenes* can develop at A_w value 0.93 (about 10% of NaCl) [6]. They are not much resistant to thermal treatments, in fact the $D_{71°C}$ is about 1-4 seconds [6]. The distribution of the serotypes of *L.monocytogenes* isolated by samples of fish-products is assembled in serotypes 1, 2 and 3a, 4 [4; 5].

The incidence of *L.monocytogenes* in the fish-products is the following:

1) processed fish (smoked and/or marinated);
2) processed shellfish (fresh or frozen);
3) fish and fresh shellfish;
4) fresh bivalve molluscs.

2.1.2.6 Virus of Hepatitis A and Virus Norwalk

The virus of Hepatitis belongs to the group of Picornavirus (RNA-virus). It is inactivated at 100°C for a time over 5 minutes, while can survive at frozen storage [4]. The infection derives from direct contact or polluted (by faeces) water and foods (bivalves molluscs) [6]. It can cause a systemic infection, with enteritis and liver lesions. The period of incubation is about 25-30 days. The simptoms are fever, weakness, nausea, abdominal pain. The illness last from a few weeks up to many months. Vehicles of the virus are: oysters and other bivalve molluscs, eaten raw or blandly cooked. The single mean of prevention is the cooking of the contaminated foods.

The Virus Norwalk is probably a Parvovirus of enteric origin. It is acid-resistant and it survives at 60°C for 30 minutes [5]. The illness is characterized by a brief period of incubation (6-48 hours). The symptoms are: nausea, vomit, abdominal pains, diarrhea, fever, anorexia, cefalea, mialgias of duration 24-48 hours. The molluscs represent the main vehicle of the virus, but also contaminated waters [6].

2.1.2.7 Fish parasites [5]

Parasites larvae can be present in fish and fish products and represent a risk for consumers, because they can cause in man symptoms of enteritis, sometimes quite serious. Between the parasites more frequently detected in fish there are the following:

Nematodes (*Anisakis simplex*...) ;

Cestodes (*Diphyllobotrium latum*, *Diphyllobotrium pacificum*...);

Trematodes (*Opistorchis felineus*, *Clonorchis sinensis*, *Heterophyes heterophyes*...) [57; 58].

2.1.2.8 Sea Toxins

Biogenic amines poisoning

The fish of family Scombridae have in their tissues a high concentration of basic amino acids (derived from himidazole) like histidine. Only the families Scombridae, Clupeidae, Engraulidae, Salmonidae, have high quantity of free histidine (100-200 mg/100 g), important qualification for the histamine production [4; 5]. It is formed by bacteria able to operate the decarboxilation of histidine by the enzyme histidine decarboxilase. Other histamine can also be obtained by not bacterial proteolisis (autolysis). The bacteria responsible of decarbosilation are Gram- : *Morganella morganii*, *Proteus vulgaris*, *Enterobacter aerogenes*, *Hafnia alvei*, *Citrobacter freundii*, *Klebsiella pneumoniae*. They are mesophile bacteria, so the best system to check the histamine production is the storage at low temperatures. The poisoning is characterized from:

- short incubation period (20 min – 2 h);
- peculiar symptoms (allergic reaction, gastro-intestinal and cardio-vascular problems...)

The duration of symptoms is usually brief. In United States every year 31000 cases of histamine poisoning are enregistered. Other poisonings caused by sea toxins are: Paralytic Shellfish Poisoning (PSP), Neurotoxic Shellfish Poisoning (NSP), Diarrhetic Shellfish Poisoning (DSP), Venerupin Shellfish Poisoning (VSP), Amnesing Shellfish Poisoning (ASP), Ciguatera Poisoning.

2.1.3 *STAPHYLOCOCCUS AUREUS* AND *SALMONELLA*

2.1.3.1 Generalities

Staphylococcus aureus and *Salmonella sp*. (fig.1 and 2) are two ubiquitarious micro-organisms (respectively a Gram+ and a Gram- bacterium), both responsible of the most important foodborne diseases in Europe.

In Italy 60-70% of foodborne illness is caused by *S.aureus* and especially the manufactured fish-products are involved. Generally the contamination of the foods by *S.aureus* is secondary to scarce hygiene of the procedures.

In France *Salmonella sp*. represents the main cause of human foodborne enteritis, followed from *S.aureus*.

Fish and fish products can be contaminated with Salmonellas, growth in sewage-polluted waters but also after circumstances of poor kitchen hygiene and practice.

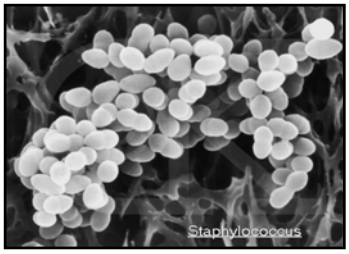


Fig.1 *S.aureus*

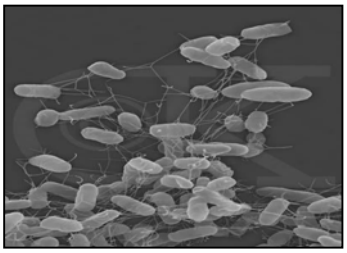


Fig.2 *Salmonella* sp.

2.1.3.2 *Staphylococcus aureus*

2.1.3.2.1 Introduction

The genus *Staphylococcus*, member of the family *Micrococcaceae*, consists of Gram+, catalase+ spherical bacteria ("cocci"), that occurs in microscopic clusters resembling grapes [7]. They grow in clusters because staphylococci divide in two planes. This configuration distinguish them from streptococci, which are slightly oblong cells that usually grow in chains, because they divide in only one plane [1]. The catalase test[1] is important in distinguishing streptococci (catalase-) from staphylococci, which are catalase producers (Table II). In 1884 ROSENBACH [1] described the two pigmented colony types of staphylococci and proposed the appropriate nomenclature: *S.aureus* (yellow) and *S.albus* (white) [7]. The latter species is now named *S.epidermidis* [1]. They usually have both an oxidative and a fermentative metabolism of glucose. There are more than 20 species (Table III), but only *S.aureus* and *S.epidermidis* have been implicated as causative agents of disease in man. *S. aureus* is a major cause of food poisoning in man as well as of a range of extraintestinal infections (toxic shock syndrome, pneumonia, meningitis, bacteraemia, boils, impetigo...) [1;2].

Table II. Differentiation of *S.aureus* from other genera

	Staphylococcus	*Micrococcus*	*Streptococccus*
Catalase	+	+	–
Fermantation of glucose	+	–	+
Cells arranged in irregular clusters	+	+	–
Sensitivity to			
Lysostaphin	+	–	/
Lysozime	–	+	/
Acid from glycerol in presence of erthyromicin	+	–	/

From "Foodborne pathogens - An illustrated text", Ed.Wolfe, England, 1991

[1] By addition of 3% hydrogen peroxide to a colony on an agar plate or slant. Catalase+ cultures produce O_2 and bubble at once.

Table III. Differentiation between the main foodborne species of *Staphylococcus* (Harvey and Gilmour,1985)

	Staphylococcus							
	aureus	*hysicus* subsp. *hysicus*	*hysicus* subsp. *chromogenes*	*simulans*	*intermedius*	*epidermidis*	*capitis*	*hominis*
Coagulase	+	–	–	–	+	–	–	–
Thermonuclease	+	+	+/–	–	+	–	–	–
Haemolysis	+	–	–	+/–	+	+/–	–	+/–
Acetoin	+	–	–	–	–	+	+/–	+/–
Pigment	+	–	+	+/–	–	–	–	+/–
Acid (aerobic)								
Sucrose	+	+	+	+	+	+	+	+
Trehalose	+	+/–	+	+/–	+	–	–	+
Mannitol	+	–	+/–	+	+	–	+	–
Cellobiose	–	–	–	–	–	–	–	–
Maltose	+	–	+/–	+/–	–	+	–	+
Mannose	+	+	+	+/–	+	+	+	–
Xylose	–	–	–	–	–	–	–	–
Phosphatase	+	+	+	+/–	+	+	+/–	–
Noviobicin	S	S	S	S	S	S	S	S

From "Foodborne pathogens - An illustrated text", Ed.Wolfe, England, 1991

Table III. (continued)

	Staphylococcus						
	warneri	*haemolitycus*	*cohnii*	*saprophyticus*	*xylosus*	*sciuri* subsp. *sciuri*	*sciuri* subsp. *lentus*
Coagulase	–	–	–	–	–	–	–
Thermonuclease	–	–	–	–	–	–	–
Haemolysis	+/–	+	+/–	–	–	–	–
Acetoin	+	+	+	+	+/–	–	–
Pigment	+	+/–	+/–	+/–	+/–	+	–
Acid (aerobic)							
Sucrose	+	+	–	+	+	+	+
Trehalose	+	+	+	+	+	+	+
Mannitol	+/–	+/–	+	+	+	+	+
Cellobiose	–	–	–	–	–	+	+
Maltose	+	+	+	+	+	+/–	+/–
Mannose	–	–	+/–	–	+	+/–	+
Xylose	–	–	–	–	+	–	+/–
Phosphatase	+/–	+/–	–	–	+	+	+/–
Noviobicin	S	S	R	R	R	R	R

From "Foodborne pathogens - An illustrated text", Ed.Wolfe, England, 1991
S = sensitivity R = resistance

2.1.3.2.2 Foods as vehicle of *S.aureus*

Staphylococcal food poisoning is one of the main foodborne infections.

In Italy 60-70% of foodborne illness are caused by *S.aureus* and especially the manufactured fish-products are involved [3]. Milk and dairy products are common sources of *S.aureus* [33]. Foods that can be contaminated from *S.aureus* are: raw meat and poultry, milk, heat processed foods, fermented foods, concentrated and dried products, bakery products [6].

2.1.3.2.3 Factors affecting enterotoxins production

The main factor affecting the growth is the temperature:

S.aureus growth temperature range: 7 - 47.8°C; optimum 37°C

Enterotoxin production temperature range: 10 – 46°C; optimum 40 – 45°C

These temperatures were determined using pure cultures cultivated in broths, and should be used only as a guide to the organism's behaviour in foods where other factors affect growth/temperature relationships. Although it is not possible to predict the relationship between the organism's growth and enterotoxin production in foods, generalised guidelines have been prepared [1].

1. In any given food, th optimum temperature for enterotoxin production is a few degrees higher than that for growth,
2. Temperature changes affect enterotoxin synthesis much more than growth.

Cells of *S.aureus* are destroyed at temperatures commonly used in food processing. There is considerable strain variation in resistance, but this is unlikely to have any consequence in processing. D_{60} values ("*D_{60} value" represents the time required to effect a 90% kill in the number of viable cells upon heating at 60°C*) range from 0.43 to 7.9 minutes, and *z* values (*"z value" is the necessary increase of temperature to reduce at 1/10 the value of D*) from 4.5°C to 10°C [1]. Heat-stressed cultures of *S.aureus* may lose the ability to synthesise enterotoxin as well as coagulase and thermonuclease. Enterotoxins are recognised as having a considerably greater level of heat resistance than *S.aureus* cells, and to survive cooking and most commercially applied heat-

treatment [6]. *S.aureus* has the ability to grow at a wide range of pH. This is about 4.0-9.8 [2]. However, the pH range for its growth and enterotoxin production is strongly modified by other factors, especially anaerobiosis. In aerobic condition growth and toxin production were possible at pH 4, whereas under anaerobic conditions, limiting values were pH 4.6 and 5.3 respectively [1]. About water activity level (A_w), the growth of *S.aureus* occurs in the range 0.83 - 0.99, while corresponding values for enterotoxin production are 0.86 to greater than 0.99 [1]. *S.aureus* is a facultative anaerobic bacteria, which can spoil meat, fresh raw eggs, chicken, ham salad, milk and milk products [4]. Also it is very salt-tolerant (100-200 g/l NaCl), and can grow fairly well in cured meats containing nitrite if other environmental conditions are favourable [2]. *S.aureus* is resistant to many preservatives used in foods, potassium sorbate (0,25%), for example, had no effect on growth of *S.aureus* in processed cheese [1].

2.1.3.2.4 Pathogenesis of *S.aureus* infections

Normally 50-60% healthy persons are carriers of potentially pathogen *Staphylococcus aureus* [3]. *S.aureus* is responsible of many different suppurative (pus-forming) infections and toxinoses in humans. It causes superficial skin lesions such as boils, styes and furuncles, more serious infections such as: pneumonia, mastitis, phlebitis, meningitis, urinary tract infections, ostemyelitis and endocarditis [2].

S.aureus is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices [1].

Human staphylococcal infections are frequent, but usually remain localized at the portal of entry by the normal host defenses. The portal may be a hair follicle, but usually it is a break in the skin which may be a minute needle-stick or a surgical wound. Foreign bodies, including sutures, are readily colonized by staphylococci, which may make infections difficult to control.

Another portal of entry is the respiratory tract. Staphylococcal pneumonia is a frequent complication of influenza [7]. The localized host response to staphylococcal infection is

inflammation, characterized by an elevated temperature at the site, swelling, the accumulation of pus, and necrosis of tissue. Around the inflamed area, a fibrin clot may form, walling off the bacteria and leukocytes as a characteristics pus-filled boil or abscess [7]. More serious infections of the skin may occur, such as furuncles or impetigo. Localized infection of the bone is called osteomyelitis.

Serious consequences of staphylococcal infections occur when the bacteria invade the blood stream. A resulting septicemia may be rapidly fatal. A bacteremia may result in seeding other internal abscesses, other skin lesions, or infections in the lung, kidney, heart, skeletal muscle or meninges.

S.aureus causes food poisoning by releasing enterotoxins into food, and toxin shock syndrome by release of pyrogenic exotoxins into blood stream [1].

S.aureus expresses many potential virulence factors:

- surface proteins that promote colonization of host tissues;
- invasins that promote bacterial spread in tissues (leukocidin, kinases, hyaluronidase);
- surface factors that inhibit phagocytic engulfment (Protein A);
- biochemical properties that permit their survival in phagocytes (carotenoids, catalase production);
- immunological disguises (Protein A, coagulase);
- membrane-damaging toxins that lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin);
- exotoxins that damage host tissues or otherwise provoke symptoms of disease (SEA-G, TSST, ET);
- inherent and acquired resistance to antimicrobial agents [1].

For the majority of diseases caused by *S.aureus*, pathogenesis is multifactorial, so it is difficult to determine precisely the role of any given factor [2].

Coagulase and, to a lesser extent, thermonucleases, produced from *S.aureus*, are used both as markers for enterotoxin production and as means of identification [7].

For many years, enterotoxin production was uniquely associated with *S.aureus*. More recently, enterotoxin production has been demonstrated by strains of *S.capitis*, *S.caprae*, *S.chromogenes*, *S.cohnii*, *S.epidermidis*, *S.haemolyticus*, *S.hysicus*, *S.intermedius*, *S.lentus*, *S.sciuri*, *S.warneri* and *S.xylosus* [1]. These species must be considered to be potential agents of food poisoning, although no cases have been reported [1].

Species of *Staphylococcus* are generally considered to be undesiderable in processed foods, but one: *S.carnosus*, which has no known pathogenicity, is used as a starter organism in fermented sausages [6].

S.aureus colonizes mainly the nasal passages, but it may be found regularly in most other anatomical locales. *S. aureus* is present in large numbers in boils, infected cuts and other skin lesions. The high rate of human carriage of *S. aureus* is an important feature of the organism with respect to its role as a foodborne pathogen. Staphylococcal intoxication is the major form of food poisoning in which food handlers play a significant role [1].

2.1.3.2.5 Symptoms of *S.aureus* intoxication

Symptoms usually appear within 4 hours of consumption of contaminated food, due only to the action of the pre-formed enterotoxins (SEs), which are a group of single-chain globular proteins, immunologically dinstinct (A, B, C_1, C_2, D, E, TST), heat-stable and water-soluble [1]. Reported symptoms include nausea, vomiting, retching, and less frequently diarrhoea [1;2;6]. Headache, dizziness and weakness are reported in a minority of cases and there have been rare, and unsubstantiated, complaints of double vision and other visual disturbances. Diarrhoea does not appear to occur in the absence of vomiting. Temperature is usually considered to be subnormal and,

indeed, the presence of subjective fever is times used to rule out the possibility of staphylococcal food poisoning.

Staphylococcal intoxication is self-limiting and symptoms usually persist for no more than 24 hours. In severe cases, dehydration leads to shock and collapse, accompanied by a weak pulse and shallow breathing. Death is rare and usually occurs only when the patient is elderly, very young, or suffering from a debilitating disease [1]. There is some evidence that both the pattern and severity of symptoms are affected by the age and physical conditions of the patient.

The symptoms of staphylococcal intoxication are readily confused with those of *Bacillus cereus* emetic syndrome. Particular care is needed to avoid confusion in cases where both organisms are isolated from the same sample of suspect food [6].

In addition to staphylococcal food poisoning, *S.aureus* has been implicated as a cause of pseudomembranous colitis in people who have received oral administration of broad-spectrum antibiotics [7]. In these situations, overgrowth by antibiotic resistant, enterotoxin producing strains of *S.aureus* may occur. Symptoms are abdominal cramps, severe diarrhoea, dehydration and electrolyte imbalance. These clinical manifestations, the isolation of antibiotic resistant *S.aureus* from stools in pure culture and necrosis of the intestinal tract, serve to differentiate this syndrome from *S.aureus* food poisoning [7].

2.1.3.2.6 Conventional cultural isolation methods

The methods, used for the search and the numeration of Staphylococci, differ for the type of selective substance utilized for the product in examination. The salty concentrations, the tellurite of sodium, the chloride of lithium, the Na-azide and the yolk of egg are at the base of many culture media for the growth and selection of the Staphylococci. In these substrate the micro-organisms, using the lipoproteins of the egg, form black colonies surrounded by a clear halo at 24 hours and inside a precipitate at 48 hours, due to the formation of salts of calcium or magnesium from the free fat acids, is obtained [1].

Among the culture media are included:

- liquid media (also used for the enrichment in the cases where the number of Staphylococci is very low): broth of Giolitti and Cantoni;
- solid media (of isolation): agar of Zebovitz or modified agar by the addition of egg's yolk (10%) or by the addition of mutton's blood (3%), agar of Baird-Parker (the selective activity of this medium is insured from the chloride of lithium and from the tellurite of potassium, which interfere with the enzymatic activities of many germs.

Techniques of isolation:

1) Direct procedure (suitable when the material is much contaminated, *S.aureus* >100 /g of product): the used medium is Baird-Parker agar (0.1 mL of material plated on agar surface of the Petri plate. Incubation at 30-37°C for 24-48 hours). The Staphylococci develop in the form of big black colonies (diameter of 2-2.5 mm or more) surrounded from a clear zone on the rest of the opaque agar [7]. Generally the Staphylococci coagulases negative do not produce the halo around the colony. The other germs do not develop at all, or if they develop, they show as small grey colonies (micrococci) or brown (protei). The colonies of suspicious *S.aureus* are collected in liquid broth (infused of heart and brain) for the following tests of identification [1].

2) Procedures for enrichment (especially in the cases of little contaminated material, Staphylococci <100/g of product and in the materials contaminated by aspecific germs):

the broth of Giolitti-Cantoni (1 mL plated onto surface) is used for the enrichment. After plating, some mL of white agar is added on plate surface. The incubation is at 37°C for 24-48 hours. The development of Staphylococci generally determines a blackening of the whole broth or only a blackish precipitate [2]. After the broth, positive for presence of *S.aureus*, is plated onto Baird-Parker agar (direct procedure), previously described.

The isolated suspicious colonies must be submitted to tests of identification [7] that consist in:

- investigations of the morphological characters,
- respiratory type,

- test of mannitole fermentation,
- fermentation of the carbohydrates,
- aceton production,
- search of the coagulase, phosphatase, haemolisine, staphyilococcal enterotoxins...

2.1.3.3 *Salmonella*

2.1.3.3.1 Introduction

The genus *Salmonella* is a typical member of the family *Enterobacteriaceae* and consists of facultatively anaerobic Gram-, oxidase-, straight-sided, rod-shaped bacteria, which are catalase+ and have both a respiratory and a fermentative metabolism of carbohydrates [1].

There are more than 2000 serotypes of Salmonellas that cause enteritis as: *S.typhimurium*, *S.enteritidis*, *S.panama*, and the Salmonellas of the group typhi-paratyphi as: *S.typhi* and *S.paratyphi* A, B and C [6].

Although members of this genus are motile by peritichous flagella, nonflagellated variants, such as *S.pullorum* and *S.gallinarum*, and nonmotile strains resulting from dysfunctional flagella do occur. The organisms grow optimally at 37°C and catabolize D-glucose and other carbohydrates, with the production of acid and gas. They generally produce hydrogen sulfide, decarboxylate lysine and ornithine, and do not hydrolyze urea [2]. Also they are lactose- bacteria.

Many of these traits have traditionally formed the basis for the presumptive biochemical identification of *Salmonella* isolates (Table IV).

Members of the genus are responsible for diseases of man and animals.

The degree of host adaptation varies and affects the pathogenicity for man in three ways.

1. Serovars adapted to man, such as *S. typhi*, *S. paratyphi* A and *S. sendai*, usually cause grave diseases with septicaemic-typhoidic syndrome (enteric fever). These serovars are not usually pathogenic to animals.

2. Ubiquitous serovars such as *S. typhimurium*, which affect both man and a range of animals, cause gastrointestinal infections of varying severity, but usually less severe than enteric fever.
3. Serovars which are highly adapted to an animal host, such as *S. abortis* (sheep) and *S. gallinarum* (poultry) usually produce no or very mild symptoms in man. However, *S. choleraesuis* which has the pig as primary host, also causes a severe systemic illness.

Salmonellas have been recognised as causes of enteric disease for many years. They belong to the most important reported causes of food poisoning and recent years have seen both massive outbreaks and a major new vehicle of infection, hens eggs, emerge.

S. typhi and other human-adapted salmonellas are less commonly transmitted by food than ubiquitous serovars with waterborne and person-to-person transmission being more important.

Table IV. Differentiation of *Salmonella* from other members of the *Enterobacteriaceae*.
From "FoodbornePathogens-An illustrated text",Ed.Wolfe,England,1991.

	Salmonella	*Shigella*	*Citrobacter*	*Edwardsiella*
β-galactosidase	-[1]	+/ -	+	-
Arginine dihydrolase	+/-	-	+/-	-
Lysine decarboxylase	+	-	-	+
Ornithine decarboxylase	+	-[2]	+/-	+
Simmon's citrate	+	-	+	-
H_2S production	+	-	-/+	+
Acid from				
Lactose	-[1]	-[3]	+/-	-
Dulcitol	+/-	-	-/+	-
Melibiose	+	-/+	-	-
Sorbitol	+	-/+	+	-
Xylose	+	-	+	-
Motilità	+	-	+	+

[1] *S. arizonae* and strains of some other serovars are positive.
[2] *Shigella sonnei* is usually positive.
[3] Delayed fermentation is a feature of some strains of *Shigella sonnei*.

2.1.3.3.2 Foods as vehicle of *Salmonella*

Fish and shellfish

Contamination of shellfish with Salmonellas due to growth in sewage-polluted waters has been a continuing problem in many parts of the world, and has led to a number of outbreaks of *Salmonella* food poisoning [6]. Fresh salmon has been an occasional cause of *Salmonella* food poisoning in the UK in recent years. In some outbreaks, the fish was probably contaminated from chicken under circumstances of poor kitchen hygiene and practice [6].

Meat and meat products

Raw meat and poultry is commonly contaminated with *Salmonella* [2;6]. Infections with Salmonellas are rarely acquired directly from raw meats. In most outbreaks, the meat is either undercooked or has been contaminated after cooking. Cooked meats may be contaminated from sources other than raw meats including food handlers and ingredients added after cooking. The continuing problems with cooked meats are illustrated by four major outbreaks which occurred during the summer of 1989 in the UK (Table V). In each case, the underlying cause was the neglet of basic principles of hygiene. Fermented meats such as salami are made from raw meat, and Salmonellas are known to survive the fermentation process. An outbreak involving a salami snack stick occurred in the UK during 1988. The causative serovar was *S. typhimurium* DT 124, and 71 cases were confirmed, of whom 55 were children under 16 years of age. Earlier outbreaks due to salami had occurred in Australia (*S. newport*) and in Italy (*S. cholerasuis*), but *Salmonella* is usually secondary to *Staphylococcus aureus* as a hazard in fermented meats [6].

Table V. *Salmonella* food poisoning and cooked meat products. UK, Summer 1989 (Communicable Disease Surveillance Centre, 1989).

Product	*Serovar*	*Number affected*
Various cold meats	*S. typhimurium* DT 12	538
Roast turkey	*S. kedougou*	61
Roast pork	*S. typhimurium* DT 193	206
Ham and other cold meats	*S. falkensee*	83

From "FoodbornePathogens-An illustrated text",Ed.Wolfe,England,1991.

Eggs and egg based-products

Raw eggs or eggs not well cooked and egg based-products not subjected to thermal treatments for the inactivation of microbial flora (cream prepared at cold, ice-creams, mayonnaise etc.) [6].

Milk and milk products

Raw milk can be contaminated by *Salmonella* and consumption of raw milk is a recognised risk factor for salmonellosis, as well as other enteric infections. The commercial distribution of raw milk in California led to continuing outbreaks caused by *S. dublin*, while in Scotland, where milkborne salmonellosis was particularly prevalent, the introduction of mandatory pasteurisation eliminated the problem. It is recognised that the mandatory pasteurisation dramatically reduces morbidity due to *Salmonella*. In recent years, there have been major outbreaks due to pasteurised milk. The largest of these occurred in 1985 in Chicago, USA, and involved 16284 known cases [1]. Milk products are a diverse group of commodities which undergo a number of different processes. In all cases, safety is dependent on heat processing the raw material and prevention of re-contamination.

Cheese

Salmonellas are destroyed or inactivated during the fermentation of high-acid products (lactic acid 1%, pH value less than 4.55) such as yoghurt and soft cheese, although the effect is less in cheese due to protection by casein. In contrast, growth of salmonellas may occur in the curd of low-acid cheese (pH value greater than 4.95). Contamination of cheese by *Salmonella* may occur during handling at any stage. An outbreak of salmonellosis, involving a Swiss cheese, due to *S. typhimurium*, was attributed to contamination of partly or fully ripened cheeses by piglets kept in a sty adjacent to the factory.

Chocolate

Chocolate (cocoa beans) are subject to contamination at source, and the heating during manufacture is insufficient to kill the organism. Two large international outbreaks of food poisoning involving *S. napoli* and *S. eastbourne* have occurred in which chocolate was the vehicle of infection. A notable feature of these outbreaks was the low infective dose of Salmonellas ingested in chocolate. Subsequently, a large outbreak occurred in Scandinavia in which *S. typhimurium* was implicated. Cocoa powder has also been implicated in *Salmonella* food poisoning [1].

Salads

Two major types of risks are presented by salads. The first involves contamination of salad vegetables either during growth or subsequent handling. The risk is greatest where nightsoil or animal manures are used as feriliser, or where polluted water is used for irrigation. Salad vegetables are not usually cooked and protection lies in thorough washing. The second risk involves the dressings applied to some types of salad. Mayonnaise is the main hazard, especially where raw eggs are used, or where the acetic acid content and acidity are too low. Mayonnaise from a salad factory caused approximately 10.000 cases in Denmark during 1975, while in 1976, outbreaks of salmonellosis among passengers on four charter flights from Las Palmas to Helsinki affected

several hundred people and caused six deaths. The vehicle of infection was mayonnaise contaminated with *S. typhimurium* [1].

2.1.3.3.3 Factors affecting the growth of *Salmonella* in foods

Temperature

The genus *Salmonella* consists of micro-organisms that readily adapt to extreme environmental conditions. Salmonellas actively grow within a wide temperature range (≤ 54°C), with an optimum of 37°C, and also exibit psychrotrophic properties, as reflected in the ability to grow in foods stored at 2 to 4°C [2]. Ability to grow at temperatures below 7°C is serovar and strain-dependent, as in the case of *S. bredeney*, *S. typhimurium* and *S. agona* that have lowest growth temperatures. *S. senftenberg* and *S. hadar* are also able to grow above 6°C. Growth rates at temperatures below 10°C are very low, but the ability to grow at relatively low temperatures may be significant where refrigeration is poor and shelf life prolonged. Salmonellas are destroyed in high a_w foods by heating at a minimum temperature of 74°C. D_{60} values for most serotypes are in the range 0.2-6.5 minutes.

S. senftenberg is considerably more resistant than other serotypes, but this is probably of limited consequence since the organism appears to be low pathogenicity to man. There is interaction between thermal resistance and pH value, and several serovars of *Salmonella* are unusual in having a greater heat resistance at pH 5.5 than pH 8.5. The heat resistance also increases markedly at low a_w levels, particularly in foods such as chocolate, which also have a high fat content.

In a study with milk chocolate the D_{70} value for a strain of *S. senftenberg* was 6 to 8 hours, and for a strain of *S. typhimurium* 12 to 18 hours. There is also evidence that successive sub-lethal heat treatments increases the resistance of *S. typhimurium* [2].

pH value

Salmonella spp. has ability to proliferate at pH values 4.5 to 9.5, with an optimum pH for growth of 6.5 to 7.5 [2]. At pH values below 4.1, inactivation and death will occur.

Water activity

Studies have shown that foods with $A_w \leq 0.93$ do not support the growth of Salmonellas [2;6].

Curing ingredients

When other growth conditions are optimal, Salmonellas are able to grow in about 4% NaCl depending on serovar and strain [1].

Survival

Although the potential growth of foodborne Salmonellas is of primary importance in safety assessments, the propensity for these pathogens to persist in hostile environments further heightens public health concerns. The survival of Salmonellas for prolonged periods of time in foods stored at freezer and ambient temperatures is well documented. Heat is widely used in food manufacturing processes to control the bacterial quality and safety of end products. Conditions that potentiate the greater heat resistance of Salmonellas and other foodborne pathogens in food ingredients and finished products have been studied extensively [1;2;4;6]. Although the heat resistance of *Salmonella* spp. increases as the menstruum A_w decreases, some studies have demonstrated that the solutes used to alter the A_w of the heating menstruum play a determinant role in the level of acquired heat resistance. For example, the heating of *S. typhimurium* in menstrua adjusted to a_w 0.90 with sucrose and glycerol conferred different levels of heat resistance as evidenced by $D_{57.2}$ values of 40 to 55 and 1.8 to 8.3 min, respectively.

Other important features associated with this adaptive response include the greater heat resistance of salmonellas grown in nutritionally rich media than of Salmonellas grown in minimal media, of cells derived from stationary- rather than logarithmic-phase cultures, and of Salmonellas previously stored in a dry environment. The ability of Salmonellas to acquire greater heat resistance following exposure to sublethal temperatures is equally notable. The phenomenon stems from a rapid adaptation of the organism to rising temperatures in the environment to a level of enhanced

thermotolerance quite distinct from that described in conventional time-temperatures curves of thermal lethality. This adaptive response has potentially serious implications with respect to the safety of thermal processes that expose or maintain food products at marginally lethal temperatures. Exposure of Salmonellas to sublethal temperatures (≤ 54°C) for 15 to 30 min enhances their heat resistance through a rapid chloramphenicol-sensitive synthesis of heat shock proteins. Changes in the fatty acid composition of cell membranes in heat-stressed Salmonellas to provide a greater proportion of membrane saturated phospholipids reduce the fluidity of the bacterial cell membrane, with an attendant increase in membrane resistance to heat damage [2].

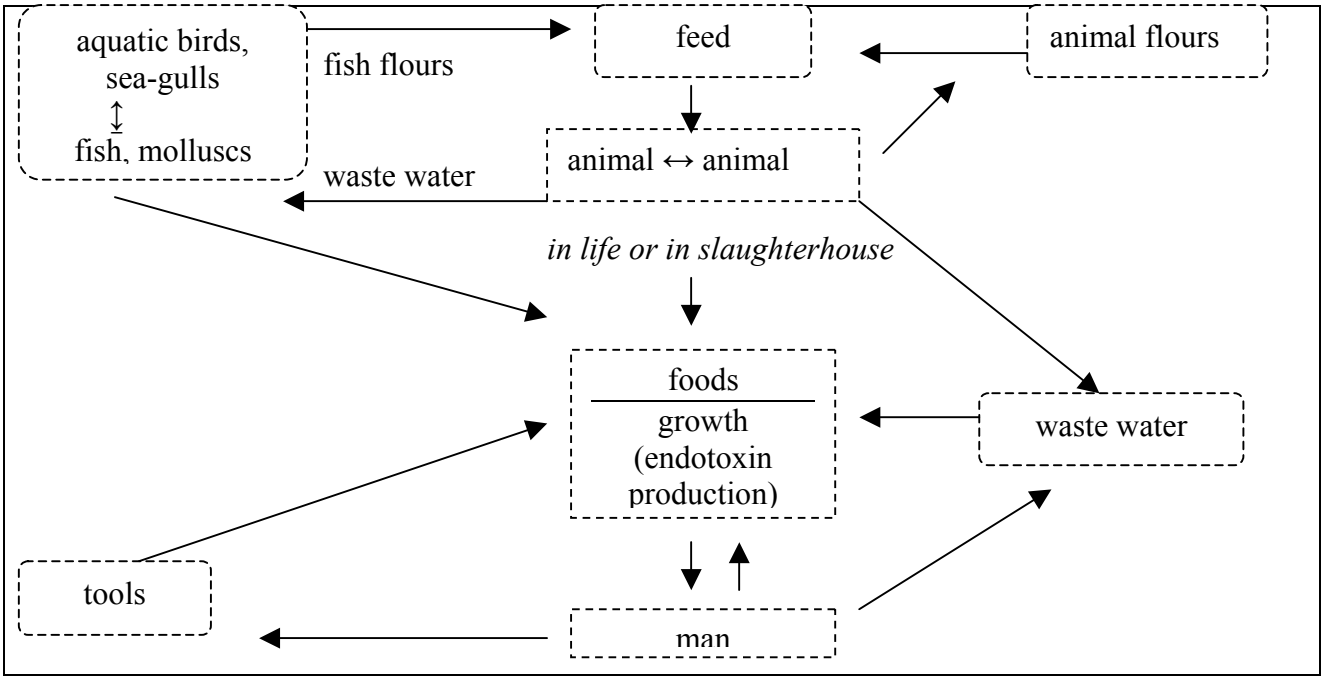
2.1.3.3.4 Ways of contamination and reservoirs of *Salmonella*

Also the healthy animals, especially poultry, pigs and calves, as well as game, is often carrier of enteric Salmonellas, so the sources of contamination for the man are primarily the foods of animal origin (Fig.3). Important source of infection is the feeds obtained by animal flours prepared with contaminated raw materials. Within this group the fish flours have an important role. Since the Salmonellas are eliminated with faeces, therefore their diffusion among the animals is favored by the breedings in mass. Starting from these contaminated animals the Salmonellas can spread during or after the slaughter in other carcasses. Poultry meat is very often contaminated by Salmonellas. The contamination chain can already start from the very young chicks, since some Salmonellas (*S.pullorum-gallinarum*, *S.typhimurium* and *S.enteritidis*) infect the ovaries of the hens and therefore the yolks can be contaminated. During the breeding further forms of contamination occur through contaminated foods, faeces, rodents... During the slaughter of the chickens many critical points exist, from the hygienic point of view, in correspondence of which, especially after the loss of faeces, it can happen cross contaminations: the boiling (80°C), the pluck, the evisceration, the final washing and the cooling. Because of the elevated presence of Salmonellas, the production of minced meat is not admitted by poultry. Also the aquatic birds (ducks) and the hens ovaioles are frequent carriers of Salmonellas, by transovarian way (vertical way) or after the deposition through

the soiling with faeces of the eggs shell (predisposing factors to the penetration of the Salmonellas are the moisture and the bad quality of the shell). In the last years it was observed an increase of the foodborne infection caused by egg-based and the most frequent serovar isolated during these episodes was *S.enteritidis* fagotype 4.

The fish, the molluscs, the mussels and the oysters, consumed raw, can be contaminated in coastal waters from the sewerages. Particularly contaminated are the molluscs, since they filter big quantities of water. A contamination of the fish, after the fishing, is also possible with faeces of sea-gulls, which are carriers of Salmonellas. The direct transmission of Salmonellas from a man to the other one is highly unlikely due to the elevated infectious dose. The man is therefore only the final ring of the infectious chain. Beginning from an infected man (sick, asymptomatic carrier) it is possible that, with dirty hands from faeces, objects and foods are contaminated.

Fig.3 Flow diagram "Ways of foods contamination by *Salmonella*".



From "Alimenti-Microbiologia e Igiene", Ed.OEMF spa,1994

The ubiquity of Salmonellas in the natural environment, coupled with the intensive husbandry practices used in the meat, fish and shellfish industries and the recycling of offal and inedible raw materials into animal feeds, has favored the continued growth of this human bacterial pathogen in

the global food chain [2;4]. Of the many sectors within the meat industry, poultry products remain the principal reservoirs of Salmonellas in many countries. The housing of birds on premises whose construction may no effectively block entry of *Salmonella* carriers, including rodents, insects and avian species, exacerbates the magnitude of the problem. Moreover, the feeding habits and close proximity of multiplier breeder, layer and broiler birds in rearing facilities encourage the rapid and widespread dissemination of vertically and horizontally introduced Salmonellas in poultry houses [2]. The continuing pandemic of human *S. enteritidis* phage type 4 (Europe) and 8 (North America) infections associated with the consumption of raw or lightly cooked shell eggs and egg-based products further bears witness to the importance of poultry products as vehicles of human Salmonellosis and to the need for sustained and stringent bacteriological control of poultry husbandry practices [2]. The problem arises from the transovarian transmission of the infective agent to the yolk before the shell deposition. The viability of these internalized *S. enteritidis* organisms thus remains unaffected by the egg surface-sanitizating practices currently applied in egg-grading stations [2]. The significant public health and societal costs associated with egg-borne outbreaks of *S. enteritidis* together with the economical losses sustained by the agricultural sectors as a result of depopulation of infected layer flocks and mandatory pasteurization of shell eggs from infected commercial layer flocks will undoubtedly result in dreadful losses.

The persistence of Salmonellas in the porcine, bovine and ovine meat industries originates from the exposure of livestock to environmental sources of contamination and contaminated feeds and from parental transmission of infection. It is abundantly clear that future improvement in the *Salmonella* status of meat animals hinges on coordinated and sustained efforts by all sectors of the meat industry to implement stringent control measures not only at the farm level but also within the processing, distribution and retailing sectors of the industry. Rapid depletion of feral stocks of fish and shellfish in recent years has greatly increased the importance of the international aquaculture industry as an alternative source for these popular food items. The high-density farming conditions required to maximize biological yields and to satisfy growing market demands open gateways to the

widespread infection of species reared in earthen ponds and other unprotected facilities that are continuously exposed to environmental contamination. Aquaculture farmers are relying heavily on antibiotics applied at subtherapeutic levels to safeguard the vigor of farmed fish and shellfish. A serious public health concern arises from the use of antimicrobial agents such as ampicillin, chloramphenicol, sulfa drugs, and quinolones, which currently provide the mainstay for the clinical management of systemic salmonellosis in humans. This antibiotic-dependent husbandry practice is shortsighted and irresponsible upon consideration that the misuse of medically important therapeutic agents in the livestock industry continues to select for antibiotic-resistant Salmonellas. The health consequences of consuming contaminated aquacultural fish in a sushi dish or lightly cooked fish or shellfish that contain viable Salmonellas are predictable.

Fruits and vegetables have gained notoriety in recent years as vehicles of human salmonellosis. The situation has developed from the increased global export of fresh and dehydrated fruits and vegetables from countries that enjoyn tropical and subtropical climates. The prevailing hygienic conditions during the production, harvesting and distribution of products in these countries do not always meet minimum standards, and they facilitate product contamination. More specifically, the fertilization of crops with untreated sludge or sewage effluents potentially contaminated with antibiotic-resistant *Salmonella* sp., the irrigation of garden plots and fields and the washing of fruits and vegetables with contaminated waters, the repeated handling of product by local workers, and the propensity for environmental contamination of spices and other condiments during drying in unprotected facilities represent weak links that undermine product safety. Operational changes in favor of field irrigation with treated effluents, washing of fruits and vegetables with disinfected waters, education of local workers on the hygienic handling of fresh produce, and greater protection of products from environmental contamination during all phases of production and marketing would markedly enhance the bacterial quality and safety of these frequently ready-to-eat products.

Major outbreaks of foodborne salmonellosis in the last few decades are of interest because they underline the multiplicity of foods and *Salmonella* serovars that have been implicated in human

disease. In 1974, temperature abuse of egg containing potato salad served at an outdoor barbecue led to an estimated 3400 human cases of *S. newport* infection; cross-contamination of the salad by an infected food handler was suspected. The large Swedish outbreak of *S. enteritidis* PT4 in 1977 was attributed to the consumption of a mayonnaise dressing in a school cafeteria. In 1984, Canada experienced its largest outbreak of foodborne salmonellosis, which was attributed to the consumption of Cheddar cheese manufactured from heat-treated and pasteurized milk. The episode resulted in no fever than 2700 confirmed cases of *S. typhimurium* PT10 infection [2]. Manual override of the low diversion valve reportedly led to the entry of raw milk into vats of thermized and pasteurized cheese-milk. The following year witnessed the largest outbreak of foodborne salmonellosis in the United States, involving 16284 confirmed cases of illness. Although the cause of this outbreak was never ascertained, cross-connection between raw and pasteurized milk was at fault. A large outbreak of salmonellosis affecting more than 10000 Japanese consumers was attributed to a cooked egg dish. In 1993, paprika imported from South America was incriminated ingredient in the manufacture of potato chips distributed in Germany. The latest major outbreak of foodborne salmonellosis occurred in the United States and involved ice cream contaminated with *S. enteritidis*. The transportation of ice cream mix in an unsanitized truck that had previously carried raw eggs was identified as the source of contamination [2].

2.1.3.3.5 Pathogenesis of *Salmonella* infections

Infective dose

It appears evident that the elderly, the newborns, infants and immunocompromised individuals are more susceptible to *Salmonella* infections than healthy adults [7]. The incompletely developed immune system in newborns and infants, the frequently weak and/or delayed immunological responses in the elderly and debilitated persons, and the generally low gastric acid production in infants and seniors facilitate the intestinal colonization and systemic spread of Salmonellas in these individuals [7]. Some investigations of foodborne outbreaks (Table VII) showed that the ingestion

of just a few *Salmonella* cells can be infectious [2]. From early reports that large numbers of Salmonellas inoculated into eggnog and fed to human volunteers produced overt disease, more recent evidence suggests that 1 to 10 cells can constitute a human infectious dose [1]. A common denominator of the food associated with low infectious doses (Table VI) is the high fat content in chocolate (cocoa butter), cheese (milk fat), and meat (animal fat). Entrapment of Salmonellas within hydrophobic lipid micelles may possibly afford protection against the bactericidal action of gastric acidity [2]. In the case of *S. typhi* infection at levels of less than 10^3 organisms ingested has occurred in a high proportion of outbreaks, confirming the generally held view of the organism's infectivity. Studies concerning non-human adapted serovars have consistently indicated that a dose of 10^5 to 10^7 organisms is required to cause infection. It has been suggested that the infective dose is lower in foods of high fat or protein content, due to protection of cells from gastric acidity [1].

Toxin production

For Salmonellas the invasive ability is essential for the development of diarrhoea and other enteric symptoms, since only invasive strains cause enteritis and fluid secretion [1].

However, invasion cannot be singly responsible for the observed symptoms, since some enteroinvasive strains do not cause fluid secretion.

Another factor, or factors, must therefore be involved and it has now been established that in addition to an endotoxin, *Salmonella* produces at least three enterotoxins and a cytotoxin [1;7].

Endotoxin

The toxicity of *Salmonella* endotoxin is derived from the lipid portion of the lipopolysaccharide molecule, lipid A, as for example in the case of "enteric fever", the endotoxin is released from the bacterial cell wall [1;2;7].

Enterotoxins

The first of three known enterotoxins was originally demonstred in *S. typhimurium* and *S. enteritidis* by Copal and Deibel (1975) [1]. This toxin, which produces fluid loss in infant mice, is a protein associated with the cell wall or outer cell membrane, but which is distinct from the endotoxin [1;7].

The other two enterotoxins are both detected by an increase in vascular permeability of rabbit skins. The first is heat stable and rapid in action, producing a response in not more than 1 hour after injection.

The second is heat labile and has a delayed response of about 18 hours [7]. This toxin further resembles cholera toxin in its mode of action and interferes with the control of adenyl cyclase activity, thus causing over-production of cyclic adenosine monophosphate (cAMP) [1].

Cytotoxin

Salmonellas produce at least one cytotoxin. This acts by inhibition of protein synthesis and may be at least partly responsible for damage to the intestinal mucosa [1].

Table VI. Human infectious doses of *Salmonella* sp.

Food	Serovar	Infectious dose*
Eggnog	*S.meleagridis*	10^6-10^7
	S.anatum	10^5-10^7
Goat cheese	*S.zanzibar*	10^5-10^{11}
Carmine dye	*S.cubana*	10^4
Imitation ice cream	*S.typhimurium*	10^4
Chocolate	*S.eastbourne*	10^2
Hamburger	*S.newport*	10^1-10^2
Cheddar cheese	*S.heidelberg*	10^2
Chocolate	*S.napoli*	10^1-10^2
Cheddar cheese	*S.typhimurium*	10^0-10^1
Chocolate	*S.typhimurium*	$\leq 10^1$
Paprika potato chips	*S.saintpaul*	$\leq 4.5 \times 10^1$
	S.javiana	
	S.rubislaw	

*cfu/ml

(from "Food Microbiology – Fundamentals and Frontiers",ASM Press, Washington D.C.,1997)

Table VII. Major foodborne outbreaks of human salmonellosis

Countries	Vehicle	Serovar	Cases	Deaths
Canada, US	Chocolate	*S. eastbourne*	217	0
Trinidad	Milk powder	*S. derby*	3000	NS[1]
US	Potato salad	*S. newport*	3400	0
Spain	Egg salad	*S. typhimurium*	702	6
Australia	Raw milk	*S. typhimurium* PT9	>500	NS
Sweden	Mustard dressing	*S. enteritidis* PT4	2865	0
The Netherlands	Salad	*S. indiana*	600	0
Scotland	Raw milk	*S. typhimurium* PT204	654	2
Canada	Cheddar cheese	*S. typhimurium* PT10	2700	0
France, England	Liver pâté	*S. goldcoast*	756	0
US	Pasteurized milk	*S. typhimurium*	16284	7
China	Egg drink	*S. typhimurium*	1113	NS
Norway	Chocolate	*S. typhimurium*	361	0
Japan	Cuttlefish	*S. champaign*	330	0
Japan	Cooked eggs	*Salmonella* spp.	10476	NS
Germany	Fruit soup	*S. enteritidis*	600	NS
France	Mayonnaise	*S.enteritidis*	751	0
Germany	Paprika chips	*S. saintpaul* *S. javiana* *S. rubislaw*	>670	0
US	Ice cream	*S. enteritidis*	>645	0
Finland, Sweden	Sprouts	*S. bovismorbificans*	492	0

[1] NS = not specified

From "Food Microbiology-Fundamentals and Frontiers". Ed.Doyle M.P.,ASM Press,Washington DC,1997.

2.1.3.3.6 Symptoms of *Salmonella* infections

Human *Salmonella* infections can lead to several clinical conditions, including enteric (typhoid) fever, uncomplicated enterocolitis, and systemic infections by non-typhoid micro-organisms [2].

Enteric fever

Enteric fever is a serious human disease associated with the typhoid and para-typhoid strains which are particularly well adapted for invasion and survival within host tissues [2]. Symptoms of

enteric fever due to *S. typhi* usually appear within 14 days of infection and include anorexia, headache, a non-productive cough and abdominal pain. These symptoms are accompanied by an increasing remitted fever which reaches as high as 40°C. In untreated patients, the fever declines during the third week, although a mortality of 10% may be expected. The enteric fever due to other human-adapted serovars such as *S. paratyphi* A is less severe [1;2].

Salmonella enteritis

Human infections with nontyphoid Salmonellas commonly result in enteritis. The symptoms of *Salmonella* enteritis (salmonellosis) usually develop within 36 hours of infection. An extremely short incubation period of 3 hours was noted in an outbreak of *S. enteritidis PT 4* infection while at the other extreme, periods of up to 72 hours are not uncommom. Extended periods of 1 to 12 days have been reported for *S. newport*, 1 to 8 days for *S. heidelberg* and 3 to 7 days for *S. typhimurium* [1]. Typical symptoms include abdominal pain, watery diarrhoea, vomiting and fever (39-40°C) [1;2;6]. The severity of the symptoms can vary considerably, particularly in newborn babies and infants, from a grave typhoid-like illness to transitory diarrhoea or asymptomatic infection [6]. Unusual prodromal symptoms have occasionally been reported. In an outbreak caused by *S. enteritidis* PT 4, diarrhoea was preceded in the previous 24 to 48 hours by severe headache, myalgia, fever, neck stiffness and photophobia [1].

Extraintestinal infection

The major symptoms of infection with *S. typhi* and other human-adapted serovars are largely extraintestinal (Table VIII). In addition to typhoid-like and septicaemic illnesses, these include other acute and chronic diseases. Some serovars of non-human adapted salmonellas have greater potential for invasiveness, serovars identified in this way are S. *choleraesuis*, *S. dublin*, *S. virchow*, *S. panama* and *S. london* [1].

Table VIII. Summary of extraintestinal infection due to *Salmonella*

Septicaemia	
Haemolytic uraemic syndrome	
Erythema nodosum	
Focal infections	Meningitis
	Osteomyelitis
	Septic arthritis
	Pneumonia
	Cholecystitis
	Peritonitis
	Pyelonephritis
	Cystisis
	Abscesses in various parts of the body
	Endocarditis
	Pericarditis
	Vasculitis

From "Foodborne pathogens-An illustrated text".Ed.Wolfe,England,1991.

2.1.3.3.7 Conventional cultural isolation methods

In foods and other non-clinical materials, Salmonellas are usually present only in small numbers and are greatly outnumbered by other bacteria. Some cells may be stressed by processing or environmental conditions. It is therefore customary to use a three-stage process-pre-enrichment, enrichment, selective plating [1;6]. These methods involve pre-enrichment, selective enrichment and selective plating followed by biochemical and/or serological confirmation of the identity of suspect colonies [1].

Pre-enrichment

A large number of media have been proposed for the resuscitation of Salmonellas, with considerable differences in performance. Lactose broth and buffered peptone water are most widely used, the latter being recommended for routine purposes [2]. Although non-selective enrichment broths are suitable for most foods, problems may occur where large number of Gram+ bacteria such as *Streptococcus lactis* are present. This problem may be overcome by the addition low levels of dyes such as brilliant green (0.002% w/v) or crystal violet (0.0004 % w/v) to the resuscitation medium. More recently, van Schothorst and Renaud (1985) proposed the addition of 0.01%

malachite green to buffered peptone water for recovery of Salmonellas from milk powder, and this is recommended in all situations where the sample material is heavily contaminated with bacteria other than members of the *Enterobacteriaceae*. Although incubation at 43°C has been used occasionally, 37°C is recommended for resuscitation of Salmonellas [1].

Selective enrichment

Many types of inhibitors have been proposed for the selective enrichment of Salmonellas, the most widely used of which are bile, tetrathionate, selenite and dyes including brilliant green and malachite green. These inhibitors have been incorporated, either singly or in combination, into an even wider range of media, the most widely used of which are summarised in Table IX.

A number of studies have been made comparing Rappapport-Vassiliadis broth (RV) with the two most widely used traditional media tetrathionate broth and selenite F broth [1]. Predictably, findings have been variable, but most have found RV to be superior for isolation of Salmonellas from human and animal food. RV broth is known to be over selective with respect to *S. typhi* and *S. dublin* [2]. Traditionally, enrichment broths have been incubated at 35 to 37°C, with loopfuls removed for streaking at 18 to 24 hours and 42 to 48 hours. A temperature of 41°C is required when using RV broth to obtain the necessary selectivity, and this temperature has also become widely used selenite F and tetrathionate broths. Incubation at 41°C should not be used with selenite cystine broth. It is accepted that, where applicable, incubation at the higher temperature increases the general efficiency of enrichment for Salmonellas. However, *S. typhi* is not recovered by enrichment at this temperature, and strains of other serovars may also be unable to grow [1].

Table IX. Examples of commonly used media for the selective enrichment of Salmonellas.

Medium	*Inhibitors*	*Applications and limitations*
Tetrathionate broth (Muller-Kauffman)	Tetrathionate, brilliant green, ox-bile	Not suitable for host-adapted serovars
Selenite F broth	Selenite	Suitable for most serovars including *S.typhi*, *S.dublin* and *S. choleraesuis*
Selenite-cystine broth	Selenite	Cystine enhances *Salmonella* growth. Widely used for foods
Brilliant green-MacConkey broth	Brilliant green, bile salts	Very effective with *S. choleraesuis* but not widely used
RV broth	Malachite green, magnesium Chloride, "low" pH value	Medium of choice for foods. May fail to recover *S. typhi* and *S. dublin*.

From "Foodborne pathogens-An illustrated text".Ed.Wolfe,England,1991.

Selective plating media

Selective plating media for *Salmonella* all contain a diagnostic system to permit differentiation of the organism from non-salmonellas. This is commonly based on the inability of most Salmonellas to ferment lactose and, in some cases, other carbohydrates such as sucrose and salicin. Bile containing media often employ a second diagnostic system based on the ability of *Salmonella* to produce hydrogen sulphide. Commonly used selective plating media for *Salmonella* are summarised in Table X. Incubation of selective plating media should be at 35 to 37°C. It is recommended that incubation should be for 24 hours since shorter periods may give false-negative results, particularly where strongly selective media are used. When necessary, incubation may be extended to 48 hours, but there is no value in extending the period of time further.

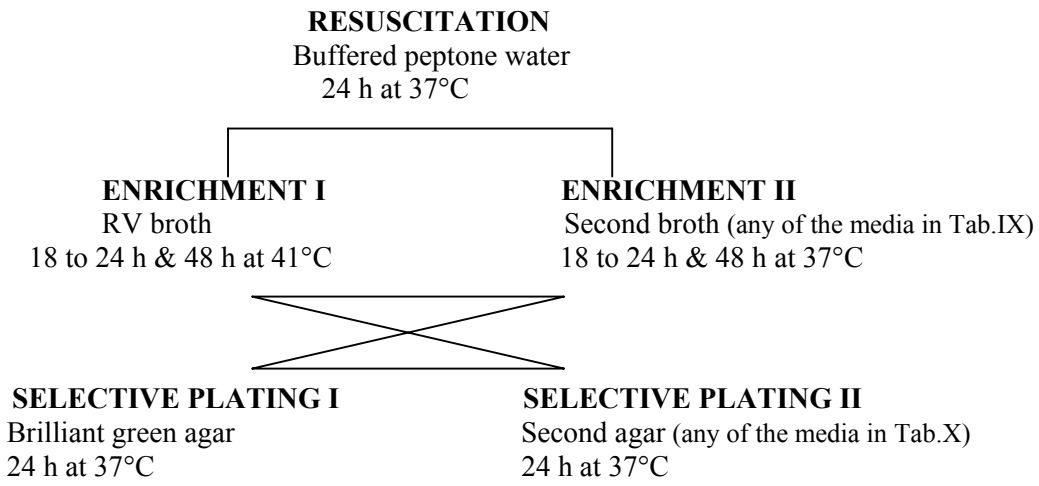
A flow diagram about a sample protocol for the recovery of Salmonellas by conventional methods is reported in Fig.4.

Table X. Examples of commonly used media for the selective plating of Salmonellas.

Brilliant green agar	
Inhibitors	Brilliant green
Diagnostic system	Fermentation of lactose and sucrose
Application and limitations	Widely used in food industry. Not suitable for *S.typhi*
Brilliant green-sulphamandelate agar	
Inhibitors	Brilliant green, Na sulphacetamide, Na mandelate
Diagnostic system	Fermentation of lactose and sucrose
Application and limitations	Effective where *Proteus* and *Pseudomonas* are present in large numbers
Deoxycholate-citrate agar	
Inhibitors	Deoxycholate
Diagnostic system	Fementation of lactose, H_2S production
Application and limitations	Relatively low selectivity
DCLS agar	
Inhibitors	Deoxycholate
Diagnostic system	Fermentation of lactose and sucrose, H_2S production
Application and limitations	Improved differentiation of *Proteus*
Salmonella-Shigella agar	
Inhibitors	Brilliant green, bile salts
Diagnostic system	Fementation of lactose, H_2S production
Application and limitations	Effective with many foods
Xylose-lysine-deoxycholate agar	
Inhibitors	Deoxycholate
Diagnostic system	Fermentation of lactose, xylose and sucrose. Decarboxylation of lysine.H_2S production
Application and limitations	Relatively low selectivity
Hektoen enteric agar	
Inhibitors	Bile salts
Diagnostic system	Lactose, salicin and sucrose fermentation. H_2S production
Application and limitations	Good differentation, relatively low selectivity
Bismuth sulphite agar	
Inhibitors	Bismuth sulphite, Na sulphite, brilliant green
Diagnostic system	Reduction of sulphite to sulphide in the presence of fermentable carbohydrate
Application and limitations	Often recommended for *S.typhi* effective with lactose-positive Salmonellas.
Rambach agar	More used medium with high selectivity.

From "Foodborne pathogens-An illustrated text".Ed.Wolfe,England,1991.

Fig.4 Flow diagram "Sample protocol for the recovery of Salmonellas by conventional methods".
(From "Foodborne pathogens-An illustrated text".Ed.Wolfe,England,1991)



2.1.3.3.8 *Salmonella enteritidis*

S. enteritidis is a specie of the genus *Salmonella*, which inhabits the intestinal tracts of a wide range of animals and can be isolated from fresh water and estuarine water contaminated with faeces. *S. enteritidis* is the cause (as other species of *Salmonella*) of the food poisoning "salmonellosis".

Fish, shellfish, fish-products, raw meat, poultry and eggs, are commonly contaminated with *Salmonella enteritidis*. In most outbreaks, the meat is undercooked or has been contaminated after cooking (food handlers, ingredients added after cooking) or contaminated by polluted waters [1; 3].

The situation has endured because of the ubiquity of Salmonellas in the natural environment and their prevalence in many sectors of the global food chain. Intense animal husbandry practices and major difficulties in controlling the spread of Salmonellas in the vertically integrated meat and poultry production and processing industries continue to render raw poultry and meats principle vehicles of human foodborne salmonellosis. The widespread administration of prophylactic doses of medically important antibiotics to reared animal species may promote on-farm selection of antibiotic-resistant strain and markedly increase the human health risks associated with handling and consumption of contaminated meat products. A similar scenario can also be drawn for the expanding aquacultural industry, where intensive rearing of fish and shelfish in generally

unprotected facilities, together with a liberal use of subtherapeutic regimens of antibiotic treatment, is common practice. The importance of *Salmonella* vehicles other than raw poultry, meats, and derived products cannot be minimized.

The incidence of human salmonellosis associated with the consumption of fresh fruits and vegetables, spices and chocolate, and milk products highlights the importance of sanitary practices during the harvesting, processing, and distribution of raw foods and food ingredients.

The arsenal of virulence factors that enable *Salmonella* species to evade the various antibacterial host defence mechanisms is remarkable and disconcerting. The physiological adaptability of Salmonellas to hostile conditions in the natural environment safeguards their survival and infectious potential.

It is clear that the occurence of Salmonellas in the global food chain and its current and projected repercussions on human health are cause for concern. Unless significant changes in agricultural and aquacultural practices are implemented, human foodborne salmonellosis will prevail in the next century [2].

2.2 High hydrostatic pressure

2.2.1 Introduction

The use of High Pressure technology and combined treatments, as pressure-temperature, constitutes a valid tool for food industry in order to preserve the foods and extend their shelf-life without changes in the nutritive value and their sensory characteristics. In recent years the growing demand for safe, fresh-like, nutritive and innovative food products, is stimulating the research into alternative technologies, one of which is high hydrostatic pressure.

Production of pressurized foods has become a reality in some countries such as: in Japan the fruit jams, sauces, fruit juices and rice cakes; in USA the avocado sauce; in France the orange juice; in Spain and Italy the fruit juices and the cooked hams.

2.2.2 The Parameter Pressure

The Pressure is defined as the ratio between a force applied on a surface and the surface unit:

$\boldsymbol{P = F / S}$ where:

$\boldsymbol{P}$ = pressure; $\boldsymbol{F}$ = force; $\boldsymbol{S}$ = surface.

The MegaPascal (MPa) is the pressure unit more used in food industry and it is a multiple of the Pascal (Pa). The equivalences with other pressure units are the following:

1 MPa = 10^6 Pa = 0.101 atm = 0.1 bar = 0.098 kg/cm^2 = 0.00689 PSI (pounds/inch^2)

2.2.3 The equipments of High Hydrostatic Pressure

2.2.3.1 Introduction

The first equipments able to reach isostatic pressures over 100 MPa have been built at the end of the nineteenth century for experimental goals. They were constituted by two essential elements present again in the currently used systems: a cylinder able to maintain the pressure in a constant volume and a pump able to produce such High Pressure. The mechanical characteristics of resistance of the materials were less good than those existing today. It happened therefore that parts of the cylinder could have fissures or that some part of the pump broke despite the safety coefficient taken in the realization of these equipments. B.HITE, the pioneer of the treatments of the foods under High Pressures, gave an example in his publication of 1899 [61]. During the experiments, having the aim the improvement of the microbial stability of milk by pressure, one of the cylinders were broken under the effect of a pressure of 900 MPa. It contained some milk contaminated by *Salmonella typhi*. A member of the research team was contaminated from the spray of milk under pressure and developed a violent typhus, that caused the interruption of the experiments on the pathogens from these researchers. The first studies on High Pressure applied in food field did not receive a great deal of attention and for this reason they did not develop. The evolution of the procedure was above all limited from the technical problems. Therefore at period it was not possible to manufacture some equipments of great size necessary for the treatment of the foods to an industrial scale. After some years (1914) P.W. BRIDGMAN [23] allowed to High Pressure application in biosciences to progress. He received the Nobel prize for the physic in 1946 for the invention of an instrument able to reach very elevated pressures, also for his works on the physical properties of the materials under High Pressures. He conceived a type of joint from which the modern joints derive. The principle is an watertight metal on metal, that combines a big mechanical

resistance to a certain possibility of deformation. The pumps to High Pressure were operated by hand, also to reach pressures over 1 GPa (10^9 Pa). In parallel of P.W. BRIDGMAN in USA, J.BASSET [20a; 20b; 20c; 20d] in France developed new High Pressure equipments and original research axis as the influence of High Pressure in Biosciences (in particular the resistance of micro-organisms), in Chemistry and in Physics.

Since 1930, the equipments of the laboratories introduced higher performances and they allow the discovery of applications of the pressure in the chemical fields and metal workers. The most important result from High Pressure works was the synthesis of diamond in 1953 (ASEA,Suède) and 1954-55 (G.G.,USA). Since 1960, the industrial installations for the production of metallic dusts, plastics or ceramics and alloys carbon/graphite, and for the synthesis of the quartz or dioxide of chrome developed. The range of pressure 50 MPa-600 MPa for non alimentary applications, but in the majority of the cases its range is 100-300 MPa. The products or the materials are pressurized in the oil, in solvents or in gas during some seconds until some weeks at temperatures between room temperature and 2200°C. The systems used in the food field derive from these installations, but they work in general at highest pressures, in the range 350 MPa-800 MPa, and at temperatures in range -20°C / +90°C. The water is the fluid of pressurization commonly used for practical, sanitary and safety reasons. Therefore the materials which oxidize are excluded by the realization of the parts in direct contact with the fluid. The cleaning of the plant is also a very important parameter in the food field, in chemistry, or in metallurgy. The times of the cycles vary between 5 minutes and tens of minutes. The cylinders therefore are conceived for bearing a big number of cycles. In effects, the work of the materials is produced more from the constraints of the deformation, to every come up as to every descent in pressure, that for the maintenance of a stable High Pressure. Because of the food products have an added value weaker than the materials and the chemical products, the conception and realization of the equipments need that the phases of opening, closing, loading, unloading and slope in pressure are very rapid. If the productivity of the plant is improved, the cost of the treatment would be less expansive.

2.2.3.2 The equipment elements

The equipments used in the domain of food-processing are composed by: a cylinder, a pump of compression, High Pressure circuits, a control and command unity and eventually of a heater-cooler group.

2.2.3.2.1 The cylinder

The body is a cylinder (the cylinder form is the most resistant one for the repartition of stresses). It is realized by use of steel blocks forged that are perforated in the length.

Two types of closings are used for the industrial cylinder. In the case where the cylinder diameter is small, the corks can be screwed on the extremities of the cylinder. But the big part of the installations is equipped of smoothed corks.

2.2.3.2.2 Pump of compression

The pressure is a force applied on a surface, all the High Pressure pumps work on the same principle. A fluid motor at low pressure (0.7 MPa-30 MPa) pushes a piston of big surface,which pushes in turn a piston of surface weaker than the surface compressing the fluid to pressurize.

The same force practicing on a smaller surface of piston, creates a more elevated pressure.

It is the relationship of the surfaces of the pistons that defines the factor by which the pressure is multiplied.

2.2.3.2.3 System of control and command

According to the dimensions of the installation and its conditions of use, different types of control-command (Fig.5) are realized: the manual command of every operation, the integral automatism...

In all the cases, some electric systems avoid the risks of false manoeuvre.

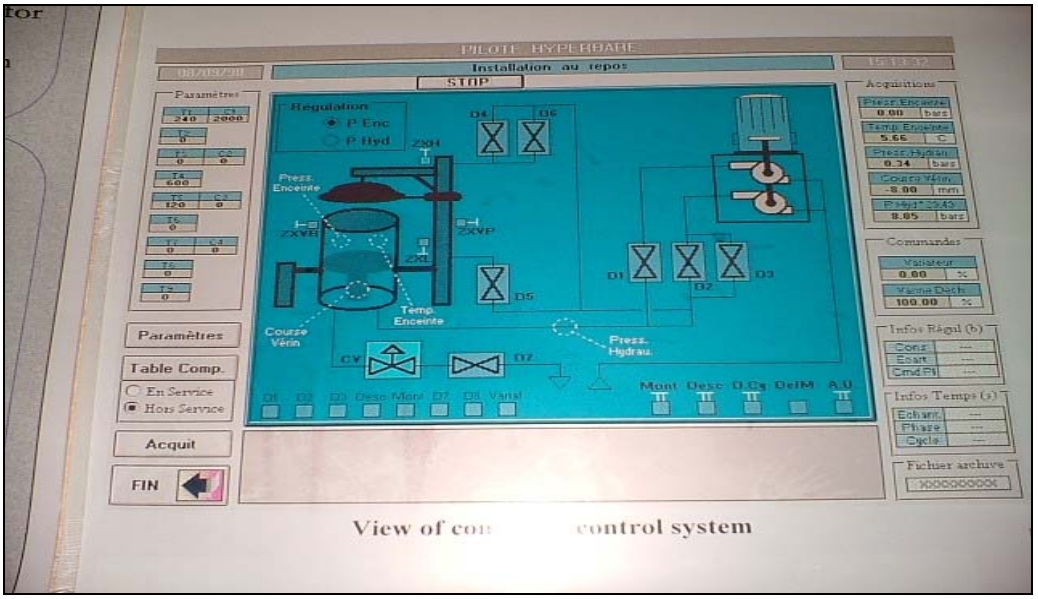


Fig.5 System of control-command of isostatic press in laboratory of group M.H.P.

2.2.3.2.4 Devices of heating or cooling

The equipments at High Pressures have to be thermally stated for some applications. The heating or the cooling can be ensured from the circulation of a fluid bringing heat among two ferrules of the cylinder and in the cylinder. The plant can be more simply heated by electric collars around the cylinder. The treatment has to be effected at refrigerated temperatures, the plant is fed with the water at the desidered temperature (4°C, 10°C, room temperature....).

2.2.3.3 The system of compression

Two systems of compression of the products exist:1) the High Pressure pump compresses the fluid of pressurization and sends it in the cylinder of constant volume; 2) the High Pressure pump compresses a transmitter fluid that pushes on a piston reducing the volume of the cylinder.

2.2.3.3.1 Indirect compression

The system of indirect compression (Fig.8) uses a pump sending a fluid of pressurization in a cylinder of constant volume. In the industrial uses it is the method often employed. The packed products are introduced in the cylinder, that is subsequently dammed. The pump of compression sends then the fluid one under pressure inside the vessel through resistant metallic pipes. In a fist step this fluid replaces the air around the products and in a second time it allows the increase of the pressure in the cylinder (at a speed of 100-200 MPa/min). The closing of a valve placed on the circuit allows to maintain the pressure inside the cylinder during the established time. The opening of this valve will make to fall the pressure. Subsequently the cylinder will need to open and to withdraw the treated products. This implicates therefore a discontinuous procedure of loading and unloading of the foods packed in the cylinder. In the classical devices, the cylinder is in vertical position, the loading of the products, to be treated, is served as the superior part of the cylinder after lifting of the cover. The company Correa in Spain has developed some types of plant (fig.6-7 and Table XI) in which the cylinder is in horizontal position. The loading is made by a side and the unload from the other side, so the march in advance of the products is respected.

Table XI. Range units of the different horizontal devices *(from Company Correa)*:

	D(mm)	Lvessel (mm)	Vuseful (l)	Production capacity*(Kg/h)
WAVE 6000/300 Tandem	300	4500	600	2000
WAVE 6000/55	200	2000	55	170
WAVE 6000/300	300	4500	300	850
WAVE 6000/150	300	2500	150	425

*Filling ratio : 50% - 3 min – 600 MPa

Fig. 6 Horizontal device Wave 6000/150 *(from Company Correa)*

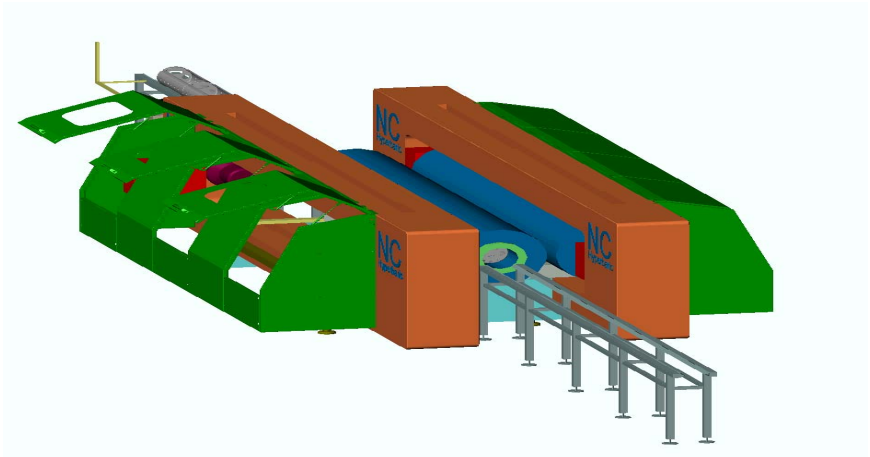


Fig. 7 Horizontal device Wave 6000/300 Tandem *(from Company Correa)*

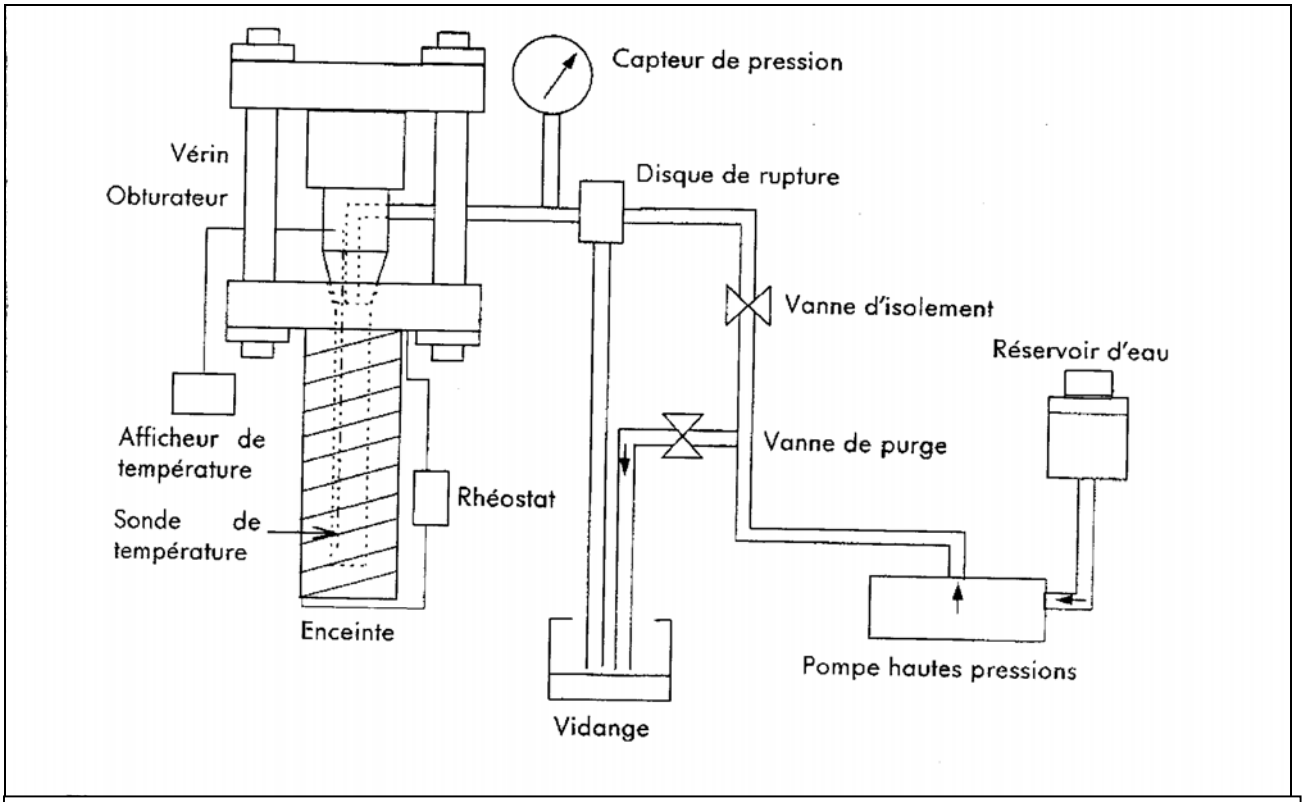


Fig.8 Scheme of the principle of indirect compression.

2.2.3.3.2 Direct compression

In such a case pressure is directly produced by a piston reducing the volume of the reaction vessel. The High Pressure is obtained by the ratio S_1/S_2, the multiplicator coefficient of low pressure created outside the vessel (Fig.9).

The products, submitted to the treatment, can be packed or, if they are liquid, they can be envoyed directly to the cylinder. The pressurized liquid is subsequently aseptically bottled.

Two industrial installations, based on this principle, are present in Japan. One allows the treatment of juice of grapefruit (600 l/h) and the other one draws 4000 l/h of juice of mandarin.

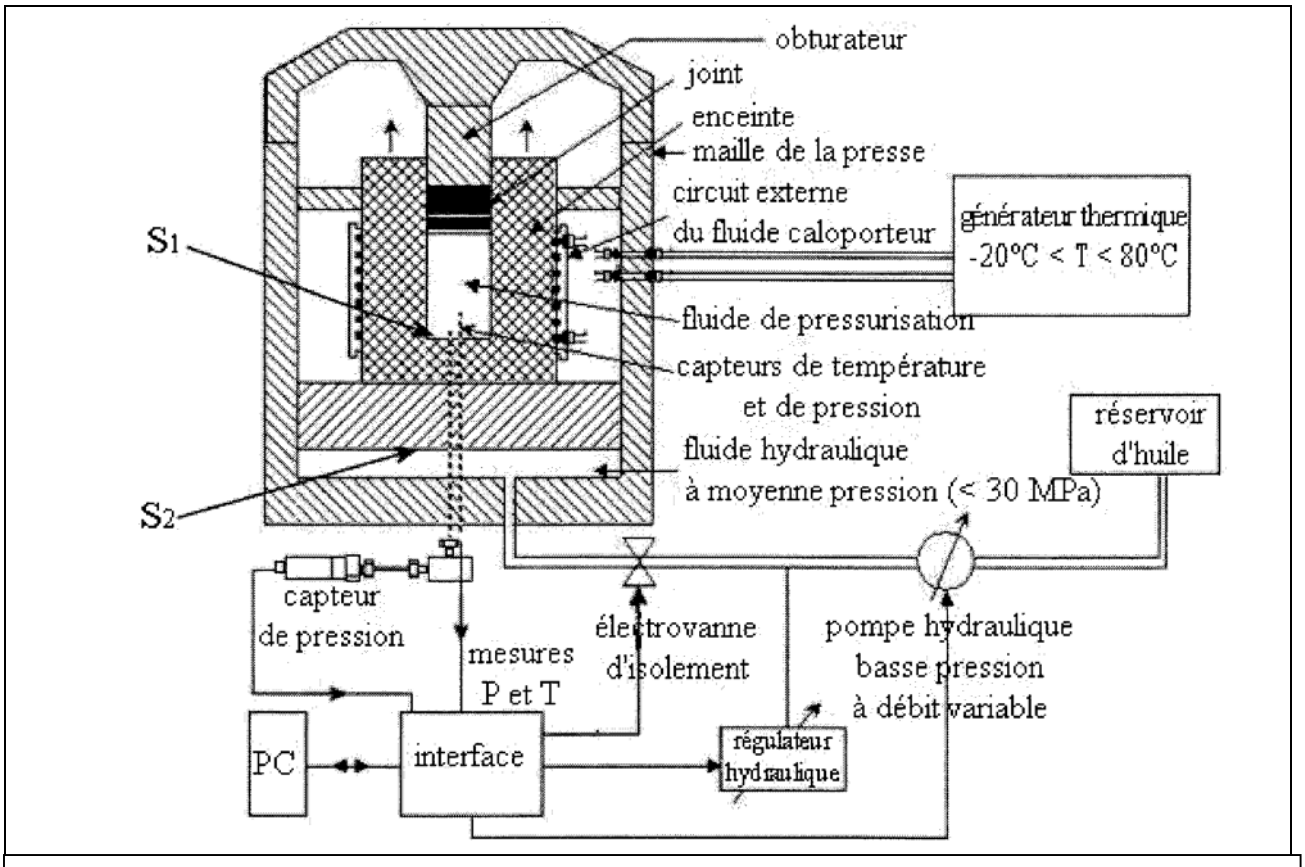


Fig.9 Scheme of the principle of direct compression.

2.2.4 Effects of High Pressure on food compounds

The effects of the High Pressure on the biological components are explained according to the "principle of Le Châtelier" which indicates that any phenomenona accompanied by a decrease in volume are enhanced by an increase in pressure and viceversa. Application of pressure shifts an equilibrium towards the state that occupies a smaller volume [133]. The changes of volume can be due to a change of molecular conformation, or to some interactions inside the molecules, or to some chemical reactions between the molecules. The weak energy bonds as the hydrogen, hydrophobe, ionic bonds, present in the food molecules, are broken from the High Pressure. Instead this treatment does not affect the strong energy bonds as the covalent bonds. So the primary structure of macromolecules and the covalent structure of biomolecules with low molecular weight (peptides,

vitamins, saccharides etc) remain unchanged. Therefore the nutrient content and some sensory properties, such as smell and taste, do not markedly change. The other scientific principle of interest for food application of High Pressure is about the isostatic transmission, that is the uniform transmission of pressure throughout the food. Thus, the product does not become deformed despite being under such High Pressure. Furthemore, the pressure is transmitted almost instantaneously. This uniform and instantaneous process, independently of product size and shape, allows to obtain very homogeneous foods.

2.2.4.1 Effects on the water

The water is the constituent principal of the foods and is also the fluid transmitter of the pressure used more frequently. Water can reach values of compression over 30% for pressures around 900 MPa. The compression of the water is less important than that of the gases. Under pressure, at a temperature of 22°C, the diminution of water volume is of 4% at 100 MPa, of 7% at 200 MPa, of 11.5% at 400 MPa and of 15% at 600 MPa.

2.2.4.1.1 The adiabatic compression

The adiabatic compression of water causes an increase of the temperature of 2-3°C for 100 MPa (according to the initial temperature and the speed of pressurization). The decompression causes a cooling of the same order of greatness. Theoretically, the water's compression at 400 MPa and 20°C is accompanied to an increase of the temperature of 10°C. These variations are reduced (among 5 and 9°C according to the speed of slope in pressure) from thermal exchanges among water, food and metallic vessel. The walls of the vessel have a high thermal conductibility, phenomenon that allows to spread a part of the heat induced by the compression. The increase of the temperature during the adiabatic compression can be valued with the formula: $\Delta T/\Delta P = T \times V \times \alpha P / C_p$

where: V is the molar volume, αP is the coefficient of thermal expansion (K^{-1}) and C_p is the calorific ability.

2.2.4.1.2 The ionic product of the water

The pressure increases the value of the ionic product of the water: $k = (H^+)(OH^-)$.

This dissociation of positive and negative charges results from the phenomenon of the elettrostriction: the molecules of water become more compact around the ions (with diminution of the total volume of the solution), because of interactions dipole-dipole and of hydrogen bonds. The dissociation: $2H_2O = H_3O^+ + OH^-$ is characterized by a value ΔV of -22 ml/moles of dissociated water. The pH of the water and of the weak acids lowers of 0.2-0.5 unities for 100 MPa. These variations of pH are, however, reversing to the decompression. The greatest part of the food products contains an important percentage of water, and this fluid generally serves from transmitter of the pressure. The knowledge of the properties of the water under pressure is therefore essential to explain certain modifications produced by the pressurization. Water compressibility is relatively weak, and it increases with the pressure: at 20°C, the diminution of volume is of 4% at 100 MPa and 15% at 600 MPa. The adiabatic compression of the water induces an increase of the temperature, variable according to the kinetics of pressurization, that it is of 2-3°C for 100 MPa. These variations of the temperature are reduced from the thermal exchanges among the water, the food and the vessel. Besides, the pressure increases the ionic product of the water and favors the dissociation of its molecules, which contributes to the diminution of the pH of the water under pressure. This phenomenon is reversing. The pH of the foods measured after the High Pressure treatment is generally identical to that initial. Finally, an important consequence of the increase of the pressure is the lowering of the point of fusion of the ice. Therefore some foods could be preserved at negative temperatures without formation of crystals of ice.

2.2.4.1.3 The phase diagram of the water

The water is liquid at -20°C under a pressure of 200 MPa, being its point of fusion lowered by the pressure until the limit of stability of the ice I (210 MPa) (Fig.10). The stabilization under

pressure of polymorphous forms, different from the ice I, without increase of volume, will be interesting since irreversible damages in the foods could be avoided. The ice can be stabilized at room temperature under 900 MPa. This phenomenon opens some economic possibilities in the food preservation becuse it will not be necessary to cool during freezing.

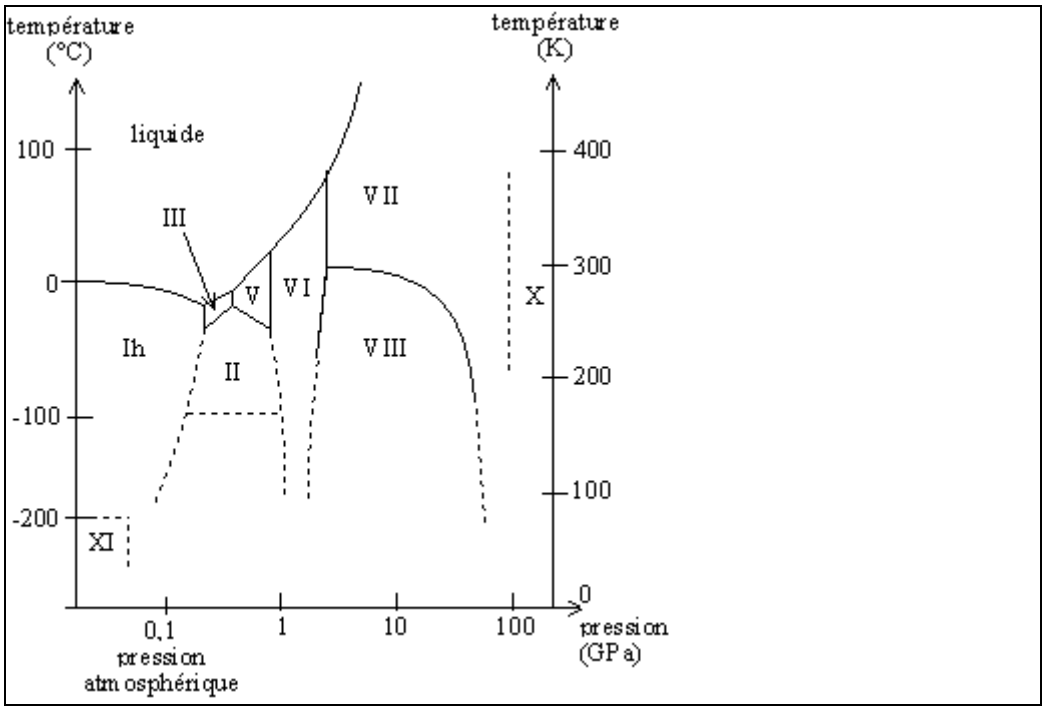


Fig.10 "The phase diagram of the water".

2.2.4.2 Effects on proteins

Due to the small energy conveyed by pressurization in liquid compression, High Pressure can modify only the weak bonds of the proteins determining some irreversible modifications of the secondary, tertiary and quaternary structures. In a recent review LULLIEN-PELLERIN and BALNY (2002) [132] described the main effects of High Pressure on the proteins.

Generally, the quaternary structure is the most sensitive to pressure, due to the hydrophobic interactions. Pressures < 150 MPa cause the dissociation of oligomeric proteins, a phenomenon always accompanied with negative and sometimes large volume changes, such as those observed for the dissociation of lactic dehydrogenase. Dissociation can be followed by subunit aggregation or

by precipitation. Oligomer dissociation occurs at pressures (150-200 MPa) lower than those at which unfolding of monomers is observed. Pressure above 150-200 MPa induces unfolding of proteins and reassociation of subunits from dissociated oligomers. Significant tertiary structure changes are observed beyond 200 MPa. However reversible unfolding of proteins can occur at higher pressure (400-800 MPa), showing that the volume and compressibility changes during denaturation are not completely dominated by hydrophobic effects. Denaturation is a complex process involving intermediate forms leading to multiple denatured forms. Secondary structure changes take place at a very high pressure (300-700 MPa) leading to non-reversible denaturation, depending on the rate of compression and on the extent of secondary structure rearrangements [132]. The High Pressure can determine some spatial changes of the proteins that modify the interactions with the solvent. The reversibility of the modifications induced by the pressure depends on the conditions of treatment applied. Pressures of 100-200 MPa induce some reversing modifications of the proteins, while above 300 MPa some irreversible effects are observed. For example the gelification of the proteins. The gels differ from the thermal gels for their texture and aspect. An application of gelification of the proteins under High Pressure is the texturization of the meat-products or fish (for example, the surimi). The protein denaturation by pressurization in some cases can be reduced by the addition of sugars or poliosides, that stabilize the proteins.

BALNY *et al.* (2002) [134] discussed the specificity of pressure effects on biological macromolecules, underlining that it is different from temperature and chemical effects. In contrast to increasing temperature, which focuses on energy and volume effects due to thermal expansivity, pressure effects are mainly on the volumetric aspects via the compressibility of the system.

2.2.4.3 Effects on enzymes

The High Pressure can cause an activation or inactivation of the enzymes versus the ΔV value. The changes have more origins: the conformation, the active site of the enzyme or the substratum.

Generally at elevated pressures the enzymes are often inactivated. In the cases of inactivation, a residual activity of the enzyme often remains after the treatment.

Pressure also modifies the rate of enzyme-catalysed reactions via changes in the structure of an enzyme or changes in reaction mechanism. Depending on the reaction under study, a reaction can be accelerated or decelerated by increasing pressure [133].

2.2.4.4 Effects on glucides

The High Pressure does not affect the "monosides" and the "oligosides", since they are constituted by covalent bonds. The High Pressure, instead, can modify the "poliosides" constituted by chains of monosides from of the weak bonds. Among the poliosides, the starch is injured from the pressurization: it causes a swelling of the wheats of starch, that is able determine a gelification similar to the gelification obtained by thermal treatment. The behavior of the starch under pressure is varying according to its origin, the composition of the medium to pressurize, the intensity of the pressure, the temperature and duration of the pressurization.

2.2.4.5 Effects on lipids

The pressure increases the temperature of fusion of the lipids: it increases by 20°C for 100 MPa.

The modification of the phase transitions of the lipids under pressure occurs equally at level of the biomembranes, being composed from lipids and proteins. The physical state of the lipids modulates their enzymatic activity and their permeability. Besides it has been shown on the extracts of fish that the pressure modifies equally the sensibility of the lipids towards the auto-oxidation.

2.2.4.6 Effects on vitamins and aromes

The vitamins are small molecules, containing in their structure especially covalent bonds, so they are not sensitive to High Pressure. Some vitamins of chicken egg, as vitamins A, E, B2 and the folic acid, did not show any molecular alteration or denaturation after pressure treatment [57].

Also in the case of aromes molecules (aldheydes, esters...) the covalent bonds are numerous and prevalent, so this can explain their barotolerance and also the preservation of the tastes and flavours of food product pressure treated [29].

2.2.4.7 Effects on nucleic acids

The High Pressure does not cause changes of the DNA, since such acid is stabilized by bonds type hydrogen that are strengthened under pressure. Therefore the technology of the High Pressure does not have mutagenic effect.

2.2.5 Effects of High Pressure on food sensory properties

2.2.5.1 Effect on the colour

The pigments of the fruit and vegetable (carotenoides, chlorophyll...) do not change under the effect of the High Pressure. The raw bovine meat showed a paler colour, instead, for treatments above 300 MPa. This change of colour was due to the denaturation of the myoglobine and of the proteins of myofibrillas.

2.2.5.2 Effect on the texture

The High Pressure modify the texture of the products in relationship to the difference of compressibility between the water and the air. For this reason the High Pressure are not adapt for the treatment of the products containing much air, as for example the whole strawberries or the bread. Besides, the higher pressures are sufficient in certain cases to cause a gelification of the polisaccharides or proteins. The pressurization of the egg albumen can cause at room temperature its gelification. The gels, obtained by this way, have some different characteristics from those obtained by heating and variable according to the physical-chemical environment and the conditions of treatment. In the particular case of the meat, the effect of the treatment on the texture depends on the muscle, on the stadium of maturation of the meat, on the temperature of the treatment.

Numerous studies showed a global effect of tenderness of the bovine meat by High Pressure. Nevertheless under industrial conditions, in post-rigor mortis and at cold temperature, the meat has tendency to harden despite the increases of liberation of enzymes proteases.

2.2.5.3 Effect on the flavours

The High Pressure preserve better the aromas of the food products in comparison to the thermal treatments. The High Pressure do not induce the reaction of Maillard in the foods, they does not cause, therefore, the appearance of the taste of "cooked". This aspect has contributed to the development of the High Pressure in Japan where the taste of "fresh" and also "raw" is very appreciated, especially concerning the animal products (fish, meat). Nevertheless, the based-fruit products, commercialized in Japan, show a very different taste from the jams traditionally consumed in Europe.

2.2.6 Effects of the High Pressure on micro-organisms

The barosensitivity of micro-organisms is highly variable. For bacteria there is a correlation with the Gram type and cell morphology. Thus, Gram+ are more resistant than Gram- bacteria and among Gram+ bacteria the cocci are more barotolerant. In general the higher the complexity of an organism the greater the sensitivity to pressure treatment. Cells in logarithmic growth phase are more sensitive than those in the dormant, stationary or death phases [63]. The spores are the most pressure-resistant microbial forms.

2.2.6.1 Morphological changes and membrane damages

Pressure treatment causes cell lengthening, separation of the cell wall from the cytoplasmatic membrane, compression of the membrane, collapse of intracellular gas vacuoles, and modifications in intracellular organelles [29; 70; 102; 128; 129]. PIERRET-CORNET *et al*.(1995) [97] measured the diameter of yeasts cells, before, during and after pressure treatment by image analysis. During

pressure treatment, the average volume gradually decreased at 250 MPa. After pressure treatment, the cells did not totally recover their original volume. SHIMADA *et al*.(1993) [108] observed a slight alteration in the outer shape of *Saccharomyces cerevisiae* with pressures below 400 MPa. Pressure treatment always involves a perturbation of the bacterial membrane. In fact, it is well-known that the primary site of pressure damage is the cytoplasmic membrane. Cell permeability and ion exchange are altered. In some cases, disruption of wall and membrane occurs. The pressure-set crystallizazion of membrane phospholipids probably contributes to the inactivation of several micro-organisms [29]. It is likely that these modifications in cell membrane are the main cause of sublethal injury generated by pressure tretament to some micro-organisms. As an increase in pressure reduces the membrane fluidity, it can be expected that cells respond to this stress by altering the composition of the membrane. For microbial cells to maintain essential cell functions, their internal pH must remain constant. High Pressure treatment can result in reduced intracellular pH, while membrane damage may impair acid efflux. Observations of mambrane damage, protein inactivation and decrease intracellular pH, along with observations on yeasts, suggest that membrane-bound enzymes associated with efflux of protons might be a target in High Pressure inactivation. Membrane-bound F_0F_1 ATPase may be such a target, as it can be inactivated or dislocated by pressure [63].

2.2.6.2 Protein synthesis

Micro-organisms contain mechanisms to resist and repair the effects of heat and other unfavorable environmental conditions, such as starvation and low pH, which enable them to survive subsequent, potentially lethal temperatures. It is not known whether they also contain mechanisms to counteract pressure. Generally, there is a narrow range between optimum temperature for growth and maximum temperature where stress proteins are induced and the cell acquires an increased resistance to heat. It is unclear if a casual relationship exists between the synthesis of heat-shock proteins and the development of thermotolerance. Increased thermotolerance after sublethal heat

shock in *Escherichia coli*, *Salmonella* and *Listeria monocytogenes* was observed in different works [79a; 79b; 80]. Many biochemical activities of bacteria such as protein synthesis, glucose utilization and L-phenylalanine utilization are affected by pressures of about 50 MPa. Transcription and translation are highly sensitive to pressure. Protein synthesis is known to be one of the most pressure-sensitive cell activities. In *Escherichia coli* cell system, protein synthesis is completely inhibited at 68 MPa, but the inhibition is completely reversible after pressure release [110].

SATO *et al*.(1996) [106] found that the expression of the genes for osmoregulatory membrane proteins was markedly reduced by High Pressure, most likely at the transcriptional level. In general, hydrostatic pressure can induce tetraploidy in *Saccharomyces cerevisiae*, indicating that High Pressure can interfere with replication of deoxyribonucleic acid (DNA). Pressure-inducible proteins have been found in *Rhodotorula rubra* and *Escherichia coli*. A pressure of 53 MPa could induce proteins in *Escherichia coli* similar to those found at elevated temperature. Resistance to heat can be brought about by sublethally low pH and vice versa, and sublethal heat can enhance pressure resistance in yeasts and in bacteria. Increased resistance to heat by sublethal pressure has not yet been observed. When yeasts or *Lactobacillus plantarum* cells were subjected to a range of sublethal pressures, no increased heat resistance or pressure resistance was found. In view of the above-mentioned findings that starvation or sublethal pH increases resistance, it is not surprising that exponentially growing cells are more sensitive to pressure than stationary-phase cells. Hydrostatic pressure also inhibits the synthesis of some membrane proteins.

2.2.6.3 Ribosomes and Enzymes

Ribosomes are affected both by heat and pressure. It may be expected that the death of the individual cell occurs when the number of functional ribosomes has dropped below a critical threshold level, beyond which the cell cannot recover. GROSS *et al*.(1993) [53] investigated the effects of High Pressure abd observed ribosome dissociation starting at 40 to 60 MPa. HURST (1984) reported that heating produces membrane damage, resulting in Mg^{++} depletion in the cells.

This, inturn, can lead to destabilization of 70S ribosomes. This phenomenon might be similar for High Pressure; HAUBEN *et al*.(1997) [55] reported that divalent cations such as Ca^{++}, Mg^{++}, Mn^{++} and Fe^{++} reduced inactivation by High Pressure. NIVEN *et al*.(1999) [87] carried out a study of the change of conformation of ribosomes by pressure in vivo and found a correlation between cell death and ribosome damage.

Both pressure and temperature have a significant effects on proteins including enzymes. There is an optimum temperature at which proteins are most resistant to pressure. A similar pattern has been observed for the inactivation of micro-organisms. Hydrostatic pressure can presumably directly affect enzymes and carriers of transport systems. Lactic dehydrogenase from rabbit muscle and glyceraldehyde-3-phosphate dehydrogenase from baker's yeast were inactivated by pressures of 200 and 100 MPa, respectively. This provides some evidence that enzyme inactivation plays an important role in pressure inactivation of micro-organisms. However, kinetic data on the inactivation of the various sites such as glycolytic enzymes, membrane-bound enzymes, ribosomes, or membranes are lacking. Pressure brings about changes in the quaternary structure rather than the tertiary structure because the quaternary structure is mainly maintained by hydrophobic interactions, which are pressure sensitive [19]. As a result, monomeric enzymes are usually more resistant to pressure than multimeric enzymes. SIMPSON and GILMOUR (1997) [109] studied the effect of high hydrostatic pressure on the activity of 13 metabolic enzymes of *Listeria monocytogenes*. They isolated the enzymes before and after pressure treatment of whole cells. Phosphoglucomutase and aconitase were particularly susceptible to pressure, and even pressures of around 200 MPa were sufficient to inactivate these enzymes. Because *Listeria monocytogenes* was hardly affected by this amount of pressure, these enzymes cannot be considered as critical sites. As in heat inactivation, it can be expected that there is more than one specific target that can affect the whole organism depending on the pressure level.

2.2.6.4 DNA

Nucleic acids, especially DNA, are relatively resistant to heat and very resistant to pressure. Their structure can remain intact even at 1,000 MPa. But as enzymes are affected by High Pressure, mechanisms of DNA replication and transcription, and translation into proteins are inhibited [29]. An extreme condensation of the nuclear material has been found for *Listeria monocytogenes*, *Salmonella typhimurium* [81]. The hypothesis is that at elevated pressures DNA comes into contact with endonucleases, which cleave DNA. This condensation, which has been found in many other instances, is reversible and presumably an enzyme responsible for renaturation is also involved. If this enzyme is deactivated by High Pressure, the cell is no longer able to multiply.

2.2.6.5 Effects on vegetative cells

2.2.6.5.1 Bacteria

The principal action of the High Pressure is a total or partial inactivation of the micro-organisms. In effects, their growth is totally inhibited at the pressures used in the food industry (100-1000 MPa). In the most greater part of the cases, at room temperature, it is necessary to apply pressures over 200 MPa to get an important reduction of the treated microbial population. The bacteria Gram+ are generally more resistant to the pressure than the bacteria Gram-. Detectable effects of High Pressure on microbial cells include an increase in the permeability of cell membranes and possible inhibition of enzymes vital for survival and reproduction of the bacterial cells. CARLEZ *et al*.(1993) [26] studied the inactivation by High Pressure of *Citrobacter freundii*, *Pseudomonas fluorescens* and *Listeria innocua* inoculated in beef. The destruction bacterial of the three bacterial types increased if the intensity of the pressure increased. At 20°C 6 $\log_{10}$ reductions of *P.fluorescens* were obtained after 200 MPa, while *C.freundii* and *L.innocua* respectively required 280 and 400 MPa for the same reduction. METRICK *et al*. (1989) [86] studied the effect of the High Pressure on *Salmonella typhimurium* and *Salmonella senftenberg*. The treatment of *S.typhimurium* at 340 MPa for 10 minutes at 23°C in buffer phosphate reduced the population of

about 2 $\log_{10}$. Similar results have been obtained when these species were subjected to the same treatment but if inoculated in the chicken. The rate of inactivated *S.senftenberg* was of 4 $\log_{10}$ in the buffer phosphate while a protecting effect was recorded when these same bacteria were treated in the chicken (>3 $\log_{10}$).

2.2.6.5.2 Moulds and yeasts

Moulds (*Byssoclamys* sp., *Eurotium* sp., *Talaromyces* sp., *Saccharomyces* sp.) are capable of growing at the typical low pH of fruit and under reduced oxygen tension, so they seem to be the most common cause of spoilage of canned fruit products. They produce very heat resistant spores, the "ascospores", and as the case of *Byssoclamys* sp., some strains are also mycotoxigenic. Very few data are available on the effect of pressure on heat resistant yeast and moulds. Ifuku *et al*.(1993) [66] observed the total inactivation of *Saccharomyces cerevisiae* and *Aspergillus niger* after a treatment of 300 MPa for 5 minutes at room temperature, while *Candida albicans* and *Penicillium citrinium* were destroyed only at 400 MPa. BUTZ *et al*.(1996) [24] investigated the response to pressure of vegetative and persistent forms of heat resistant moulds *Byssochlamys nivea*, *Byssochlamys fulva*, *Aspergillus fischeri*, *Eupenicillum* sp. and *Paecilomyces* sp.. All the ascospores (except for *B.nivea* ascospores), pressure treated, were strongly reduced by treatment in the range of 300-600 MPa at 10 to 60°C. *B.nivea* ascospores needed pressures above 600 MPa and temperatures above 60°C for their inactivation. EICHER *et al*. (1997) [37] observed that conidiospores of *Penicillium expansum* and ascospores of *Eurotium respens* were protected against pressure, of respectively 350 and 500 MPa, in high osmolalic media (with NaCl and sucrose). OXEN and KNORR (1993) [94] reported similar results for the yeast *Rhodotorula rubra* where high concentrations of different sugars and NaCl led to a higher pressure resistance.

2.2.6.6 Effects on spores

The bacterial spores are the most resistant forms to the pressure, withstanding pressures above 900 MPa as in the case of *Clostridium sporogenes* spores. In the work of Gola et al. (1996) a pressure of 900 MPa at 30°C for 10 min was not sufficient to inactivate totally spores of *Cl. sporogenes* PA3679 ($8.4\ 10^2$ spores/ml) [72]. This exceptional resistance of the spores seems depend on different factors.

Three principal factors seem involved:

- their morphology characterized by rich thick wraps in proteins that protect them from the external agents;
- their weak content in water (20%) in comparison to a vegetative cell (80%) causes a strong diminution of the metabolism and a dehydration of the proteins, become heat-resistent;
- their chemical composition characterized by an elevated content in dipicolinic acid, that can reach 10% of dry weight, contributes to the dehydration and accordingly to the resistance to the external physical-chemical agents (temperature, pH, pressure, acids, UV).

This general behavior of resistance various in relation to the nature of the treated spores (generally the spores of *Clostridium* sp. are more resistant to pressure that the spores of *Bacillaceae*). Such exceptional resistance represents a serious problem in the High Pressure procedure for the foods preservation. In order to decontaminate by pressure the foods from the spores various researches have been carried out with different approaches. Sometimes weak pressures are been used (under the 300 MPa) to induce the germination of the majority of the spores, that become so more sensitive and can easily be inactivated by the pressure or by the heat. Also, strong pressures are been used (>600 MPa) applied in continuous or by cycles. Further High Pressure (200-400 MPa) were combined with high temperatures (70-90°C) in some experiments in order to reduce the spore population of *Bacillus stearothermophilus* significantly [70]. In this case the formalities of action are not entirely clarified. The spores inactivation increases with the duration and the intensity of the

applied pressure, and contrarily to the results obtained with the vegetative bacteria, the maximal destructions of the spores are recorded at neutral pH.

2.2.6.7 Effects on viruses

SHIGEHISA *et al*.(1996) [107b] studied the possibility to inactivate by High Pressure the HIV virus and using pressures of 350 MPa at room temperature for 10 minutes they achieved more than 5 $\log_{10}$ of reductions in virus population. Titres of Haepatitis A Virus (HAV) were reduced significantly at 300-450 MPa and a feline calicivirus (a Norwalk virus surrogate) was completely inactivated after 5-minutes treatments at 275 MPa [69]. In a recent study GASPAR L.P. *et al*. (2002) [131] observed that High Pressure inactivates two membrane-enveloped viruses, influenza and Sindbis, by trapping the particles in a fusion-intermediate state. Viruses supsensions were pressurized at 2.5 kbar for up to 8 hours. The pressure treatment permitted to reduce the infectivity by 10^5.

2.2.6.8 Effects on parasites

Pressurization can inactivate some parasites usually found in fish, meat or poultry. The complete inactivation of *Trichinella spiralis* inoculated in pork samples was achieved at 100 MPa at 5°C for 10-30 minutes [88]. The destruction of larvae of *Anisakis simples* in salmon fillets was obtained at 207 MPa after only 3 minutes [36].

2.2.6.9 Factors affecting microbial inactivation

The processing conditions (initial sample temperature, circulating water temperature, pressurizing medium, holding time, cycles) under which High Pressure is applied significantly influence the level of inactivation as well as the overall effect on the nutritional and sensory characteristics of food.

ALPAS *et al.* (2000) [12] studied the pressure tolerance of different strains of *S.aureus* and *Salmonella* in solutions with various pH (4.5; 5.5; 6.5) at many combinations of temperature (25-

45°C) and pressures (207-345 MPa). They reported that media pH played a very important role in the destruction of microbs. The pressurization at high acidity of medium increased the viability loss by 1.2-3.9 $\log_{10}$ at 345 MPa.

Variations in the pH and water activity of foods can result in different levels of lethality of a bacterium for the same High Pressure processing parameters. Studies conducted by TIMSON and SHORT (1965) [115] on *B.subtilis* showed that the pressure resistance of the bacterium (when subjected to 483 MPa for 30 min) was decreased as the pH in milk medium was lowered or raised from a pH value of 8.

The type of culture medium can also have a significant impact on the pressure resistance of any micro-organism. In general, the richer the growth medium, the better the baroresistance of the micro-organism, maybe because of the increased availability of essential nutrients and amino acids to the stressed cells.

PATTERSON *et al.* (1995) [96] studied the sensitivity of vegetative pathogens (*Yersinia enterocolitica* 11174, *Salmonella typhimurium* NCTC74, *Salmonella enteritidis*, *Staphylococcus aureus* NCTC10652, *Listeria monocytogenes*, *Escherichia coli* O157:H7) in buffer (pH 7), UHT milk and poultry meat at High Pressure up to 700 MPa at 20°C. A 5 $\log_{10}$ reduction in counts was obtained in all cases when pressures in the range of 275-700 MPa for 15 min were applied. In buffer the pathogens showed less resistance to the pressure than in milk and poultry. Differences among the strains of the same bacterium were also found. The water activity (A_w) at which the cells are pressure-treated also affects their pressure resistance. It is reported that the lower the A_w, the higher the pressure resistance of cells.

The resistance to the pressure varies between bacteria, yeasts, moulds, viruses and spores. When the behaviour to the pressure of different bacteria is compared, it can be observed that the rate of inactivation changes strongly according to the bacterium, but also to different species of a same genus (for example *Salmonella*), and to different strains of same species (*E.coli*, *Listeria monocytogenes*, *S.aureus*).

Generally the cells in stationary phase are more resistant to the pressure that the cells in exponential phase of growth. The number of cells surviving to a pressure treatment quickly decreases at the beginning of the process, but if the treatment continues a reduction of inactivation effect is obtained. Then it is clear that the inactivation of the micro-organisms by pressure does not follow a first order cinetic model. This phenomenon, defined "tailing" can be explained with the existence of a small fraction, inside microbial population, more resistant to the treatment, or well protected from the dead cells and/or from the products of their destruction. But if these cells are isolated, cultivated and submitted to a second treatment, the same proportion of resistant cells will result. It is the reason for which this phenomenon is generally interpreted as some phenotypic variations of a small fraction of the population [78; 111b].

Another factor of variation is the temperature. For every bacterial type an optimal temperature exists at which it is more pressure resistant. The study of CARLEZ *et al.* (1993) [26] showed, for the different micro-organisms inoculated in the meat or in buffer solution, that their resistance to the pressure is greater at 20°C that at low temperatures (3-5°C). A hypothesis, often considered to explain this behavior, is the modification of the fluidity of membrane at low temperatures. In effects it is observed a weakening of the hydrophobe interactions and a crystallization of the lipids that make the membrane structure more fragile.

The composition of the medium of treatment can also influence the behaviour of the micro-organisms to the pressure. The tolerance to the pressure is generally higher in a rich medium that in a simple salty solution. This phenomenon is probably due to the greater availability of the elements (amino acids, vitamins etc.) necessary to the reparation of the stressed cells.

Also the presence in the medium of antimicrobial compounds, as the bacteriocins, can have a synergistic effect with High Pressure on inactivation of micro-organisms. Sorbic acid, which acts as an organic acid but also interferes with the microbial membrane, is more active in combination with the pressure. Microbs are particularly sensitive to nisin ("*antimicrobial compound*") during or after pressure treatment. Kalchayanand *et al.*(1994) [68a] reported that sublethal injury sensitized not

only *Listeria monocytogenes*, but also *Escherichia coli* and *Salmonella typhimurium* to nisin when pressure treated. Apparently Gram- bacteria such as *E.coli* and *Salmonella*, which are normally resistant to nisin, can be sensitized to this agent when pressurized. This might be explained by the specific action of nisin, which interacts with the cell membrane, and it could possibly penetrate inside the cell. During pressure treatment, *Escherichia coli* was found to be sensitive to lysozyme, nisin and EDTA separately and even more sensitive in a combination of these compounds [55]. OGAWA *et al.*(1990) [90] observed that the addition of 5-10g allyl-isothiocyanate incresead the reduction of *Salmonella* at 200 MPa by 5 log cycles. KALCHAYANAND *et al*.(1998) [68b] investigated the combined effect of High Pressure, temperature and pediocin AcH (3000 units/mL) ("*antimicrobial compound*") and found that the presence of pediocin during pressure treatment resulted in an additional inactivation of 2 log cycles. PONCE *et al*.(1998) [98] studied the combined effect of nisin and High Pressure on the destruction of *Listeria monocytogenes* and *Escherichia coli* in liquid whole egg. An additional effect of nisin was found after a relatively severe pressure treatment (400 MPa for 15 minutes at 20°C).

2.2.7 Effect of High Pressure on *S.aureus* and *S.enteritidis*

Processing conditions (pressure, temperature, time), the strain of micro-organism, the kind of the substrate, in relation to its composition (since some molecules, such as carbohydrates and fats, can give a protection to the micro-organisms against the pressure), pH and A_w, influence obviously the inactivation caused by pressure treatment. In general the sensitivity of micro-organisms to pressure processing is lower in foods than in buffer suspensions [48; 96] and can depend on the food type [95].

2.2.7.1 *S.aureus* under High Pressure

Several researches were achieved using many pressurized products inoculated with *S.aureus* and obtained different and interesting results concerning the decrease in counts of these pathogens after

processing (Table XII). O'REILLY *et al.* (2000) [93] observed a reduction > 4 log cycle of *S.aureus* ATCC 6538 counts, inoculated in cheese, after a treatment at 600 MPa and room temperature for a duration of 20 minutes. At 800 MPa and 10°C only 1.5 log cycle of reduction in counts was obtained. ERKMEN and KARATAŞ (1997) [38] inoculated *S.aureus* ATCC 27690 into pasteurized cows'whole milk and submitted it to a High Pressure treatment in the range 0.5-3.5 kbar at 20°C. No cells survival was indicated at 3.0 kbar for 8 minutes. The decimal reduction time values at 20°C from pressurized samples were estimated to be 211.8, 15, 3.7 and 2.56 min at 2.0, 2.5, 3.0 and 3.5 kbar [41].

Generally *S. aureus* showed a higher resistance to High Pressure processing achieved at low temperatures compared to room and higher temperatures [48; 56; 89; 93]. GERVILLA *et al.*(2000) [48] demonstrated that *S.aureus* CECT 534 was the micro-organism with highest resistance to High Pressure treatments at low temperature (4°C), compared to the other tested micro-organisms (*P.fluorescens, E.coli, L.helveticus, L.innocua*) inoculated in ovine milk. Also they observed that ovine milk had a baroprotective effect on micro-organisms compared to inoculated Ringer solution samples, pressurized in the same conditions of parameters.

For *S.aureus* NCTC 10652 a 15 min treatment of 500 MPa at 50°C was required to achieve a 5 $\log_{10}$ reduction in poultry meat and a 6 $\log_{10}$ reduction in UHT milk [95]. In a previous work PATTERSON *et al.* (1995) [96] needed a treatment of 700 MPa at room temperature to achieve a 5 $\log_{10}$ reduction in numbers of cells of *S.aureus* NCTC 10652 inoculated in PBS. The organisms were more resistant to the pressure when treated in UHT milk and in poultry meat than in buffer [95; 96].

In a recent work [11] seven strains of *S.aureus* were subjected to a pressure of 345 MPa for 5 minutes at room temperature in a peptone-based medium and showed different behaviour versus pressure. Among these strains the viability losses ranged between 0.7 and 7.8 $\log_{10}$, the great part resulted very pressure resistant.

In many works the combining effect of High Pressure and bacteriocins on *S.aureus* was studied [10; 68a; 82]. A 2.1 log_{10} reduction of *S.aureus* ATCC 6538, inoculated in a milk medium with addition of lacticin 3147, pressurized at 150 MPa for 30 minutes (25°C) occured. Instead, after the same treatment without the bacteriocin, the viability loss in counts was less than 0.5 log_{10} [Morgan et al.,2000].

Table XII. Viability Loss of *Staphylococcus aureus* treated by High Pressure in different media.

Strain	Medium	T (°C)	t (min)	P (MPa)	V.L. (log_{10}CFU)	Reference
NCTC10652	PBS	20	5-30	700	> 5	Patterson *et al*.,1995
NCTC10652	poultry meat	50	15	500	5	Patterson *et al*.,1998
NCTC10652	milk	50	15	500	6	Patterson *et al*.,1998
ATCC25923	pork slurries	25	10	>600	ND	Shigehisa *et al*.,1991
778	peptone solution	25	5	345	0.70	Alpas *et al*.,1999
485	peptone solution	25	5	345	0.70	Alpas *et al*.,1999
743	peptone solution	25	5	345	0.86	Alpas *et al*.,1999
315	peptone solution	25	5	345	0.90	Alpas *et al*.,1999
565	peptone solution	25	5	345	1.12	Alpas *et al*.,1999
765	peptone solution	25	5	345	1.51	Alpas *et al*.,1999
582	peptone solution	25	5	345	7.8	Alpas *et al*.,1999

T = Temperature
t = time
P = Pressure
V.L. = Viability Loss in counts
CFU = Colony Forming Unit

2.2.7.2 *S.enteritidis* under High Pressure

Not many studies have been done about the effect of High Pressure processing on *S.enteritidis* (Tab.XIII), which is the main food poisoning *Salmonella* serotype [58].

Furthemore, among the species of genus *Salmonella, S.enteritidis* is the most resistant to High Pressure processing especially at low temperatures [99; 11]. PONCE *et al.* (1999) [99] tested in their work various behaviours of *S. enteritidis* inoculated in egg-based medium for different pressure (350 and 450 MPa), temperature and time levels. The strongest effectiveness of the process

was observed at 50°C, followed by 20°C, 2°C and –15°C. Cycles of 5 minutes (2 or 3 times) achieved higher destruction than continuous treatment of the same total time (10 and 15 min). The effect of continuous pressure at 50°C was equivalent to that by alternating cycles at 20°C for treatments at 450 MPa. Pressure cycles enhanced *S.enteritidis* sensitivity [99].

S.seftenberg, an extremely heat-resistant salmonella ($D_{57.5°C}$ = 15 minutes), resulted less barotolerant than *S.typhimurium* when it was treated at the same conditions [86]. The counts suspended in a buffer solution were decreased 4 log cycles after 10 minutes at 340 MPa.

YUSTE *et al.* (2000) [125] found a reduction in *S.enteritidis* counts of between 7.2 and 7.7 $\log_{10}$ g^{-1} in poultry sausages processed at 500 MPa by combining different times (10 and 30 min) and temperatures (50, 60 and 70°C).

PATTERSON *et al.* (1995) [96] had to apply a pressure of 450 MPa for 15 minutes at 20°C to obtain a 5 $\log_{10}$ reduction in *S.enteritidis* counts in PBS suspension.

ALPAS *et al.* (2000) [12] studied the effect of High Pressure processing at different time, temperature and pressure conditions on *S.enteritidis* FDA and *S.aureus* (strains 485 and 765) counts in peptone solution. Increasing the temperature, at lower pressure (207 MPa) the viability loss of bacteria raised significantly only for *S.enteritidis* FDA (5.46 $\log_{10}$ reduction after 10 min-treatment), while to achieve the same inactivation rate for *S.aureus* (strains 485) the authors applied a pressure of 345 MPa at 50°C. *S.aureus* strains showed to be, in this work, more resistant to High Pressure compared to *Salmonella sp*.

In a previous work ALPAS *et al.* (1999) [11] tested the sensitivity to High Pressure of different species of Salmonella (*S.enteritidis*, *S.typhimurium* and *S.choleraesuis*) but also of different strains inside the same specie. These micro-organisms were inoculated in a peptone solution and treated by a pressure of 345 MPa for 5 minutes at 25°C. *S.enteritidis* showed the highest resistance to the pressure, while *S.choleraesuis* was the most sensitive with 8.34 $\log_{10}$ reduction in counts.

Table XIII. Viability Loss of *Salmonella* sp. treated by High Pressure in different media.

Strain	Medium	T (°C)	t (min)	P (MPa)	V.L. (log_{10}CFU)	Reference
S.typhimurium VL	Peptone Solution(PS)	25	5	345	7.48	Alpas *et al*.,1999
S.typhimurium ATCC14028	PS	25	5	345	7.30	Alpas *et al*.,1999
S.choleraesuis ATCC6539	PS	25	5	345	7.70	Alpas *et al*.,1999
S.choleraesuis ATCC10708	PS	25	5	345	8.34	Alpas *et al*.,1999
S.enteritidis FDA	PS	25	5	345	5.45	Alpas *et al*.,1999
S.enteritidis	poultry sausages	50-70	10-30	500	7.2-7.7	Yuste *et al*.,2000
S.enteritidis	PBS	20	15	450	> 5	Patterson *et al*.,1995
S.enteritidis	liquid whole egg	20	15	350	1.9	Ponce *et al*.,1999
S.enteritidis	liquid whole egg	-15	15	350	1.8	Ponce *et al*.,1999
S.enteritidis	liquid whole egg	2	15	350	4	Ponce *et al*.,1999
S.enteritidis	liquid whole egg	50	15	350	4.7	Ponce *et al*.,1999

T = Temperature
t = time
P = Pressure
V.L. = Viability Loss in counts
CFU = Colony Forming Unit

2.2.8 Fish products under High Pressure

Not many works, having as objective the study under pressure of fish products, were performed. In a recent study concerning the application of High Pressure processing (300-600 MPa at 20°C for 2 minutes) on bivalves and prawns, LINTON *et al*.(2003) [75] verified that psychrotrophic bacteria are more sensitive to High Pressure than mesophiles and they lose the ability to grow at low temperature after pressure treatment (500 MPa at 20°C for 2 min). This can lead to an extension of shelf-life of seafoods during storage at refrigeration temperatures.

H_2S producers bacteria (*Shewanella putrefaciens* is the predominant species), which are involved in spoilage of fish and fish-products, due to the reduction of TMAO to TMA, have showed great sensitivity to High Pressure in different studies [77a; 77b]. After pressurization of oysters and prawns at 400 MPa and 7°C for 10 minutes, counts of H_2S producers bacteria were reduced to below the detection levels [77a; 77b]. High Pressure processing could extend the shelf-life of fish

and seafoods by the inactivation of spoilage microflora and the control of the endogenous enzyme activity.

LOPEZ-CABALLERO *et al.*(2000) [77b] found levels of TVB (total volatile nitrogen bases) in pressurized oysters (400 MPa for 10 min) lower than unpressurized samples, probably due to inactivation of spoilage flora. Then High Pressure processing could be useful to improve microbial safety of fish and seafoods by the inactivation of harmful microrganisms (such as *Vibrio parahaemolyticus*, *Salmonella* sp, *Aeromonas hydrophila*…).

Detrimental changes in sensory properties of fish muscle (texture, colour, consistence...) can be derived and depend on the levels of pressure, temperature and time of the treatment and the consequential oxidation of lipids and denaturation of muscle proteins.

HURTADO *et al.*(2000) [65] applied on hake fillets a procedure of 200 MPa and 400 MPa (by 3 cycles of 5 minutes) at 7°C. They observed that total counts after 200 MPa and 400 MPa-treatments were 1 and 2 $\log_{10}$ lower, respectively. In pressurized samples the increase in TMA was significantly smaller compared to the control until day 9°, after it increased faster in 200 MPa-pressurized samples than in 400 MPa-pressurized ones, probably for higher concentration of microbs able to convert TMAO to TMA in 200 MPa-pressurized samples. The control samples were rejected after 9 days. The shelf-life of 200 MPa-pressurized ones was prolonged by about 1 week and the hake fillets retained a raw appearence. The shelf-life of 400 MPa-pressurized ones was prolonged by about 2 weeks, but the hake fillets showed a cooked appearence.

CARPI et al. (1995) inoculated smoked creamed salmon samples with a variety of micro-organisms: *Salmonella typhimurium*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, *Penicillium expansum*, *Rhizopus oryzae*, *Clostridium sporogenes*, *Lactobacillus casei*, and *Enterobacteriaceae*. They reported that an extended shelf life from 60 to 180 days at 3 or 8°C (without significant chemical, microbiological or sensory changes) was obtained for high pressure-treated samples. They also suggested that high pressure-treated samples

must be stored at temperatures <3°C to prevent outgrowth of surviving *Clostridium botulinum* spores.

MYLLYMAKI *et al*.(1997) [129] treated pike perch fillets, raw salmon, smoked salmon, marinated salmon and eggs of baltic herring by pressures of 500 MPa and 700 MPa at 15-25°C for 5 minutes.

It resulted that Total Flora and H_2S producers bacteria were inactivated by 700 MPa effectively.

After 500 MPa a pink colour and a texture like ham were obtained in marinated and raw salmon, while in smoked salmon this level of pressure did not affect the appereance, only the texture was slightly harder than the reference.

Concerning the eggs of baltic herrings, after 500 MPa the structure became firmer, the eggs remained separated and the taste was mild and salty. The sensory qualities were still acceptable for at least 3 weeks.

Minced meat of mackerel (*Scomber japonicus*), treated at 200 MPa for 60 min, showed a decrease in bacterial counts by 80%, *Bacillus* sp., *Moraxella* sp., *Pseudomonas* sp., *Flavobacterium* sp. disappeared after pressurization [44]. Also a retard of 4 days in bacterial growth occurred when it was stored at 5°C. So from freshness indexes and sensory evaluation, the shelf life of pressurized samples was judged to have been prolonged by about 4 days.

Different studies were achieved on freezing and thawing of fish products by High Pressure [73; 85]. The lowering of melting point of the ice by the increase in pressure up to 220 MPa permits the thawing under High Pressure at low temperatures. The main advantages of pressure-thawing, compared to the conventional thawing at room pressure, consist in a reduction of procedure duration and of exudation volume. Sometimes an improving of microbial quality of the products occured [73].

The pressure-freezing permits to obtain small ice crystals in the whole product, then the preservation of the texture is guaranteed. Also the pressurization has the capacity to reduce the exudation phenomenon compared to the conventional methods of congelation.

Actually the industrial applications on fish products concern the fish in Japan and the oysters in USA. The fish sold in Japan (herring) is treated by High Pressure as frozen (185 MPa, -17°C) and an effect of pasteurization on surface or of inactivation of parasites larvae is obtained. The application on oysters is made at pressures of about 400 MPa in a short time directly in marine water in order to destroy pathogens as *Vibrio* sp. The treated oysters can be stored at refrigeration temperatures for about two weeks.

2.2.9 Regulation on "novel foods" [128]

The High Pressure treatment, being a non traditional procedure, has to be submitted, contrary to the other physical treatments, to a system of preliminary authorization.

This represents a non negligible obstacle to the development of their use.

The regulation of the foods is based on two essential points: the safety of the consumers and the loyalty of the commercial transactions, this last aspect including the consumers' right to the information.

As for the ionizing treatment, the regulation of the employment of the High Pressure in the treatment of the foods is marked by these two principles.

They concern some dispositions on the authorization conditions of these treatments and dispositions on the labelling.

In the case of the non traditional treatments as the High Pressure, the European Community, in the Rule 258/97 of January 27th 1997 related to the novels foods and to the new food ingredients, imposes a preliminary authorization.

The employment of the procedure is valued on basis of the modifications involving the foods. The foods, obtained by a new procedure, or a method not traditionally used in the elaboration of the foods, are considered as "novel" and for such reason submitted to specific obligations.

The status of the regulation, so conferred to the treatment by High Pressure, does not concern only it, but interests all non traditional treatments, that could develop in the future.

2.3 THE CAVIAR [21; 22; 112]

2.3.1 The sturgeon

The small black eggs manufactured to obtain the precious caviar, come from the two families of fish, the sturgeons (*Acipenseridae*) and the spatules (*Polyodontides*). The families of sturgeons include four genera: the "beluga" (*Huso*), the sturgeon in "stricto sensu" (*Acipenser*), the "scaphiryncus" (*Scaphirhynchus*) and the "false scaphiryncus" (*Pseudoscaphirhynchus*). The family of the spatules includes (*Polyodontides*) two genera, every genus has a single specie: the "American spatula" (*Polyodon spathula*), present in the Mississippi, and the "Chinese spatula" (*Persephurus gladius*) living in the Yang-Tse Kiang.

Nomenclature of sturgeon species

Acipenser stellatus, the "starry sturgeon", gives a caviar well-known on the market with the name "**sevruga**". *A.nudiventris*, the sturgeon with bare abdomen, is called more often "chip" than "Glattdick". *A.gueldenstaedti*, known also as "Waxdick", is well-known as "**oscietre**". The Russian names beluga, sevruga and oscietre correspond to the genera more used in the production of the caviar.

2.3.1.1 The genus *Huso*

The beluga (genus *Huso*) and the sturgeon "sticto sensu" (*Acipenser*) include some species of sweet water sturgeons and the maritime genus ("anadrome": the fish goes up the sea to depose in sweet waters) have in common the fact to depose in sweet waters. The five lines of bony shields, whose their body is gifted, are their principal characteristic. Their lanky body has cylindrical form and the lengthened face is provided of a pair of wattles, installed between the mouth and the extremity of the face. The females are bigger than the males. The genus beluga, that the Russian call the "great sturgeon", includes: the beluga in "stricto sensu" (*Huso huso*) and the kaluga (*H.dauricus*) also called "great siberian sturgeon". The size of the eggs of kaluga, as beluga eggs,

ranges between 2.5 mm and 4 mm. Their weight, at the maturity, can represent up to 25% of the total weight of the female. The kaluga (*Huso dauricus*) and the beluga provide the most precious caviar, whose big grey eggs are traditionally packed in blue boxes under the commercial name "beluga malossol". The beluga is present in the Caspian, Black, of Azov seas and in the oriental part of the Mediterranean. It is one of the greatest fish in the world (it can overcome 5 meters of length) and alives more than one hundred years. It reaches maturity between the 12 and 18 years and it goes up again the rivers to depose the eggs. The principal rivers where it deposes are: Turns, Ural, Koura, Don, Terek, Dniestr, Desna, Dniepr, Bug, Rhine, Danube and Po. The kaluga is a sweet water specie of the river Amur, which constitutes a part of the oriental frontier between Russia and China.

2.3.1.2 The genus *Acipenser*

The genus *Acipenser* (sturgeon in "stricto sensu"), which includes more than 16 species present in the European, Asian and American continents, is geographically the most diffused.

Acipenser sturio (known as "sturgeon of Europe", but also "sturgeon of the Atlantic", "sturgeon of the Baltic", "common sturgeon" or "créac", of the south-west of France) was present along the European coasts from the island of Nordkapp in Norway until the north of the Mediterranean, on the coast of north Africa and in the Black and Baltic sea, the English, Irish and south Iceland coasts. Once it abounded in the fiords of Varanger in Norway, in the lake Ladoga in Russia, in Moselle, Gironda and Guadalquivir in Spain, in Danube in Rumania, in Thames in England. Only the Danube has maintained some fisheries of sturgeon and continues to produce small quantities of caviar. It is an anadrome fish, which does not live much time in sweet waters. It can reach 3 meters of length and a weight of 200 kg.

Acipenser gueldenstaedti, the oscietre, also called "Russian sturgeon", is present only in Black, Azov and Caspian seas, where it alives in separate and well located groups. It includes two sub-species: the Iranian sturgeon (*A.gueldenstaedti persicus*), which deposes the eggs in the rivers

Babol, Gorgan and Safid-Rud and Koura in Russia and the sturgeon *A.gueldenstaedti colchicus*, less known.

The oscietre, anadrome fish, alives only in sweet waters and can overcome 2 meters of length. The oscietre, whose massive artificial propagation allows to safeguard the stocks of the Caspian, is the genus that provide the most important quantities of sturgeon and caviar in the world. The eggs of oscietre, smaller than beluga's eggs, measure 1.5-3 mm of diameter. Their color can considerably vary to such a point that sometimes the greatest black eggs are commercialized as eggs of beluga in the blue boxes. But in the commerce, the caviar oscietre is mentioned as "Oscietre malossol" and is conditioned in the yellow-orange boxes.

The sevruga or " starry sturgeon" (*A.stellatus*) is the third great specie used in Russia for the caviar. Sevruga is the name commercial correspondent to the smaller and black eggs of caviar, that certain experts also consider as the tastiest. The genus is peculiar for its long face, which constitutes the 60% of the length of the head and for the numerous small starry points scattered on the body among the abdominal and dorsal fins. The maximum size of the sevruga is 2.20 meters, its weight 80 kg and its duration of life around 30 years. The fertility of this fish various between 50,000 and 700,000 eggs of the diameter of 1.2-2.5 mm. Its caviar is packed in boxes with red cover. The "chip", sturgeon with bare abdomen (*A.nudiventris*) have some differences concerning the inferior lip and wattles compared to the other sturgeons. It is a fish with rapid growth, anadrome, that reaches a length of 2 meters and alives in Aral, Caspian and Black seas. Although its commercial value is recognized, its name has not resulted in an official appellation of the caviar. It is packed in yellow or red boxes in relation to the color of the eggs. The chip often produces some hybrids with the sevruga, the oscietre and the beluga.

A.ruthenus, the "sterlet", is a sweet water fish of small size (maximum 1 meter). It reaches maturity at 5 years and its fertility various between 5,000 and 100,000 eggs with diameter between 1.5 and 1.9 mm. It brings on its sides more than about fifty bony shields, that clearly distinguish it from the other sturgeons. Although it does not provide important quantities of caviar and shows only a

modest part of the commercial captures, the sterlet is well-known for its delicate taste. The soup of sterlet, note as "tsarskaya ukha", was the traditional dish of the Tzars and the Russian nobility. The sterlet occupies a vast zone of the Caspian, Black, Azov seas.

The sturgeon of Siberia (*A.baeri*) that populates the great rivers of Siberia, possesses a certain commercial value. It includes more anadrome sub-species living in the rivers and lakes having a life duration of 60 years. Its size ranges between 1 and 3 meters. The eggs have a 2-3 mm diameter.The sturgeon of the Amur (*A.schrencki*), which lives the river Amur, is allied with the oscietre. It is a sweet water fish, diffused in Russia and China for its meat and for its caviar. It is present in all the tributaries of the river Amur, it has also been observed in the rivers Chita and Sungari and in the lake Khanka. The sturgeon of the Adriatic or "Italian sturgeon" (*A.naccarii*) is a anadrome specie present mainly in the Po and the Adige. It is a rare and a little known specie.

The "green sturgeon" or "sturgeon of Sakhaline" (*A.medirostris*) is also a rare specie. This fish alives on the two slopes of the Pacific. Its different sub-species include three Japanese sturgeons: *A.medirostris mikadoi*, *A.medirostris multiscutatus*, *A.medirostris kikuchii*. The last sub-specie is Korean: *A.medirostris dabryanus*. As its name points out, the "green sturgeon", that can reach 2 meters of length, brings some greenish rays on the body. But this rare specie has never justified a real commercial diffusion. The "white sturgeon" (*A.transmontanus*) is one of the most important species of the american continent. This peculiar member of the family of the sturgeons is easily recognizable from the wattles on the face with very advanced position. It can reach 6 meters of length and more than 680 kg of the weight. Its area extends from Monterrey, in California, until Alaska on the Pacific coast. It has produced one of the more prosperous commercial peaches of the century, but the reserves are now fallen to extremely low levels. The "black sturgeon of America" (*A.oxyrhynchus*), the "sturgeon with short face" (*A.brevirostrum*) and the "yellow sturgeon of America" (*A.fulvescens*), that have determined a big fishing in the past, are the representatives species of the east coast of the American continent.

The "bester" is the most recent member of the family of the sturgeons. It is a hybrid obtained by the intersection among a beluga female and a sterlet male.

2.3.1.3 The spatules

Polyodon spathula, the "American spatula", had in the past a big commercial value. It can reach 2 meters of length and lives in the basins of the Mississippi, from Dakota of the North until the Gulf of Mexico, and until the State of New York and the South Carolina, toward the east.

2.3.1.4 The scaphiryncus

The only specie of scaphiryncus, among the single two present in the Mississippi, which has had in the past a good commercial value, is the *Scaphirhynchus platorhynchus*. It does not overcome 1 meter of length (modest size for a sturgeon). The second specie, *S.albus*, thicker, is nowadays very rare. It lives in the low waters of the Missouri-Mississippi and the river Kansas and its tributaries.

2.3.1.5 The false scaphiryncus

Psudoscaphirhynchus occupies the basin of the Aral sea. This specie is composed of three species: *Pseudoscaphirhynchus kaufmanni* or "great false scaphiryncus" and *Pseudoscaphirhynchus hermanni* or "small false scaphiryncus", which live in the Amur-Daria, *Pseudoscaphirhynchus fedtschenkoi*, today very rare, alives in the Syr-Daria. Some of these sturgeons have never had any commercial value and they seem voted to the disappearance because of the catastrophic damage that the sea of Aral has suffered. In URSS numerous laws control the fishing to the sturgeon, they establish the quotas, the zones of fishing, the calendar of opening of the fishing etc. Currently the commercial and the sporting fishing are severely limited in all the European countries as in Iran, Canada and United States. But all these measures are not enough to resolve the reduction in reserves due to the poaching's development. The sophistication of the modern means at disposition of the poachers and the disappearance of all the forms of authority in certain regions can give some explanation of the phenomenon. During this century many countries have tried to interdict totally the fishing to the sturgeon in certain regions. Such measures have known an undeniable success in countries as Russia, the United States or Canada, and for a certain period of time, the stocks seemed

able to occur again. But subsequently the pressures for the reestablishment of the fishing, to which are added the effects of the pollution, of the construction of dikes and other interventions of the man on the environment, have not stopped the risks for the populations of sturgeon. Therefore certain species are completely disappeared and the among them some species are on the list of the species close to the disappearance. All these problems have induced the development of fit techniques as the artificial repopulation of the sturgeons through techniques of insemination of the eggs and techniques of hybridization (especially in the Russia). Also the increases of sturgeons breeding in Europe ands USA (aquaculture) is much developed. Big part of the caviar today commercialized on a large scale derives from aquacultured sturgeons.

2.3.2 The caviar [21; 22; 112]

The term “caviar”, perhaps of Italian ("*caviare*") or Turkish ("*khavyar*") origin, appeared for the first time in 1458 in the writings of Platina, administrator of the pope Pio II. It appeared also later in the XVI century in different documents in Italy, France, Spain and England. In Russian, the caviar is designate with the word "ikra" that means "egg" and "ovaries" in biological sense. but it corresponds equally to a whole series of products derived by the fish eggs, among which the same caviar, but also the ovary-based products, whole or cut in pieces. The Japanese call the salmon caviar "ikura", exclusively to define the salty caviar of salmon, while the whole and salty ovaries of salmon are designated with the term "sujiko."

2.3.2.1 Different types of caviar

The fish eggs provide a large number of alimentary products. They represents, in weight, the part most important of the mature female fish. Their nutritional value is superior to that of the meat of the same fish, since they are systematically richer in proteins and in lipids. The fish eggs, with a pleasant taste, are protected from the membrane of the ovary and they are naturally sterile. Because of the connective tissue that maintains them, they forms a compact mass, solidly attached to the

membrane of the ovary. When the maturation of the eggs is reached, the connective tissue disappears and the eggs detach, it is what happens when the female deposes. The fish eggs provide three types of food specialities: the whole ovary-based products, the patè and the caviar. The whole ovaries can be cut in pieces or not. Subsequently they are salty, dried, marinated, smoked, fried or pressed, according to the cases. In the case of the patè and the other culinary products, the eggs are mixed to the salt some aromas, then pasteurized or cooked. The caviar is composed of fish eggs that pass in a sieve to be separated from the connective tissue of the ovary before the salting. The caviar is always composed of individual grains. It can be pressed, and in this case it is defined "pressed caviar". The term caviar can be linked to a series of names that qualify it both in relation to the origin specie (sturgeon, salmon, lumpfish...) or the aquatic environment where the fish has been fished (caviar of the Caspian, of the Amur, of the Danube...). Other references are to the methods of manufacture, by which it is subjeceted, as "caviar in grains", "pressed", "pasteurized" or "spicy." The labelling is subjected to various rules from a country to another country. On March 1917, in the United States, the FDA, called to decree on the use of the term caviar, has established in one statement that the term "caviar" can appear only on the packages of sturgeon eggs, while all the other products prepared from eggs of other species have on the labels the written "caviar of... " to complete with the name of the fish. The rules of the labelling demand also that they show up the weight, the origin's country of the product, as the name and address of the society, responsible of the packing, or the address of the distributor. All the labels containing words, expressions, figures or symbols referring wrong informations about the origin of the product, its quality or used technology, constitute a violation of the rules in vigor.

a) Salmon caviar

The five great anadrome species of Pacific ocean salmon are the principal usable resource for the production of salmon caviar. They are respectively: the "salmon keta" (*Oncorhinchus keta*), the "pink salmon" (*O.gorbuscha*), the "silvered salmon" (*O.kisutch*), the "royal salmon" (*O.tscawytscha*) and the "red salmon" (*O.nerka*). The "salmon masou" or "Japanese salmon"

(*O.masou*) represents a resource of weak importance in some regions of Japan and Korea. The "salmon of the Atlantic ocean" is object of a breeding on large scale (its production was estimated 250,000 tons in 1991), but it is commercially exploited before having reached sexual maturity. Aquaculture-reared rainbow trout (*O.mykiss* formerly *Salmo gairdneri*), specifically the ocean run cariant "steelhead" trout, often referred to as salmon trout in Europe, is also a popular source of red caviar. The USA exports over 90% of the salmon eggs it produces to Japan, France, Spain, Germany, Taiwan, Holland, China, Korea and Scandinavia. Salmon eggs are primarily exported to Japan as "sujiko" (salted and cured whole ovaries or skeins), "ikura" (cured single egg product), or frozen green or unprocessed eggs. Farmed salmon is most commonly harvested before reaching sexual maturity.

b) Lumpfish caviar

Lumpfish (*Cyclopterus lumpus*) roe is an extremely popular, moderately priced caviar. The small eggs (2-5 mm diameter) have a colour purple-red at reached maturity. When they are still immature the colour is green or gray-white. Traditionally the eggs of lumpfish were salty abundantly after the unloading. The product, so obtained, could be preserved one year or more. The salty eggs were turned into caviar in a second time. In past, Iceland, the greatest producer of ovaries of lumpfish, salted the eggs of lumpfish, while the caviar of lumpfish was manufactured subsequently in the buyers countries of north Europe. The tendency to the manufacture of the caviar of lumpfish next to the zones of fishing has had more recently the effect to strengthen the quality of the product. Lumpfish roe can survive pasteurization better than other caviar products and can be produced at a high enough salt content and low enough water activity to make it shelf stable.

c) Whitefish Roe (fish eggs)

Other fish with roe of small size that are sold commercially are mullet (*Mugil cephalus*) and the roe from various whitefish: Pacific herring (*Clupea pallasii*), Atlantic herring (*Clupea harengus*), perch (*Perca fluviatilis*), Atlantic whitefish (*Coregonus huntsmani*), lake whitefish (*Coregonus clupeaformis*). These roes are brined, cured and sometimes smoked.

d) Cod Roe

Salted cod roe (*Gadus morhua* and *Gadus macrocephalus*, respectively Atlantic and Pacific cod) and the roe from other whitefish are sometimes flavored with sugar, treated with nitrite, and smoked. Oil, potato flour and other ingredients may also be added to these products. These roe may be aged for several months before they are sold. A common Scandinavian spread is produced from cod or other roe that is blended with cream cheese and consumed by younger individuals much as peanut butter is in the USA.

e) Other fish roe products: kazunoko and kazunoko kombu

"Kazunoko" or "yellow diamond" roe is cured whole herring egg skeins. The herring roe is not immediately removed from the fish. Instead, the fish are frozen with the objective to preserve the natural shape and form of the roe sacs within the fish. The frozen herring is then shipped to processing plants where the kazunoko is produced. A most interesting herring roe product is "kazunoko kombu". The product is used in a variety of dishes, most commonly soups and can be very expensive, often times over $100.00 per pound. For the highest quality kazunoko kombu, an uniform dense layer of herring eggs of similar size and color covers both sides of a piece of kelp.

f) The "artificial caviar"

The increasing success that the caviar has received and the elevated level of the prices, to which the unequal division of the world resources of fish eggs is added, has induced the researchers and engineers of the food industry to find out new caviar-like products. During last years, the industry of the artificial caviar is very developed in countries as Spain, Russia, Japan and Israel, with the purpose to imitate the two types of caviar more appreciated: the sturgeon and salmon caviar. Since more than 2000 years Spain produces the "mujol", a traditional commodity obtained drying at the sun the ovaries of mullet. Currently the country produces an artificial caviar called "Mujol Shikran", composed by the 40% of eggs of mullet and commercialized under the appellative "Eurocaviar". The other commodities quite similar to this product, eggs-based, freeze or fresh, are the eggs of herring and salmon. From Russia two types of artificial caviar are produced, whose commercial

name is respectively "red caviar in grains" and "caviar of protein" both consumed inside the country. In the composition of the red caviar in grains, obtained from salty eggs, the vegetable or fish oil, agar or glaze, aromas and colourings (carotenoides) make part. Instead the caviar of protein does not contain fish products, for this reason it probably represents the most artificial of the “artificial caviars”. In fact the caseine of the yolk of chicken, the glaze, extracts of tannin, aromas and artificial colourings enter in its composition.

2.3.2.2 Sensory characteristics of caviar

While the morphology of the eggs of fish is generally the same (every egg being composed of an pigmented emulsion included inside a membrane), the size, the colour, the form, the resistance and the taste of the eggs varies according to the species. The emulsion, a colloidal solution, that confines a germinal vesicle and the globules of lipids of different size. The external membrane of the egg is porous and has a resistance and thickness depending on the number of its layers. The colloidal liquid contained inside the egg ("vitellum") possesses an own membrane and the space which separates this membrane from the external membrane has called "space perivitellinum." During the salting this space absorbs the brine and swells, determining the round form and the stability of fish egg. If all the eggs of a same fish have an identical colour, some tonalities can be observed by an individual to the other within a same specie. This occurs particularly in the sturgeons, that show numerous tonalities of grey, from the grey clear to the grey dark passing through the black, the grey-yellow, the grey-brown, the green-grey, even a dark gilded color. The salmon eggs vary from the clear orange to the red passing through the red-orange, while the eggs of the other species of fish have very pale color in the shades of the grey, yellow, pink, green or brown. The lumpfish eggs can have purple or brown colour. The eggs, at reached maturity, have a brighter aspect than the immature and opaque eggs. The resistance is habitually valued through the crushing of the egg up to the breakup. Fragility can reveal a missed maturity, a lack of freshness, a preceding frozing. This technique is valid only for the eggs of big size as the sturgeon or salmon

eggs. The smallest eggs of herring or carp have often a more solid external membrane. The taste is specific for every specie, although the diet of the fish and waters nature of its habitat are able, in some cases, to modify it a little. Sometimes the sturgeon can have a back-taste of grass. Instead, the red salmon or the silvered salmon can manifest a light bitterness, while the pink salmon eggs are sweet. The polluting products can also produce some parasitic tastes in the fish eggs. Salt is able to disappear this taste or at least to disguise it.

2.3.2.3 Chemical composition of fish eggs

The chemical composition of caviar varies from species to species, with the condition and maturity of the eggs, within and among harvests areas, and from year to year. Like poultry eggs, fish eggs have high concentrations of lipids (5-20%) and proteins (16-30%). As the egg matures, the mass increases and the ratio of moisture to lipid increases. After the eggs are brined, the percentage of moisture decreases, the protein content increases because the water is removed during salting process. The ash content increases because of the added salt, and the lipid content increases proportionately as moisture is removed during curing. The predominant lipid components in fish eggs are triglycerides or phospholipids. Triacylglycerols and phospholipids are the major class of lipids in cured trout (*O.mykiss*) eggs. The higher lipid content, in general, of the Salmonidae eggs compared with Clupeidae eggs may be a results of differences in energy needs for the fertilized eggs, even though the muscle tissue of the Clupeidae is among the food fish with the higher muscle fat content. The cholesterol content of fish eggs is about one-fourth of that of chicken eggs. It ranges from 300 to 500 mg/100 g. The fatty acid composition of the neutral lipid fraction in fish eggs contain about 24% saturated fatty acids and about 37% of ω–3 fatty acid content. Fish eggs are also a rich source of vitamins (A, D, B1, B2, B12, C, E) and contain minerals as calcium, iron, magnesium, manganese, phosphorus, potassium, copper and zinc. The protein quality of fish eggs is high because of its amino acids profile, in fact fish proteins contain all the essential amino acids (cystine, aspartic acid, threonine, serine, glutamic acid, tyrosine, histidine, leucine, isoleucine,

proline...). Specific protein fractions can be recovered particularly in fish viscera. One such example are the basic proteins or protamines which can contain as much as 65 percent of arginine [130].

The caloric content of caviar is between 370 and 320 calories/100 g and can vary with the fat content. Salmon caviar products are highly digestible and have been used traditionally in Russia to aid patients recovering from surgery as well as to treat rickets in children. The proximate composition of eggs from different fish is provided in Table XIV.

Table XIV – Chemical composition of fish eggs

Specie	Moisture (%)	Proteins (%)	Lipids (%)	Ash (%)
Caspian Sturgeon (*Huso* sp.)	57-65	17-32	11-18	1.0-2.2
Salmon Keta (*O.keta*)	50-56	27-35	12-20	1.5-1.7
Pink Salmon (*O.gorbuscha*)	50-60 F	23-38	10-15	1.9-2.0
Red Salmon (O.*nerka*)	56-58	20-29	10-13	0.7-1.7
Rainbow trout (*O.mykiss*)	51-70	21-34	7-9	1.2-1.9
Pike (*Esox* sp.)	64-67	14-27	1.5-2.4	-
Cod (*Gadus morhua*)	78-80	16-20	0.3-0.7	1.7-2.3
Herring (*Clupea* sp.)	70-80	14-25	0.7-4.8	1.8-3.7
Mackerel (*Scomber japonicus*)	65-71	21-30	4.4-6.3	1.0-1.9
Lumpfish (C*yclopterus lumpus*)	80-85	10-12	4.4-4.7	1.5-2.0

From "Caviars and Fish roe products",Bledsoe G.E.et al., Crit.Rev.Food Sc.Nutr.,2003.

2.3.2.4 Microbial quality of fish eggs

Eggs that are aseptically removed from fish are microbiologically sterile. However, when the eggs are used in commercial products or for home use, they do not remain sterile. It is well-known that fish carry bacteria on the surface of their bodies if the water they live in is microbiologically contaminated. During the process of excerption of the eggs from the fish, the bacteria on the surface of the fish may be transferred to the eggs. Thus, these can form great risk for the finished product. To protect the caviar from bacteriological spoilage from the raw product to the finished product, it is very important to do continual control of hygiene and sanitation by bacteriological analysis throughout the flow of the process. Also the product composition (pH, salt concentration...) and storage at suitable conditions, according to product type, are other important factors [14]. Black

caviar can be contaminated with various species of opportunistic bacteria, cause of its spoilage (*Proteus* sp., *Flavobacterium* sp., *Moraxella* sp., *Pseudomonas* sp., *Lactobacillus* sp., *Enterococcus* sp., *Micrococcus* sp., yeasts and moulds), but also contaminated with various pathogens (*Vibrio* sp., *Aeromonas* sp., *Clostridium botulinum, Staphylococcus aureus, Listeria monocytogenes, Salmonella* sp.). All these micro-organisms arise from the flora of the fish and can be trasmitted to fish eggs in the course of processing and it can affect the product negatively due to the lack of hygiene and sanitation during caviar production [14]. Tools and utensils used especially in the old technology had not suitable aseptic conditions. Because this possibility it has been stated that *Clostridium botulinum* was frequently isolated in the periods when the fish eggs products were produced through the old technology. Although this production is carried out with the latest technologies nowadays, it is still possible to observe microbial contaminations [14; 60]. ALTUG & BAYRAK (2003) examined by microbial analysis caviar samples from Russia and Iran. In their results FMAT ranged from 10^3 to 2.6 x 10^6 cfu/g, coliforms from $<10^1$ to 2.4 x 10^4 cfu/g, yeasts from 10^1 to 6 x 10^5 cfu/g, *E.coli* from $<10^1$ to 3 x 10^2 cfu/g. *S.aureus* was detected as 5 x 10^2 cfu/g only in one sample. *Salmonella* sp., *C.perfringens* and moulds have not been isolated in any samples. Generally the FMAT level is never below 10^5 cfu/g for the foods leading to food-poisoning. Although the FMAT is considered as a quality standard for the food samples, there is not any direct correlation of it with pathogenic micro-organisms present. However FMAT is an important indicator for the life cycle of the product and the potential for growth of the micro-organisms present. It constitutes a criterion in the determination of the general microbiological quality of the product [42]. FMAT values observed above [14] indicate that the caviar samples were close to exceed the critical limits specified in respect of microbiological quality. High counts of FMAT are associated to a product with poor quality [84; 51]. Furthemore the existence of the coliforms in caviar samples was the sign that the product was subjected to process under inefficient hygiene conditions. Among different species of micro-organisms causing food spoilage there are the yeasts.The yeasts cause organoleptic spoilage, that visually observed, depends on the time and

reproduction intensity and affects the commercial quality of the product in a negative manner. It is very inportant to limit their development in the food using the best techniques that prevent this development and assure the food preservation and in consequence the food safety. For example a correct salt concentration (4% in first quality black caviar, maximum 5% in second quality caviar), an acidic pH value (pH 4-5), pasteurization thermal process protect the product from bacterial spoilage. The samples with high counts of FMAT, coliforms and yeasts are products processed under deficient hygiene and sanitation. So the caviar production requires attention related to the bacterial growth, because this product is an optimum mean for bacterial growth [14].

Caviar can pose food safety risks if not properly processed and handled. Fish eggs can only be heated to a temperature under 70°C without the eggs becoming opaque or loosing color. Irreversible protein denaturation occurs between 70 to 80°C. Caviar can be pasteurized, but this is not yet a widely used practice, because the changes in sensorial properties of the thermically treated product, not appreciated from consumers [21]. Refrigerated caviar products are often packaged in the same way as pasteurized products (in glass jars), and the possibility of unintentional thermal abuse can easily occur, because products are often not adequately labeled. The salt concentration and storage temperature should be key preventive measures to preserve and make caviar safe. Salt concentration varies widely among caviar products of the same type, but consumers prefer caviar with a lower salt content [21]. The FDA requires a high salt content to inhibit the germination of *Clostridium botulinum* spores [43]. In general, the salt content alone may not be high enough to inhibit the germination of *C.botulinum* spores. *C.botulinum type E* can grow at 4.5 to 6% salt and at temperatures of 3.3°C [43]. Although pH can sometimes be adjusted downward as a preventive measure for *Clostridium botulinum*, this is not recommended because of detrimental changes to product flavor. Preservatives can be added to caviar because their antimicrobial effect. Some chemical preservatives, like borax, urotropin and formaldehyde, can be added to Russian black caviar. In Europe the borax is the additive more used during the caviar processing.

Caviar is generally distributed at refrigeration temperatures. However, the refrigeration temperatures may not be low enough to inhibit the growth of microbial pathogens such as *Listeria* sp. Therefore, there is a strong incentive to develop pasteurization processes that could improve product safety. Because protein denaturation occurs in caviars between 70 to 80°C, long pasteurization times at 50 to 70°C are realizable, but cause loss of product quality.

2.3.3 Processing of fish eggs into caviar

Often hundrends or thousands of individual eggs are enveloped within ovarian membranes. These skeins of eggs can be processed into fish eggs products, or the individual eggs can be recoverd separately and the processed. Fish eggs are generally processed into three products: whole ovaries (such as "sujiko" from Pacific salmon), individual eggs ("caviar", and pate or other products such as "dried mullet eggs". Eggs must be an optimal level of maturity to produce caviar. Immature eggs tend to produce caviar that is bitter or which does not take up salt uniformly.Overly mature eggs may be soft and lose their elasticity. The main steps of fish eggs processing for the production of caviar are: the screening, the salting, the dripping and the packaging.

2.3.3.1 The screening of fish eggs

The distinctive line of the caviar, in comparison to the other products manufactured from fish eggs, consists in the fact that the eggs are separated from the ovary and from the surrounding tissues. The technique of eggs separation is the "screening", often effected by hand. As more precious are the eggs, more it is probable that they will be sieved manually rather than mechanically. The first procedure allows to minimize the losses than the second. The mechanical screening is mainly justified in the case of the large captures during a short period of time or when the value of the eggs is particularly weak (for example, in the case of the eggs of ordinary fish). The ovaries are initially rinses, then dripped and examined with the purpose to discover the possible presence of blood clots or other extraneous bodies. They are pressed subsequently on a sieve in

metallic thread. The person, responsible of this job, has to give to the ovary a very sweet circular movement until to force the eggs to pass through the grate, while the connective tissue remains in surface. If the movement is too much abrupt in comparison to the resistance of the eggs the losses are notable.

The use of the enzymes, to decompose the connective tissue of the salmon or trout ovaries, is a technique well-known since 1960. It advantage is to allow the use of ovaries at less advanced maturity stadium in guarantee of the outputs in more elevated eggs than the others obtained by the manual sifting. The supporters of this technique also insist on the diminution of the costs of manpower that it implicates. The used enzymes are generally proteases from vegetable or animal origin. The time of treatment can vary from a few minutes to a half hour and the enzymatic concentration of the solution ranges between 0.02% and 0.1%. This procedure is used for the manufacture of the trout caviar from breeding.

2.3.3.2 The salting

Salt is the principal, and sometimes the single agent of the caviar preservation, even if it resorts to the pasteurization or to chemical preservatives. Salt inhibits the growth of the bacteria, that degrade the product and can be dangerous for the human health. It also contrasts the action of the enzymes. But the evolution of the public taste does not permit to incorporate to the products the doses of salt necessary to a good preservation and stabilization. The rate of salinity of the caviar is generally 3.5-4% for weight, while the necessary rate for a reasonable preservation is 10%. The pH of the caviar products is generally placed among 4.3 and 5.9, that it is not sufficiently acid to stop the growth of all the harmful bacteria. Some micro-organisms are particularly well adapted to the environments at high concentration in salt. It is the case of a yeast, *Torulopsis candida*, present in the brines, in the fish and sometimes in the caviar. It is able to develop in an environment with 20% of salinity and at temperatures next to the point of congelation. Instead it does not withstand to a traditional pasteurization at 60°C for 20 minutes. Some micro-organisms, resistant to the salt, can

be present in the same medium, if its rate of impurity is important. It is the cause for which the producers of caviar have to use always the best quality of available salt on the market. The caviar of sturgeon is salty by crystalline salt (4.5-5% of the eggs weight). The rate of salinity is about 3 and 3.5%, after all the useless water, naturally present in the product has been eliminated. The salinity of the final product is varying according to the maturity and the freshness of the eggs, the temperature of the mixture eggs/salt and the time of salting. The attainment of a specific salinity of the caviar depends largely on the experience. The eggs of salmon, whose rate of final salinity is about 3.5-3.8%, are salty in a saturated brine, that is a saturated solution of salt. It is not rare to find on the market some lotteries of caviar insufficiently or excessively salted. The societies, that assure a further packing of the products, are forced sometimes to effect a second salting or rinse, according to the case, with the purpose to correct the rate of salinity. But this kind of procedure can be prejudicial to the product and can simply make it unsuitable to the consumption. Therefore it is essential to reach the objective of salinity during the first salting procedure.

2.3.3.3 The dripping

The dripping and the dry of the salty eggs have an important effect on the viscosity of the liquid inside the eggs. Once salted, the eggs of sturgeon have started dripping in layers of 3-4 cm of thickness on a sieve during a period of 5-15 minutes. They are subsequently packed. When the caviar of sturgeon is packed in the regulation metallic boxes of 1.8 kg, the drip of the eggs can be achieved by inclination of the boxes. These boxes are traditionally filled by hand up to 10-15 mm below the edge of the cover, then the cover is set to the summit and progressively sunk. The boxes are settled then on a side during about two hours to eliminate the last drops of liquid but also the present air among the eggs. When this operation is finished, the boxes are clean and they are covered a layer of caoutchouc destined to assure the fixation of the cover. The presence of juice to the fund of the container is index of insufficient drip. In case of drip or excessive dry the eggs risk to have tendency to aggregate.

2.3.3.4 Packaging of caviar products

The 50% of the Russian production of sturgeon caviar are vacuum packed and pasteurized in containers of glass (of 56 and 113 g) and in metallic boxes of 90 g. The rest is packed in metallic boxes of 1.8 kg and 600 g. The Iranian exporters assure the packing of a small volume of sturgeon caviar in the metallic boxes of 100, 200 and 300 g. The rest of the production is, also, packed in metallic boxes of 1.8 kg and 600 g as the Russian ones. In Russia the salmon caviar is packed in boxes of 150 g and in plastic buckets of 5 pounds. In Japan the salmon caviar is packed in boxes of wood containing 1-3 kg of product. The inside of the boxes is covered by many layers of cotton cloth smeared of saturated brine and vegetable oil. Despite this protection, the external layers of the caviar are sometimes too dried before reaching the table of the consumer. In Japan the great stores pack again the salmon caviar in the containers of plastics of 50 g. The type of packing and its size are function of the commercial sector. The most popular model is the small container of glass with wide opening and slightly conic. The sizes used more frequently are: 25, 50 and 100 g. The other types of detail container are the boxes in aluminum of 100-150 g. There are also some metallic boxes vacuum packed and provided of a cover, which can be easily opened. After opening of a packing of detail caviar, the product must to be consumed within two or three days.

2.3.3.5 The chemical preservatives, the aromes, the colourings

<u>The preservatives</u>

The preservatives are an effective mean against the alteration of an expensive and perishable product as the caviar. The Russian norms, destined to guarantee the quality of the products to export, interdict the manufacture of caviar without addition of preservatives. But the used preservatives are subjected to the rules present in the importing countries, that evolve frequently.

USA have adopted the principle GRAS (Generally Recognised AS Safe), that means “recognized not dangerous”. Instead European Community has opted for a classification that consists of a number corresponding to the additive, preceded from “E”. The use of the borax is forbidden in USA, but in Russia is authorized for the products destined to the national market. Here the different mixtures of preservatives traditionally used for different types of caviar in USSR:

- Borax and boric acid (0.3% and 0.1% respectively);
- Borax and urotropine (0.3% and 0.1% respectively);
- Nitrate of potassium (0.16%);
- Sorbic acid and urotropine (0.1% every compound);
- Urotropine and tripolyphosphate (0.2% and 0.15% respectively);
- Urotropine and benzoate of sodium (0.1% every);
- Benzoate of sodium (0.1%);
- Nisin (0.1%).

The nisin is an antibacterial substance produced by some strains of the genus Lactococcus (*L.lactis*). It is much used in the manufacture of the cheese. But it has also given good results for the preservation of the caviar. The nisin specifically sticks to the spores and inhibits their germination. France authorizes the employment of hexamethylenetetramine or urotropine and benzoate of sodium in equal proportions, the addition of the two substances doesn't have to overcome 0.1%. Switzerland authorizes the employment of the formic acid at rate 0.05 %. The addition of the preservative can be made in different ways. When the eggs are salty in dry, as in the case of the sturgeon caviar or the lumpfish eggs, the salt and the preservatives, under crystalline form, have to be mixed and administered at the same time. The residual level of the preservative in the final product is in relation to the degree of salt absorption during the dripping that follows the salting. In the case of the caviar salty in brine, the preservative is added after the salting and before the end of the dripping. The used preservatives are often soluble in the water. The antimicrobial power of a preservative is tied up to the pH of the product. For example the benzoate, effective against yeasts,

moulds and bacteria, is active up to pH 4 and it is not more valid beyond 4.5. Then this means that the effectiveness of the sodium benzoate in the antibacterial struggle is very limited in the case of the caviar. Contrary the sorbic acid and the sorbate of potassium are more effective against yeasts, moulds and bacteria until to a pH of 6.5. A mixture of the two additives would be ideal against an larger range of bacteria, but this type of product is not authorized by the regulation, that forbids the use of the sorbates in the caviar preservation. The additives generally introduce the drawback of a back-taste, even if the modern techniques of aromas addition allow to eliminate the undesirable tastes. The benzoate of sodium is instead characterized by a sweet taste. The urotropine has a very pronounced sweet-bitter back-taste, while the sorbate of potassium is neutral from the point of view gustatory.

The aromas

In the 1854 R.G. Westacott of Worcester, in the Massachussets, has deposited a brevet (recorded as n.7895) based on: “the improvement of the taste of the sturgeon caviar with the incorporation of oil extract from the liver of male sturgeon”. Currently the caviar of sturgeon doesn't contain aromas anymore. The caviar of salmon contains oil and agents destined to give it a more sweetish taste. The mixed glicerol/oil is used for the red salmon caviar, where it compensates the traditional light bitterness. The vegetable oil avoids that the eggs clump the one to the others and it confers them a bright aspect. The aromas are often used for the insipid eggs of ordinary fishes (codfish, herring, lumpfish, pike caviar) and among the aromas are used: the extract of laurel, the sugar, the sauce of soy, the spices, the vinegar, the juice of lemon, the anchovy and the juice of meat. On the market aromas of salmon, also of sea fruits, and also caviar can be found. The very particular taste of the sturgeon caviar has been confirmed from numerous organoleptic tests. It can be defined: delicate, sweet and pleasant, similar to that of the egg yolk.

The colourings

They are entirely used for the caviar from ordinary fish, to the eggs of small size in order to make them more attractive and with shining colour, or in the case of lumpfish eggs with the purpose to

imitate the caviar of sturgeon. As the preservatives, the colourings are object of a narrow regulation in every country, and in operation of the product and of the authorized doses. In USA the code of good practice, which fixes the least rate of a colouring, exists. The colourings of vegetable origin are not sometimes considered by the regulation of some countries. The colouring added to the carp caviar is based on an extract of beet that gives its sweetish taste. The employment of synthesis alimentary colourings is systematically subdued to regulation. Certain colourings can have both a natural and artificial origin. It is the case of the caramel and the carotenoids. All the colourings are on the label in the form of a name or of the corresponding code.

2.3.3.6 Pasteurization of caviar

The containers of caviar in detail are often submitted to a pasteurization. The pasteurization has as purpose of destroy the majority of the micro-organisms and not the totality, as in the case of the sterilization. The pasteurization aims to lengthen the shelf-life of the caviar, even the storage at room temperature during a short period. As technique of caviar preservation can be completed by the addition of preservatives. The pasteurization is expensive for times and energy. It can have negative effects on the organoleptic qualities of the product if it does not respect certain technological criterions. The range of temperatures is relatively low, between 50 and 70°C, and the proteins present in fish eggs don't suffer an important coagulation, this explains the fact that their aspect is not modified. Some changes of colour can be observed: the salmon caviar appears paler, while the caviar of white fish slightly darkens and takes a yellowish color. The different types of caviar don't react all equally to the pasteurization: the eggs of sturgeon, very delicate, have tendency to harden, while the eggs of salmon become softer and, if they are immature, they have the tendency to get deformed. Only the caviar, whose freshness and maturity are real, can be pasteurized. In products of scarce quality the eggs are susceptible to break and to free bad odors, that can make the unsuitable caviar to the consumption.

2.3.3.7 Frozen caviar

The freezing of the caviar allows to lengthen considerably its shelf-life (up to two years). The quality of the product does not hear again of this procedure if the rules of freezing and thawing are respected. The eggs of small size (from white fish or herring) are lent well to the freezing, being endowed with a resistant membrane to the breakup, possible phenomenon during the freezing or thawing. The foods containing salt must have frozen to a lower temperature than the others since the present liquid inside the cells freezes to an inferior temperature to that of the non salty water. For example the salmon caviar with 3.5% of salinity starts to freeze at -7.5°C. The temperature of freezing and storage never has to overcome -20°C. For a prolonged stored the Japanese researchers recommend a temperature of -40°C. The frozen caviar has a paler color than the fresh caviar, although in the case of the salmon caviar, the taste and the texture do not change compared to the fresh product. The thawing is a delicate procedure, because if effected too much rapidly can provoke the breakup of the eggs membranes. This operation must to be sufficiently long in order to minimize the losses in eggs and the production of liquid that is accompanied to their breakup. The best results are obtained at temperature of 2°C. Despite the advantages of the freezing, the European and American consumers continue to be reticent respect to the frozen salmon caviar, contrarily to the Japanese people that do a big consumption of it.

2.4 AIM OF THE WORK

The aim of this work was to test the behaviour of different types of caviar under High Pressure both from a point of view of the sensorial quality and of the microbiological quality.

In the first case the analysis of the changes in parameters as colour, structure, texture consistence, was made at different treatment conditions in relation to pressure, temperature and time.

In the second case the study of the inactivation rates by High Pressure of two foodborne pathogens, as *S.aureus* and *S.enteritidis*, inoculated in TSB medium and fish eggs samples, was performed.

This work founds its basis from the emerging interest of consumers about the sturgeon caviar and also the caviar from other fish eggs, and the high request of safe products having fresh-like organoleptic qualities.

3. MATERIALS AND METHODS

3.1 EQUIPMENTS HIGH PRESSURE

The High Pressure treatments of the samples were achieved by the use of two isostatic equipments, destinated to applications in food science field, belong to the laboratory M.H.P. ("Matériaux et Hautes Pressions"), interface of research between an Institute of C.N.R.S. (Institut de Chimie de la Matière Condensée de Bordeaux - I.C.M.C.B.- UPR.CNRS.9048) and an engineer school (Ecole Nationale Supérieure de Chimie et de Physique de Bordeaux – E.N.S.C.P.B.), both on the Campus of the University Bordeaux 1 "Sciences and technology".

3.1.1 Device of "indirect compression"

This hyperbar apparatus was realized from the research group "Matériaux et Hautes Pressions" for the applications in the food field. It is a device of "indirect compression".

The equipment is constitued of two parts (Fig.11):

- a generator of High Pressure using water as fluid placed outside of the vessel,
- a vessel with constant volume.

The vessel, built in steel with high mechanic characteristics (elastic resistance = 800 MPa, mechanic resistance = 1000 MPa at 300°K), has an useful height of 430 mm, an useful diameter of 50 mm, and an useful volume of 0.85 l. It is closed from a tubular obturator where the pressure of a hydraulic jack is applied (maximum force: 1800 kN). The obturator and the neck of the vessel are in direct contact, metal on metal. A light difference of conicity and the mechanic resistance of the their materials ensure the watertightness.

Fig.11 : Apparatus for producing isostatic pressure through indirect compression developed at the M.H.P.

The water is compressed by a hydraulic pump supplied in pressed air (up to 700 MPa). A piezo-electric sensor, with a precision +/-5 MP, permits to control the pressure. The procedure of its funtion is entirely manual. After placing the prepacked sample in the vessel and fill it with water, the obturator is put on the neck of the vessel. The hydraulic jack is put down and maintains a sufficient force on the obturator for ensuring the watertightness between the neck the vessel and obturator. The water under pressure is transported from the exit of the High Pressure pump to the internal part of the vessel, by rigid tubular tubes in stainless of internal diameter of 2.4 mm and external diameter of 6.35 mm. The rise speed in maximal pressure is 200 MPa/min, manually setting. A disk of rupture, sited on the circuit of water under pressure, represents the safety system in the case of exceeding of the pressure 700 MPa. The pressure is maintained constant by the closer of the gate of isolation between the pump and the vessel. After treatment, the depressurization is obtained stopping of the High Pressure pump, opening the gate of isolation and then the gate of draining. The minimum time of depressurization is < 3 sec, it can be increased but without precise

control. The heating unit (up to 120°C) is constitued of heating resistances around the vessel. The temperature is regulated at +/- 2°C of the instruction, by a rheostat. It is measured inside the vessel by a thermocouple, slided through the obturator in a metallic shaft with a form of "finger of glove", linked to an electronic table. This device permits to achieve experiments at 4°C, but cooling the vessel by immersion in a water bain additioned of glace.

3.1.2 Device of "direct compression"

The hyperbar apparatus (Fig.12), constructed by NFM-Technologies (Le Creusot, France) and FRAMATOME (Paris, France) and commercialized by CLEXTRAL (Firminy, France), is based on the modifications of the volume of the high pressure vessel.

The pilot has the following features:

- capacity of the pressurization surrounding wall: 3 l,
- internal diameter of the pressurization surrounding wall: 12 cm,
- maximal pressure: 800 MPa,
- fluid transmitter of the pressure (water or water + glycol),
- rise speed in maximal pressure: 500 MPa/min,
- range of temperature: -20°C to +80°C.

The pressure is generated by a hydraulic pump and by the reduction of the volume of the pressure vessel, this one being moved by a hydraulic jack.

A piezo-electric sensor of precision +/- 1 MPa permits to control the pressure directly inside the pressure vessel. This sensor is joined to an electronic billposter. A thermocouple in Iconel placed inside the pressure vessel permits to measure the temperature of the pressurization fluid. The temperature is controled by an external cooling unit. The system of order of this pilot is automated and the parameters time, pressure and temperature are controled automatically by the computer system that also permits to record the evolution of all the parameters during the treatment. Pressure and temperature are constantly monitored and recorded during the process.

Fig. 12: Apparatus for producing isostatic pressure through direct compression developed at the M.H.P.

3.1.3 Settling on temperature of vessel "High Pressure"

It is possible to carry out high pressure cycles at temperatures between –20°C and 80°C. The temperature is regulated by an external command on the thermal generator. The heating liquid circulates around the vessel and transfers its heat to the fluid of pressurization by the effect of conduction through the wall of the vessel. To obtain temperatures of –20°C, the water (the more used fluid transmitting pressure) has to be substitued from the glycol, which remains liquid at –20°C and 0.1 MPa. A serpentine of copper placed directly inside the vessel and connected in derivation to the external circuit of cooling of the vessel, improves the slope down in temperature of glycol and permits to attain –20°C.

3.1.4 Parameters of High Pressure Treatment

For duplicate samples of *S.aureus* in TSB suspension, pressure treatments were carried out in the range 150-550 MPa at room temperature for different times (15 min, 5 min x 3 cycles, 3 min x 5 cycles, 2 min x 7 cycles). Duplicate samples of *S.enteritidis* suspension in TSB were treated in the pressure range 150-450 MPa at room temperature for various times (15 min, 5 min x 3 cycles, 3 min x 5 cycles, 2 min x 7 cycles). All the High Pressure treatments were tested twice. Control samples were held at atmospheric pressure. After processing, all the samples were stored at refrigeration temperature until analysis. The come-up times of the pressure were: 60 sec, 80 sec, 100 sec, 120 sec, 140 sec, 160 sec, 180 sec, 200 sec, respectively at 150 MPa, 200 MPa, 250 MPa, 300 MPa, 350 MPa, 400 MPa, 450 MPa and 500 MPa.

The pressure used for the treatment of caviar samples, inoculated with *S.aureus* ATCC 6538*,* was 500 MPa at room temperature for 15 minutes and 450 MPa for 3 cycles of 5 minutes. Instead the caviar samples, inoculated with *S.enteritidis* ATCC 13076*,* were pressurized at 400 MPa and room temperature for 15 minutes and 350 MPa for 5 minutes for 3 cycles.

For these experiments, the choice of the pressure values followed the analysis of preliminary results of TSB suspensions for only two pressurization times (15 min, 5 min x 3 cycles).

500 MPa and 400 MPa were the pressures able to destroy respectively *S.aureus* and *S.enteritidis* after a treatment of 15 minutes, while for the treatment for 3 cycles of 5 minutes, 450 MPa and 350 MPa were the lethal pressures.

The aim of this work was to study the application of High Pressure treatments able to destroy an elevated charge of pathogens, potentially present in fish eggs, and to find the optimal pressure levels, necessary to preserve the organoleptic characteristics of the fresh caviar.

3.2 THE CAVIAR

3.2.1 Sturgeon caviar

Samples of "caviar de Aquitaine" (eggs from Siberian sturgeon: *Acipenser baerii*) (Fig.13), free from additive borax, packaged into 2-part metal tins of 30 g, were obtained from S.C.E.A. Sturgeon, St Fort sur Gironde, France. The technical schede concerning the characteristics of the caviar are reported in Table XV.

Fig.13 Samples of "caviar de Aquitaine".

3.2.2 Trout Caviar

Samples of aquacultured trout caviar (eggs from Rainbow trouts: *Onchorinchus mykiss*) (Fig.14) in jars of glass (about 30 g) were provided from Viviers de France, Sarrance (France).

The trout caviar samples were stored at 0-4°C until use.

Fig.14 Samples of trout caviar.

3.2.3 Salmon Caviar

Samples of red salmon caviar (eggs from aquacultured salmon: *Onchorinchus nerka*), were obtained from S.C.E.A. Sturgeon, St Fort Sur Gironde, France. The salmon caviar samples were stored at 0-4°C until use.

Table XV. Fiche technique : « Caviar d'Aquitaine »*(From S.C.E.A. Sturgeon,St.Fort sur Gironde,France)*

Espèce du poisson d'origine	A*cipenser baerii* (esturgeon Sibérien)
Appelation:	Caviar d'Aquitaine
Composition:	œuf d'esturgeon, sel gemme 200/400 microns (3.5%), borax (0.5%)(tétra borate de sodium décahydraté: $Na_2Bo_410H_2O$) grammage: 30g-50g-100g-200g-500g-1000g

Mode de production: technique traditionelle, entièrement manuelle. Les locaux sont aux normes européennes (N° 17-331-003) et fréquemment contrôlés par les services vétérinaires. Le personnel est formé à l'hygiène. La production est controlée par la méthode HACCP.

Caractéristiques techniques:

Taille du grain: de 2.5 mm à 3.3 mm Moyenne: 2.7 mm
Couleur: gamme de brun clair doré à brun très foncé, majorité: brun foncé
Valeur énergétique: 268 kcal/100g
Glucides totaux: 0 g/100 g
Lipides (après hydrolyse acide): 17.6 g/ 100 g
Protéines: 27.44 g/ 100 g
Taux d'humidité: 49.1 g/ 100 g
Cendre: 6.03 g/ 100 g
Taux AG poly insaturé (% d'AG) : 37%
Taux Acide Gras Oméga-3 (%AG): 26.94%
Taux Acide Gras Oméga-6 (%AG): 10.1%

Remarque:
le caviar d'Aquitaine peut etre proposé a différents stades de maturité:
- de 1 mois à 4 mois d'age (saison de production uniquement), caviar très frais, gout fruité de noisette ou beurre marqué, court en bouche, gout de sel marqué, grain ferme ;
- de 5 à 9 mois d'age, caviar plus onctueux, plus long en bouche, sel moins marqué ;
- de 9 à 24 mois d'age, caviar plus corsé, long en bouche, gout de type anchois plus marqué (similaire caviar russe) grain plus souple.

3.3 METHODS FOR ORGANOLEPTIC TESTS

3.3.1 Preparation of caviar samples

In order to study the behaviour of the fish eggs under High Pressure and in particular the sensory attributes (structure, texture, consistence, colour, juice), some tests at different conditions of pressure, time and temperature were achieved.

For each test three samples were prepared: two were subjected to the pressurization and one remained at room pressure as control.

3.3.2 Packaging of caviar samples

Caviar samples (about 30 g) were not vacuum packaged but simply sealed in polyethylene pouches (150 x 120 mm, PA/PE 20/70 μm, Siegelrand-Beutel, France) for better observing the possible presence of juice inside the pocket and its characteristics.

3.3.3 Organoleptic tests

After High Pressure treatment a study of behaviour under pressure of fish eggs was done and concerned some organoleptic characteristics as: the structure, the texture, the colour, the consistence and the potential presence of juice.

All the changes in sensory parameters were evaluated by comparison with the untreated sample.

3.4 THE MICRO-ORGANISMS

3.4.1 The strains

S.aureus ATCC 6538 ("***ATCC"****: American Type Culture Collection. The number ATCC corresponds to the reference of the strain in the american collection*) and *S.enteritidis* ATCC 13067 were obtained as freeze-dried pellets cultures in thermosealed vials from Institut Pasteur, Paris, France. These cultures were selected because they are well-defined typed strains which have been used in many previous investigations including High Pressure processing [83a; 83b; 93; 101; 116]. The vials were stored at freezing temperature until use.

3.4.1.1 *Staphylococcus aureus* ATCC 6538

Staphylococcus aureus ATCC 6538 (Gram+ bacterium, genus *Staphylococcus*, fam. *Micrococcaceae*) is a strain from animal source, used in the methods of European and American Pharmacopea for the validation of culture media and sterility assays, for the antimicrobial preservation and for antibiotic dosages.

The high rate of human carriage of *S. aureus* is an important feature of the organism with respect to its role as a foodborne pathogen. Staphylococcal intoxication is the major form of food poisoning in which food handlers play a significant role.

3.4.1.2 *Salmonella enteritidis* ATCC 13076

S. enteritidis ATCC 13076 (Gram- bacterium, genus *Salmonella*, fam. *Enterobacteriaceae*) is a strain isolated from many different foods of animal origin.

S. enteritidis is one of the most important cause of the foodborne disease known as "salmonellosis". Fish, shellfish, fish-products, raw meat, poultry and eggs, are commonly contaminated with *Salmonella enteritidis*. In most outbreaks, the fish products is undercooked or has been contaminated after cooking (food handlers, ingredients added after cooking) or contaminated by polluted waters.

3.5 MICROBIOLOGICAL METHODS

3.5.1 Unit of microbiology

All the microbiological analyses were carried out inside the microbiological unit of the laboratory ERAP (Equipe de Recherche Agroalimentaire Perigourdine), University of Bordeaux IV, at Périgueux. The laboratory is equiped with all the devices indispensable for our work (fig.15-16-17) as: thermostats, Bunsen becs, sterile Petri plates, autoclave, fridge, bain-marie, vortex, spectrophotometer...).

Fig. 15 View of the thermostats.

Fig.16 View of the spectrophotometer.

Fig. 17 View of bain-marie.

3.5.2 Stockage of the strains

Before the preparation of the strains for the High Pressure treatments, some stock cultures of *Staphylococcus aureus* ATCC 6538 and *Salmonella enteritidis* ATCC 13076 were prepared on TSA (Tryptic Soy Agar, from Difco), which was incubated at 37°C for 24 hours and then stored at 4°C. TSA is the more used solid medium for stockage of micro-organisms [99]. After a refrigerated storage, the tubes containing the developed colonies were preserved 2 months and they represented our stock cultures. Each 2 months the stockage was achieved again in order to obtain young colonies for the assays.

3.5.3 Preparation of the strains

3.5.3.1 Culture of the strains

During the preparation the handlings have to remain sterile, for this reason they were achieved in a sterile atmosphere.

At the reception, the cultures of *S.aureus* ATCC 6538 and *S.enteritidis* ATCC 13076 (in form of lyophilized pellets) were rehydrated with addition of 0.3 ml of TSB (Tryptic Soy Broth, from Merck), a nutritive culture broth, largely used in pressure treatments from many authors [99; 125]. The choice of TSB medium, for the culture of the strains and also for the model suspension subjected to High Pressure treatment, can be explained on basis of the features of this medium (neutre pH, isotonic diluter), that respect the entireness of micro-organisms.

3.5.3.2 Preparation of bacterial suspension in TSB solution

For the revival of the micro-organisms, a single pellet of the cultures was resuspended in 5 mL of TSB, followed by incubation at 37°C for 48 hours.

This procedure was carried out in duplicate for each culture.

Subsequently, 3 ml of each cultured broth were inoculated in 100 ml of TSB and incubated at 37°C for 48 hours on a rotary shaker (Fig.18).

A further dilution in the same broth was necessary to achieve a concentration of approximately 10^8-10^9 cfu/ml of *S.aureus* ATCC 6538 and of *S.enteritidis* ATCC 13076 at the beginning of the High Pressure processing .

To verify that the microbial charge was in the same range for each assay, a control of OD (optical density) at 600 nm was made by a spectrophotometer.

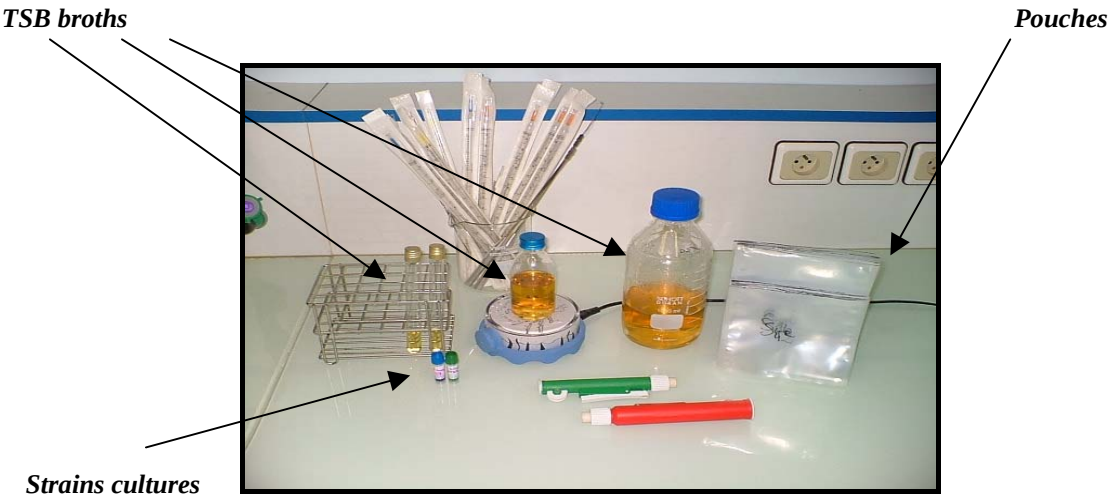


Fig.18 Preparation of bacterial suspension in TSB.

3.5.3.3 Packaging of the suspension samples

The cell suspensions (25 ml) were vacuum sealed in double sterile polyethylene pouches (150 x 120 mm and 150 x 190 mm PA/PE 20/70 µm Siegelrand-Beutel, France) (Fig.19-20) and subjected to High Pressure treatments.

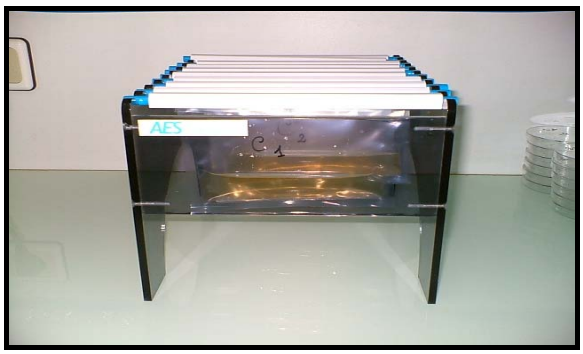


Fig.19 Packaging of TSB suspensions.

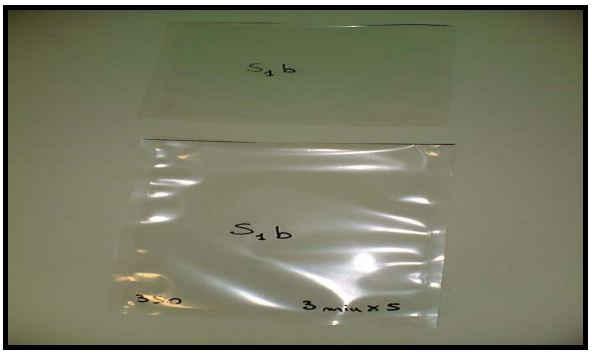


Fig.20 Different package sizes.

3.5.4 Enumeration of viable cells

Starting from the sample, appropriate 10-fold serial dilutions (10^{-8}) were made in sterile buffered peptone-water (AES Laboratoire).

1 ml of each dilution was plated onto a solid non-selective medium, as the PCA (Plate Count Agar, AES Laboratoire), in duplicate. This technique of plating consists in pouring about 15 mL of PCA (maintained liquid at 45°C in a bain-marie) inside the Petri plates (where we have added 1 ml of each dilution) and after mixing up uniformly. The plates were incubated at 37°C for 48 hours.

The colonies could grow in surface and in depth.

The average number of colonies from the duplicate plates was then recorded for each sample. The number of survivors was expressed as log cfu/ml (cfu = colony forming units).

3.5.5 Microbiological methods for analysis of caviar

3.5.5.1 Preparation of caviar samples for microbiological analysis

After reception, the caviar samples (sturgeon and trout caviar) were stored at refrigerated temperature until use.

During all the steps of the experiment the eggs were handled in sterile conditions to avoid secondary contaminations.

For each analysis two caviar samples were prepared.

A first step of the microbiological analysis concerned the pre-enrichment (37°C per 24h) for detection of endogenous *Salmonella* sp. by addition of 225 ml of buffered peptone water to 25 g of caviar and mixing.

After, 10-fold serial dilutions (10^{-8}) were made and used for the plating onto the different media by the following way (according to the standards of French Authority "AFNOR"):

- 1 ml onto PCA for the detection of total aerobe mesophile flora (30°C per 24-72 h);
- 1 ml onto Desox for detection of total coliforms (30°C per 24 h);
- 0.1 ml onto OGA (20°C per 5 days) for the detection of fungi flora;
- 0.1 ml onto Baird Parker for the detection of *Staphylococcus aureus* (37°C per 48 h);
- 5 ml onto VFSR (at 37°C and 46°C per 48h) for the detection of anaerobic sulphito reductor flora.

For endogenous *Salmonella* sp. the next procedure consisted in addition of 2 ml of the pre-enrichment to a selective enrichment broth, Rappapport-Vassiliadis (RV), stored at 37°C and 43°C per 24 h.

After, starting from RV, 0.1 ml were plated in parallel onto two selective solid media, Salmonella-Shigella agar and Hektoen enteric agar (37°C and 43°C per 24h).

3.5.5.2 Enumeration of viable cells

After the storage, the colonies of micro-organisms, developed on the different media, were counted. The average number of colonies from the duplicate plates was then recorded for each sample. The number of survivors was expressed as log cfu/g (cfu = colony forming units).

For *Salmonella* sp. it was determined the presence or the absence.

3.5.5.3 Preparation of caviar samples inoculated with *S.aureus* and *S.enteritidis*

The tests were achieved separately for the broth culture of each strain.

The cell suspension (1 ml) were added to 25 g of caviar. The mix was gently shaken by hand for 5 minutes.

3.5.5.4 Packaging of caviar samples

The inoculated samples were vacuum sealed in double sterile polyethylene pouches (150 x 120 and 150 x 190 mm, PA/PE 20/70 µm, Siegelrand-Beutel, France) and subjected to High Pressure procedure. For each treatment duplicate of the samples were prepared.

3.5.5.5 Enumeration of viable cells

25 g of each sample and 225 ml buffered peptone-water were homogenized using a stomacher. Further 10-fold dilutions (10^{-8}) were made in sterile buffered peptone-water (from AES Laboratoire).

0,1 ml of each dilution was plated in duplicate onto selective mediums RPF and Baird-Parker (from AES Laboratoire) used to enumerate *S.aureus* ATCC 6538, Rambach (from Merck) and Hektoen (from AES Laboratoire) used to enumerate *S.enteritidis* ATCC 13076.

The plates were incubated at 37°C for 24-48 hours.

The average number of colonies from the duplicate plates was then recorded for each sample. The number of survivors was expressed as log cfu/g (cfu = colony forming units).

4. RESULTS AND DISCUSSION

4.1 Behaviour of caviar under High Pressure

The changes of sensory properties of caviar samples, as structure, consistence, colour, texture of eggs, were examined after High Pressure treatments at different pressures, temperatures and time of processing. The eggs belonged to the fish: sturgeon, trout and salmon. The behaviour of the eggs to High Pressure was different between the three types of caviar, so showing to depend on the caviar and not only on the conditions of treatment.

4.1.1 Sturgeon caviar

Sturgeon eggs samples were subjected to pressure treatments in the range 100 – 250 MPa, at 4°C and 20°C, and for continuous times (15 and 30 min) and pressure cycles (5 min x 3 cycles; 3 min x 5 cycles). The structure of eggs was preserved for pressures up to 150 MPa, at room temperature, only after 15 minutes (both for continuous and cycled treatments). It was observed a slight exudation in the packages. The grey colour and the normal consistence were retained. After 30 minutes at 150 MPa a large exudation occured and the consistence of the eggs became very soft. Starting from a pressure of 250 MPa and for all the times of the procedure, caviar became gelatinous, due to the denaturation of the proteins. Also at 4°C the pressure of 250 MPa induced the gel formation in caviar samples after 10 minutes. Only at 150 MPa, both for continuous and cycled treatments, the characteristics of eggs resulted preserved (Fig.24).

4.1.2 Trout caviar

Trout eggs samples were submitted to the pressure range of 100–350 MPa at room temperature and at –10°C, for testing the effect of subzero temperature combined to the pressure on the eggs. The times of exposure were 15 minutes in continuous and in cycles. At room temperature the trout

eggs showed higher resistance to pressure up 300 MPa and important changes in their texture, structure and consistence were observed only at 350 MPa (the eggs appeared gelatinous). At subzero temperature (Fig.21,22,23) the best quality of the trout caviar was preserved after 150 MPa only for cycling pressure processing (5 min x 3 cycles; 3 min x 5 cycles). In fact for continuous treatment of 15 min a large exudation appeared.

At 250 MPa important changes in colour and texture of the eggs occured, also a large presence of juice after 15 min-treatment and the beginning of gel formation after 3 cycles of 5 min.

At 300 MPa and for continuous treatment of 15 min the eggs became paler, softer and with a greater exudation.

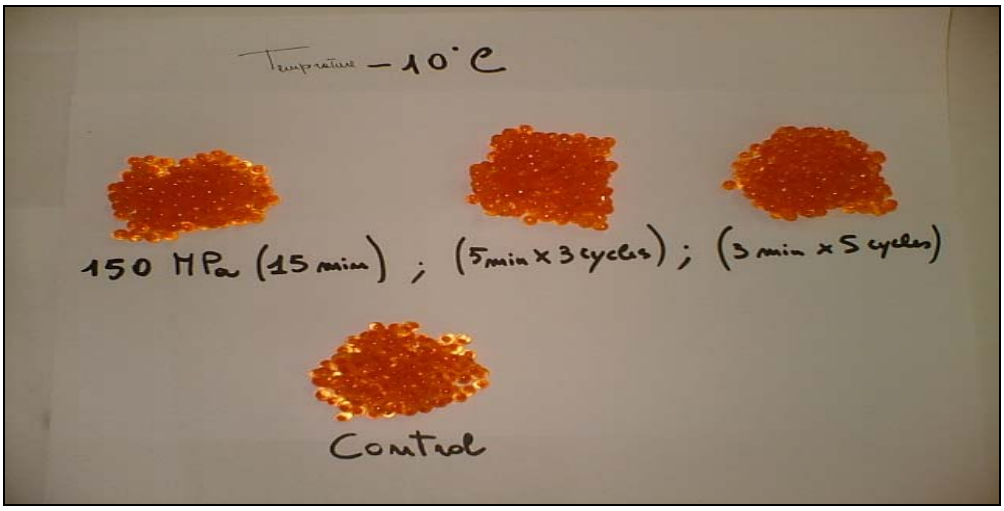


Fig.21 Trout caviar after 150 MPa at –10°C.

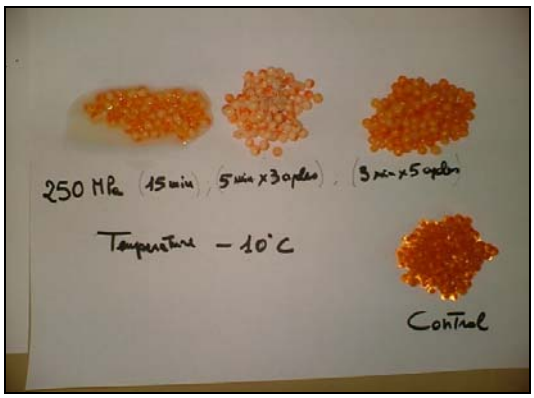


Fig.22 Trout caviar after 250 MPa at –10°C.

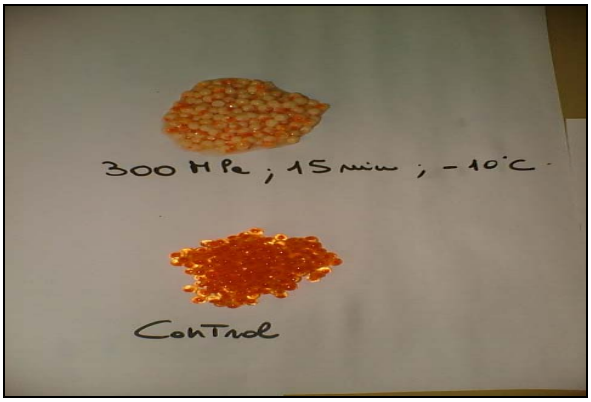


Fig.23 Trout caviar after 300 MPa at –10°C.

4.1.3 Salmon caviar

Salmon eggs were treated at pressures up to 450 MPa at 20°C for 15 minutes. They retained a good aspect, similar to fresh product for pressure treatments up to 250 MPa. The structure, the colour and texture of the eggs were well preserved and no exudation was observed.

After 250 MPa and for the times of treatment, gel formation and changes in colour and structure of the eggs occured (Fig.24).

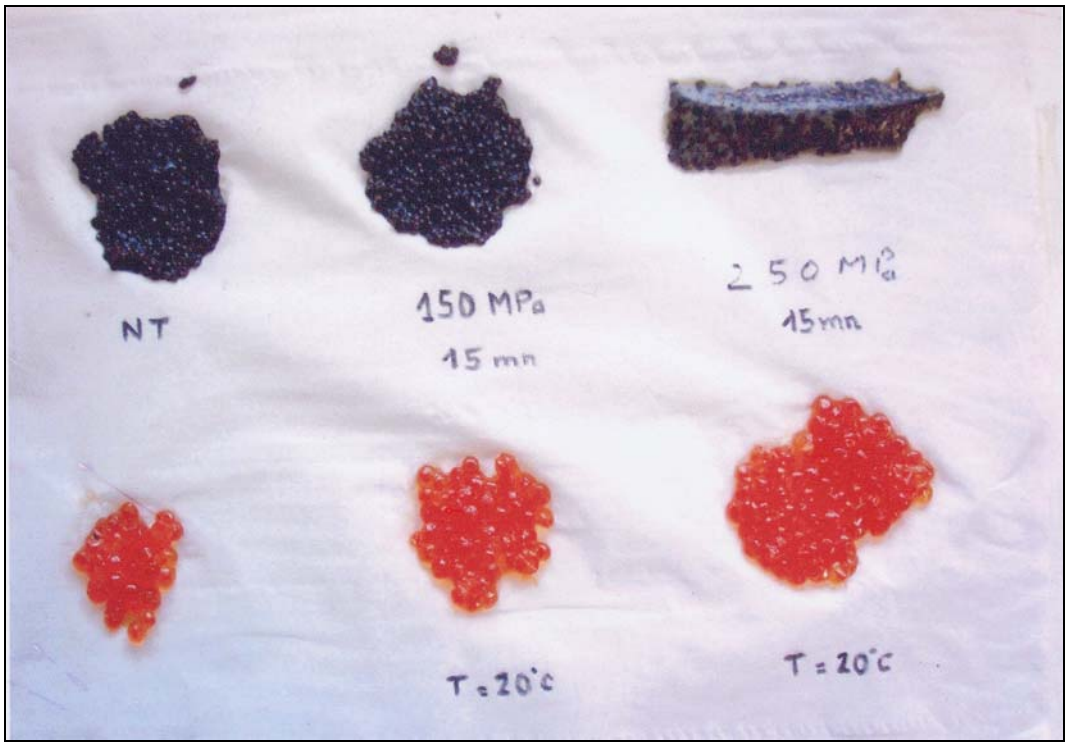


Fig.24 Sturgeon and salmon caviar under High Pressure at 20°C.

4.2 Destruction by High Pressure of *S.aureus* and *S.enteritidis* in TSB suspension

In order to study the effect of High Pressure on pathogens, *Staphylococcus aureus* ATCC 6538 and *Salmonella enteritidis* ATCC 13076, and to value their inactivation rate, bacterial TSB suspensions were subjected to the treatment at different conditions of pressure and time. The number of viable cells in the untreated suspensions (N_0) and the number of viable cells in the pressurized suspensions (N) was calculated during every experiment. They represent the average of the values obtained for samples in duplicate. So the effectiveness (DE, Destructive Effectiveness) of High Pressure to inactivate the pathogen strains is described and expressed by the following formula:

$(DE) = - \log (N/N_0) = \log N_0 - \log N.$

The rate of micro-organisms able to survive to the treatment is expressed by the ratio N/N_0.

In our study it ranges from 1 to 10^9.

The goal of our work was to find the conditions (of pressure and time) necessary to obtain a high DE of the process versus every strain, and also to preserve the sensory properties of fresh caviar, well-known to be a fragile and high nutritive value foodstuff. The influence of different parameters, as pressure intensity, time of treatment, temperature and type of treatment (continuous and cycles), on DE of High Pressure was studied. The "tryptic soy broth" (TSB) was chosen in this work because it is frequently used as culture medium for the microbial suspensions submitted to High Pressure Processing. TSB is a sterile and isotonic solution with a neutre pH. All these characteristics permit to protect the physiological entireness of the micro-organisms.

4.2.1 Effect of High Pressure on *S. aureus* ATCC 6538 in TSB suspension

4.2.1.1 Effect of High Pressure treatment in continuous

At first we have studied the Destructive Effectiveness (DE) of High Pressure versus *S. aureus* ATCC 6538, suspended in TSB medium, at room temperature in the range 150-550 MPa for a treatment of 15 minutes. These preliminary results were used as a starting-point in order to find the pressure level able to inactivate completely the pathogen.

The Fig.25 and the Table XVI show the inactivation of *S.aureus* correlated to the pressure for a treatment time of 15 minutes at room temperature (20°C).

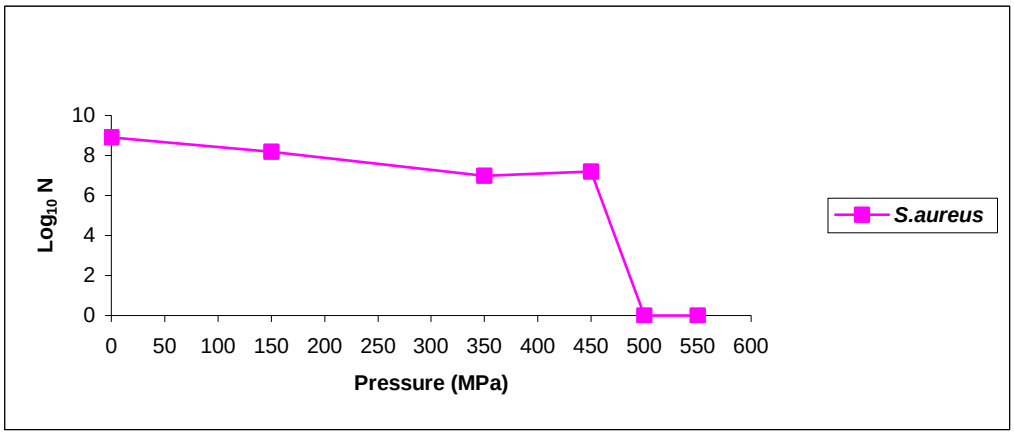


Fig.25 Inactivation of *S.aureus* ATCC 6538 in TSB suspension treated for 15 min at 20°C.

Table XVI. Inactivation of *S.aureus* ATCC 6538 in TSB suspension treated for 15 min at 20°C

Pressure(MPa)	**0**	**150**	**350**	**450**	**500**	**550**
N_0(Log cfu/ml)	8.9	8.9	8.9	8.9	8.9	8.9
N(Log cfu/ml)	8.9	8.2	7	7.2	0	0
Log N/N_0	0	-0.7	-1.9	-1.7	-8.9	-8.9

N_0 = number of viable micro-organisms in untreated suspension
N = number of viable micro-organisms in pressurized suspension
Log N/N_0 = ED = effectiveness destructive of treatment

The inactivation rates after treatment of 15 minutes at 150 MPa, 350 MPa, 450MPa and 500 MPa were respectively: 0.7 $\log_{10}$, 1.9 $\log_{10}$,1.7 $\log_{10}$ and 8.9 $\log_{10}$.

It can be observed the existence of a destruction threshold-value of 500 MPa, because, up to this pressure level, no significant destruction occurs. We observed in the range 150-450 MPa only some threshold-values of weak inactivation of *S.aureus*, while the total inactivation of micro-organisms present in TSB suspension, at an initial charge of about 10^8-10^9 cfu/ml, occured only at 500 MPa. This result indicates the high baroresistance of *S.aureus* ATCC 6538.

Some studies also showed the high resistence to continuous pressure of different strains of *S.aureus*. PATTERSON *et al.*(1995) [96] applied 700 MPa for 15 minutes at room temperature to *S.aureus* NCTC 10652 in PB solution and they obtained an inactivation over 5 $\log_{10}$. GERVILLA *et al.* (1999) [49] showed that an increase in pressure was necessary to obtain a higher inactivation of *S.aureus* 534 CECT, inoculated in ovine milk, after a pressure treatment in the range 300-500 MPa at room temperature for 15 minutes. The strain *S.aureus* ATCC 6538, used in our work, showed a more significant baroresistence. Also the results of the works of O'REILLY *et al.*(2000) and RIGALDIE (2002) gave a similar conclusion. O'REILLY et al. (2000) [93] applied a pressure of 600 MPa at 20°C for 20 minutes on the micro-organism and they observed only a 4 $\log_{10}$ reduction in counts. RIGALDIE (2002) [101] treated TS suspensions *S.aureus* ATCC 6538 in the range of pressure 200-600 MPa at room temperature for 10 minutes and observed only 2.26 $\log_{10}$ of destruction of micro-organism.

On basis of our results, confirming the high baroresistance of *S.aureus* ATCC 6538, in a second step of our work different treatments in cycles were achieved in order to determine that treatment combining the features of high Destruction Effectiveness (DE) and of the ability to preserve the organoleptic qualities of fresh caviar.

4.2.1.2 Effect of High Pressure treatment in cycles

Cycles of pressurization-depressurization were carried out in the range 150-550 MPa at room temperature in order to improve the DE of High Pressure treatment.

The inactivation rates for 3 pressure cycles of 5 minutes (20°C) were: 1 $\log_{10}$, 2.2 $\log_{10}$, 8.85 $\log_{10}$, 8.85 $\log_{10}$, 8.85 $\log_{10}$ respectively at 150 MPa, 350 MPa, 450 MPa, 500 MPa, 550 MPa.

So the total destruction occured at 450 MPa for this cycle (Tab.XVII).

For 5 pressure cycles of 3 minutes (20°C) the inactivation rates increased only at 350 MPa (Tab.XVIII) and resulted 4.15 $\log_{10}$.

Increasing further the number of cycles the inactivation rate of *S.aureus* resulted greater also at lower pressures. In fact after 7 cycles of 2 minutes the inactivation rates were: 3.1 $\log_{10}$, 8.85 $\log_{10}$, 8.85 $\log_{10}$, 8.85 $\log_{10}$, respectively at 250 MPa, 350 MPa, 450 MPa, 500 MPa. So the total destruction in counts occured at a pressure 350 MPa (Tab.XIX).

In the Fig.26 the inactivation rates of *S.aureus* for continuous and cycling pressure treatments are represented. The Fig.27 shows the inactivation rates of *S.aureus* correlated to the different cycles at the pressure of 350 MPa.

A similar conclusion on effectiveness of pressure cycles resulted in the work of Rigaldie (2002) [101]. The author showed that 3 cycles of 10 minutes at 500 MPa and room temperature were sufficient to obtain a reduction in the counts of 6.6 $\log_{10}$, while 6 cycles of 5 minutes inactivated completely *S.aureus* (8.6 $\log_{10}$). At the same conditions of pressure and temperature for a continuous treatment of 30 minutes the viability loss of micro-organisms was only 1.42 $\log_{10}$.

After the analysis, by electronic microscopy, of the changes occuring in cells after pressurization, the author observed irreversible modifications of the intracellular material and also a perturbation in cell division concerning the septum and the cell membrane. These phenomena can explain the lethal effect of pressure cycles on the micro-organisms.

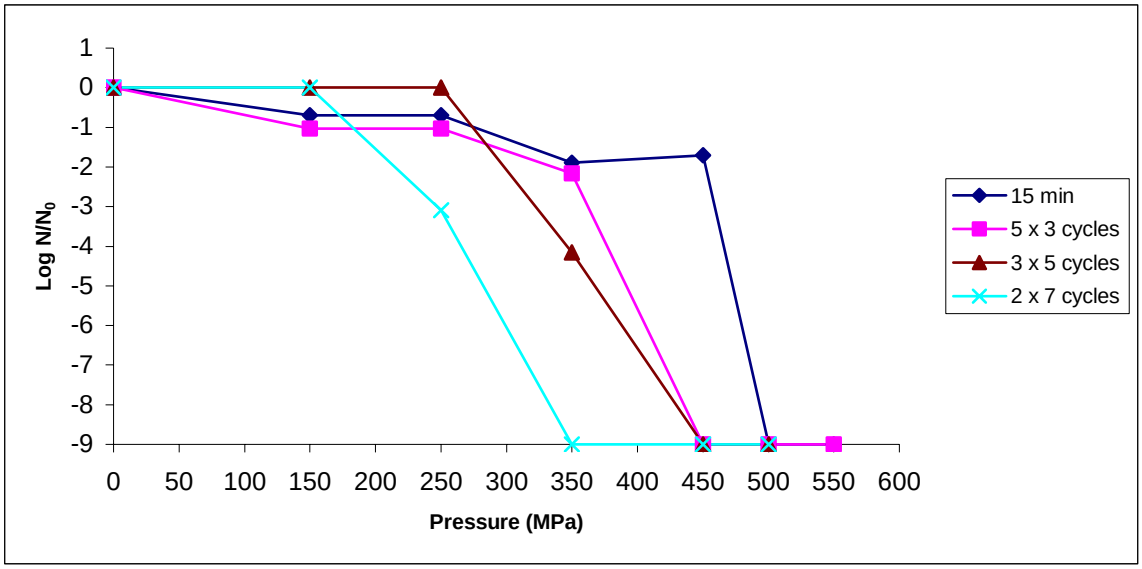


Fig.26 Inactivation of *S. aureus* ATCC 6538 in TSB at different pressures at 20°C.

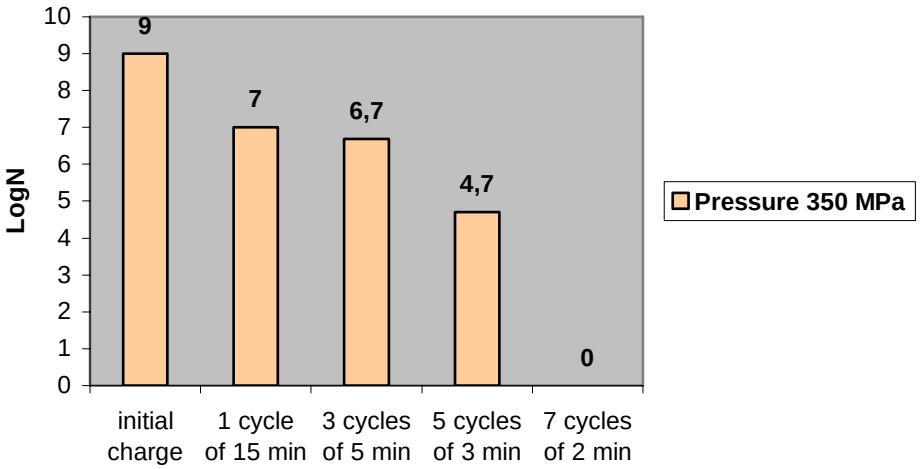


Fig.27 Inactivation of *S.aureus* in TSB at 20°C for different cycles at 350 MPa.

At 350 MPa increasing the number of cycles the viability loss in counts of *S.aureus* increased significantly and resulted 2.2 $\log_{10}$ for 3 cycles, 4.15 $\log_{10}$ for 5 cycles and 9 $\log_{10}$ for 7 cycles.

Table XVII. Inactivation of *S.aureus* ATCC 6538 in TSB suspension treated for 5 min x 3 cycles at 20°C

Pressure(MPa)	0	150	350	450	500	550
N_0(Log cfu/ml)	8.85	8.85	8.85	8.85	8.85	8.85
N(Log cfu/ml)	8.85	7.82	6.69	0	0	0
Log N/N_0	0	-1.03	-2.16	-8.85	-8.85	-8.85

Table XVIII. Inactivation of *S.aureus* ATCC 6538 in TSB suspension treated for 3 min x 5 cycles at 20°C

Pressure (MPa)	0	150	350	450	500
N_0 (Log cfu/ml)	8.85	8.85	8.85	8.85	8.85
N (Log cfu/ml)	8.85	8.85	4.7	0	0
Log N/N_0	0	0	-4.15	-8.85	-8.85

Table XIX. Inactivation of *S.aureus* ATCC 6538 in TSB suspension treated for 2 min x 7 cycles at 20°C

Pressure (MPa)	0	250	350	450	500
N_0 (Log cfu/ml)	8.85	8.85	8.85	8.85	8.85
N (Log cfu/ml)	8.85	4.76	0	0	0
Log N/N_0	0	3.09	-8.85	-8.85	-8.85

N_0 = number of viable micro-organisms in untreated suspension
N = number of viable micro-organisms in pressurized suspension
Log N/N_0 = ED = effectiveness destructive of treatment

4.2.2 Effect of High Pressure on *S. enteritidis* ATCC 13076 in TSB suspension

4.2.2.1 Effect of High Pressure treatment in continuous

As in the case of *S.aureus* model, we also studied for *S.enteritidis* ATCC 13076 in TSB suspension the effect of High Pressure for a continuous treatment of 15 minutes, but in the pressure range 150-450 MPa.

The choice of this range followed data published in the literature [11; 95; 99], showing that *S.enteritidis* is less baroresistant than *S.aureus*. From these preliminary results the study developed in the same way as in the experiments on *S.aureus*, with the application of various pressure cycles.

The Fig.28 and the Table XX show the inactivation rates of *S.enteritidis* counts obtained at room temperature for pressures up to 450 MPa and for an exposure time to the pressure of 15 minutes. A

5.36 $\log_{10}$ reduction in counts was obtained only at a pressure of 350 MPa, while the total inactivation occured at 400 MPa. PONCE *et al.*(1999) [99] observed a higher baroresistance of *S.enteritidis* inoculated in liquid whole egg for 350 MPa-treatment of 15 minutes at room temperature. Only 2 $\log_{10}$ reduction in counts was achieved. Increasing the pressure (450 MPa) the viability loss in counts increased (5 $\log_{10}$). So for the continuous treatments the pressure levels resulted important to the inactivate higher cell numbers.

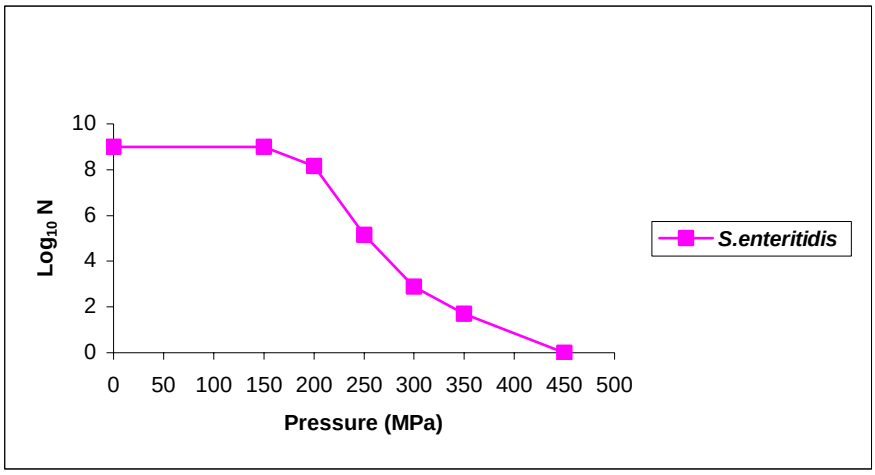


Fig.28 Inactivation of *S.enteritidis* ATCC 13076 in TSB suspension treated for 15 min at 20°C.

TableXX. Inactivation of *S.enteritidis ATCC 13076* in TSB suspension treated for 15 min at 20°C

Pressure (MPa)	**0**	**150**	**200**	**250**	**300**	**350**	**400**	**450**
N_0 (Log cfu/ml)	8.95	8.95	9.09	9.09	8.24	8.95	9.09	8.95
N (Log cfu/ml)	8.95	9	8.15	5.15	2.88	1.69	0	0
Log N/N_0	0	0	-0.94	-3.94	-5.36	-7.26	-9.09	-8.95

N_0 = number of micro-organisms in untreated samples
N = number of viable micro-organisms in treated samples
Log N/N_0 = DE = destructive effectiveness of the treatment

From 200 MPa to 250 MPa the viability loss in counts of *S.enteritidis* increased of about 3 $\log_{10}$ and up to 350 MPa it was over 6 $\log_{10}$, after it resulted total at 400 MPa.

4.2.2.2 Effect of High Pressure treatment in cycles

The Fig.29 the inactivation rates of *S.enteritidis* for continuous and cycling pressure treatments are represented. The effectiveness of pressure cycles is evident if it is compared to continuous pressure of the same conditions of treatment. The effect of different cycles at 250 MPa on destruction of *S.enteritidis* is showed in the Fig.30.

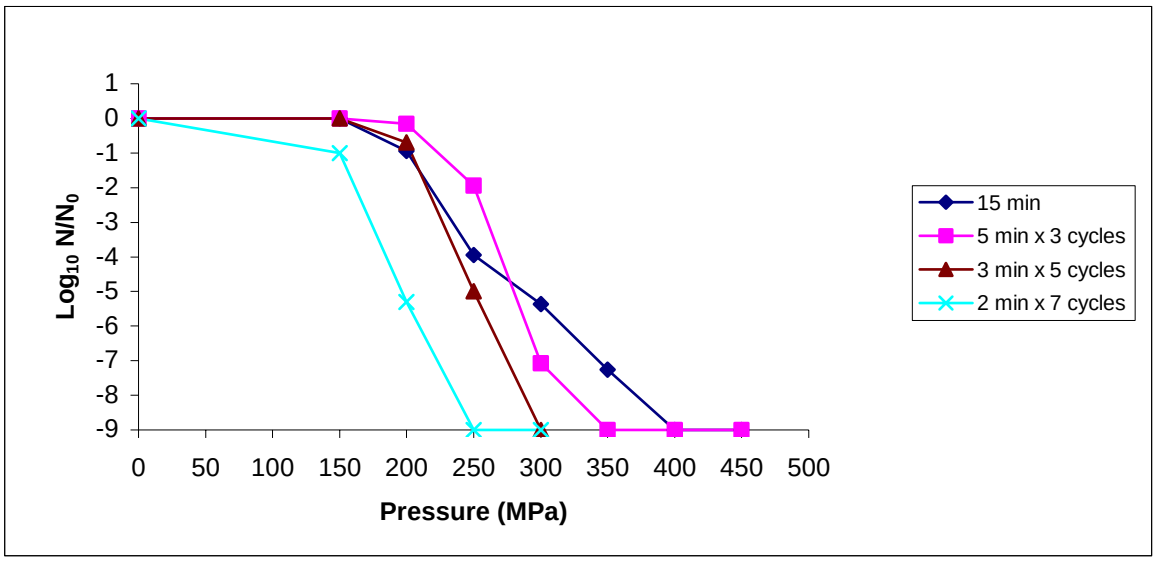


Fig.29 Inactivation of *S. enteritidis* ATCC 13076 in TSB at different pressures at 20°C.

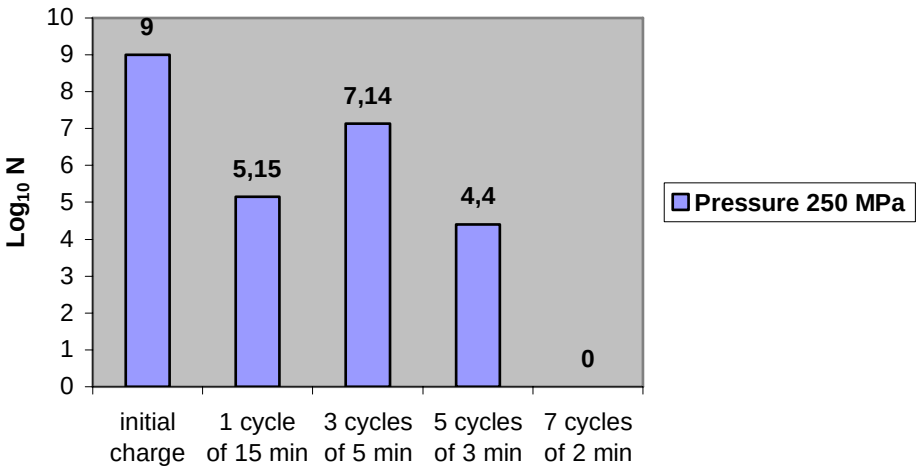


Fig.30 Inactivation of *S.enteritidis* in TSB at 20°C for different cycles at 250 MPa.

After 250 MPa the viability losses for 3, 5 and 7 cycles were respectively: 1.95 log_{10}, 5 $log_{10,}$ 9 log_{10}. The total inactivation of *S.enteritidis* was obtained at 250 MPa after 7 cycles of 2 minutes. Therefore the increase of the number of cycles improved the Destructive Effectiveness (DE) of High Pressure treatment. The threshold-value in destruction of counts was 250 MPa for 7 cycles. Further we observed that the inactivation levels were more significant also at other pressures.
In the Tables XXI-XXIII the data concerning the inactivation rates, the final and initial counts for the cycled pressure treatments are reported.

Also PONCE *et al*.(1999) [99] verified the greater effectiveness of treatment in cycles than continuous treatments of the same total time. Compared to the work of PONCE *et al.*(1999), having the same conditions of treatment concerning pressure (350-450 MPa), time (3 cycles of 5 min, 5 cycles of 3 min) and temperature (20°C), we obtained an higher cells destruction at 350 MPa.

The difference in respective results could depend on different strains (*S. enteritidis serotype enteritidis*, *S. enteritidis* ATCC 13076) used for the experiment and their different sensitivity to High Pressure process, also depend on different medium, which was inoculated to obtain the cell suspension (liquid whole egg, TSB). The egg could have a protective effect on micro-organism versus High Pressure treatment.

Table XXI Inactivation of *S.enteritidis ATCC 13076* in TSB suspension treated for 5 min x 3 cycles at 20°C

Pressure (MPa)	0	150	200	250	300	350	450
N_0 (Log cfu/ml)	8.95	8.95	9.09	9.09	8.24	8.95	8.95
N (Log cfu/ml)	8.95	9.04	8.94	7.14	1.17	0	0
Log N/N_0	0	0	-0.15	-1.95	-7.07	-8.95	-8.95

Table XXII Inactivation of *S.enteritidis ATCC 13076* in TSB suspension treated for 3 min x 5 cycles at 20°C

Pressure (MPa)	0	150	200	250	300
N_0 (Log cfu/ml)	8.9	8.9	9.4	9.4	8.9
N (Log cfu/ml)	8.9	7.9	8.7	4.4	0
Log N/N_0	0	-1	-0.7	-5	-8.9

Table XXIII Inactivation of *S.enteritidis ATCC 13076* in TSB suspension treated for 2 min x 7 cycles at 20°C

Pressure (MPa)	0	150	200	250	300
N_0 (Log cfu/ml)	9.5	9.5	9.5	9.5	9.5
N (Log cfu/ml)	9.5	8.5	4.2	0	0
Log N/N_0	0	-1	-5.3	-9.5	-9.5

N_0 = number of micro-organisms in untreated samples
N = number of viable micro-organisms in treated samples
Log N/N_0 = DE = destructive effectiveness of the treatment

The effect of pressure on inactivation of *S.enteritidis* was significant at 200 MPa only for 7 cycles of 2 min. The inactivation rate was 5.3 $\log_{10}$ at these conditions.

For lower pressure levels not important reduction in counts occured. The total destruction of *S.enteritidis* was obtained for 3, 5 and 7 cycles respectively at 350 MPa, 300 MPa and 250 MPa. The increase of cycles number permited to reduce the pressure level effective to destroy *S.enteritidis*.

4.3 Destruction by High Pressure of *S.aureus* and *S.enteritidis* inoculated in caviar

4.3.1 Results about microbiological quality of caviar samples

The results about the microbiological analysis of caviar samples showed the presence of not high counts of *S.aureus* (3 $\log_{10}$) and FMAT (2 $\log_{10}$), sign of proper handling during processing and manufacture of the product.

The same observation, limited only to *S.aureus*, can be done for trout caviar samples, where no detection of *S.aureus* counts occured. For these samples a higher detection of FMAT and Fungi Flora counts was observed.

The results concerning the microbiological analysis of sturgeon caviar and trout caviar samples are summarized in the Tables XXIV and XXV.

Table XXIV Results of microbiological analysis of Sturgeon caviar samples

Micro-organism	AFNOR code	cfu/g	Log_{10} cfu/g
Total coliforms	NFV08-015	0	
FMAT 30°C	NFV08-051	10^2	2
S.aureus	NFV08-014	10^3	3
Salmonella sp.	NFV08-013	absence	
H_2S producers bacteria	NFV08-029	0	
Fungi Flora	NFV08-036	2×10^2	2.3

c*fu = colony forming unit*

Table XXV Results of microbiological analysis of Trout caviar samples

Micro-organism	AFNOR code	cfu/g	Log_{10} cfu/g
Total coliforms	NFV08-015	0	
FMAT 30°C	NFV08-051	3.34×10^9	9.52
S.aureus	NFV08-014	0	
Salmonella sp.	NFV08-013	absence	
H_2S producers bacteria	NFV08-029	0	
Fungi Flora	NFV08-036	19×10^2	3.28

c*fu = colony forming unit*

4.3.2 Effect of High Pressure on *S.aureus* and *S. enteritidis* inoculated in caviar

Starting from the analysis of preliminary results of the TSB models, the pressurization of caviar samples inoculated with *S. aureus* ATCC 6538 and *S. enteritidis* ATCC 13076 was carried out at the threshold-values obtained for the suspensions for the 15 min-treatment and 3 cycles of 5 minutes, at room temperature.

The effect of High Pressure treatments on the strains, inoculated in caviar, is shown in Tables XXVI-XXIX.

For the samples inoculated with *S. aureus* ATCC 6538, both pressure treatments (450 MPa/5 min x 3 cycles and 500 MPa/15 min) decreased significantly the counts.

Also the counts of *S.enteritidis* ATCC 13076 inoculated in caviar samples, obtained after pressurization, were < 1 log cfu/g for the treatments in continuous (400 MPa/15 min) and in cycles (350 MPa/5 min x 3 cycles).

So at the pressure levels used for the experiments the effect of pressure in continuous and in cycles was similar. Besides the substrate did not affect the effect of High Pressure Processing on micro-organisms.

There were not substantial differences between the results of High Pressure treatment of pathogens inoculated in caviar and those in TSB suspension at the same condition of procedure (pressure, time and temperature).

Table XXVI *S.aureus* inoculated in sturgeon caviar samples

Sample	MPa/min	Counts (cfu/g)	Log_{10} cfu	Method
Control	0	$30.5\ 10^3$	4.5	NF V08051
Treated	500 / 15	< 10	< 1	NF V08051
Treated	450 / 5 x 3 cycles	< 10	< 1	NF V08051

cfu = colony forming unit

Table XXVII *S.enteritidis* inoculated in sturgeon caviar samples

Sample	MPa/min	Counts (cfu/g)	Log_{10} cfu	Method
Control	0	$13.5\ 10^5$	6.1	NF V08051
Treated	400 / 15	< 100	< 2	NF V08051
Treated	350 / 5 x 3 cycles	< 100	< 2	NF V08051

cfu = colony forming unit

The inactivation rates both for *S.aureus* and *S.enteritidis*, inoculated in sturgeon caviar, resulted over 4 log_{10} after the respective pressure treatments. There were not considerable differences concerning the pressure sensitivity of the two micro-organisms inoculated in sturgeon caviar.

Table XXVIII *S.aureus* inoculated in trout caviar samples

Sample	MPa/min	Counts (cfu/g)	Counts Log_{10} cfu	Method
Control	0	$46\ 10^3$	4.66	NF V08051
Treated	500 / 15	< 10	< 1	NF V08051
Treated	450 / 5 x 3 cycles	< 10	< 1	NF V08051

cfu = colony forming unit

Table XXIX *S.enteritidis* inoculated in trout caviar samples

Sample	MPa/min	Counts (cfu/g)	Counts Log_{10} cfu	Method
Control	0	$56\ 10^3$	4.74	NF V08051
Treated	400 / 15	< 100	< 2	NF V08051
Treated	350 / 5 x 3 cycles	< 100	< 2	NF V08051

cfu = colony forming unit

The inactivation rates both for *S.aureus* and *S.enteritidis*, inoculated in trout caviar, were over 4 log_{10} after the respective pressure treatments. There were not considerable differences concerning the pressure sensitivity of the two micro-organisms inoculated in trout caviar.

5. CONCLUSION

High Pressure Processing represents an interesting technique for the preservation of fish products, especially the caviar, not only because it inactivates micro-organisms, pathogens or cause of spoilage, potentially present in the product, but also because it could avoid the use of chemical additives, that in many cases are resulted to be nocive.

For example the additive borax added to the caviar in order to control the growth of pathogens, is permitted by EU legislation only in this product because it is consumed at low doses, having potential risks not well precised [28].

Further, the development of an alternative treatment to thermal pasteurization as the mean of eliminating pathogenic and spoilage micro-organisms is substantial especially for the processing of caviar and other fish eggs.

The product treated by High Pressure retain its natural color and texture, typical of fresh product, while by thermal processing these sensorial parameters change and are typical of pasteurized caviar. In this thesis it can be observed 3 important parts of the work on the application of High Pressure treatment on caviar.

A first part concerned the study of the behaviour of fish eggs, from fish sturgeon, trout and salmon, under High Pressure at various temperatures, pressures and times, was achieved, in order to evaluate the feasibility of the pressure treatment of this fragile matrix.

In the second part some models on the baroresistance of *Staphylococcus aureus* ATCC 6538 and *Salmonella enteritidis* ATCC 13076 were achieved in TSB suspension at different pressure levels and for continuous and cycling pressurization at 20°C.

The results on the destructive effectiveness of the High Pressure on these pathogens were used for the applications of treatment on artificially contaminated caviar, objective of the last part of the thesis.

Concerning the effects of High Pressure on the sensory characteristics of fish eggs, we observed that at all the temperatures, used in the experiments, the preservation of caviar was guaranteed up to 150-200 MPa and for treatment times not exceeding 15 minutes. Above these conditions in parameters a solidification of the caviar occured because of the gel formation from the proteins.

Therefore the aim of following steps of the work was to attain these treatment conditions in inactivation of high charges of *Staphylococcus aureus* ATCC 6538 and *Salmonella enteritidis* ATCC 13076 inoculated in the caviar and the preservation of the caviar.

The results of the second part of the thesis underlined the higher baroresistance of *Staphylococcus aureus* ATCC 6538 compared to *Salmonella enteritidis* ATCC 13076 and the effectiveness of pressure cycles in destruction of both pathogens at weak pressure levels.
By some cycles it was possible to reduce the pressure level able to inactivate high charges of *S.aureus* and *S.enteritidis* (10^8-10^9 cfu/ml), in fact after 7 pressure cycles of 2 min at room temperature the total destruction of the pathogens in TSB suspension was obtained respectively at 350 MPa and 250 MPa

The existance of thresholds-values of inactivation in cycling treatments were observed and resulted significant if compared to the continuous treatments.

The destruction thresholds-value was marked especially in the case of *S.aureus* ATCC 6538 for the continuous treatment, while for *S.enteritidis* ATCC 13076 this phenomenon did not occur. The higher baroresistance of *S.aureus* ATCC 6538 could be explain the different results.

The caviar did not affect the resistance of both pathogens to High Pressure at the conditions in parameters studied and between continuous and cycling treatments any difference was found. There were not considerable differences between the pressure sensitivities of the two studied micro-organisms inoculated in the same caviar and also in different caviar types. We observed only a slightly higher capacity of baroresistance of *S.enteritidis* ATCC 13076 both in sturgeon and trout caviar.

But it would be important to develop this study by application of other treatment conditions about pressure levels, temperature and other cycles of pressure.

Also the analysis of the shelf-life of caviar, treated by pressure, could result interesting in order to evaluate the efficacy of the pressurization as technique of microbial stabilization of the product.

Further, the understanding of the phenomena connected to pressure inactivation and pressure destruction of micro-organisms would be interesting by electronic microscopy.

So the application of High Pressure is a potential method for the preservation of caviar, but the understanding of the critical limits of the process and the extent to which this might ensure appropriate treatment of this fragile foodstuffs is very important.

In order to the stabilization of caviar and to attain the pressure values necessary to preserve the organoleptic qualities of caviar we suggest further increase in cycles number during pressure treatment.

There is no doubt that High Pressure Processing represents another interesting and promising technique for fish products not only because it inactivates micro-organisms, but also because foods so treated retain their natural flavor, color and texture without loss in vitamin or nutrient content.

There can be no possibility of extensive implementation of this technology unless specific processing parameters are established for each food material treated by High Pressure.

Further a possible commercialization of the products (so-treated) also depends on economic viability. Therefore, a detailed analysis of the costs of High Pressure Processing needs to be done in order to compare the process costs with those of processing by conventional means and at the same time taking in count the added value of the improved sensory characteristics of High Pressure treated foods.

It is important that standard operating criteria and processing conditions be established that will ensure the reliability of High Pressure Processing as an alternative to thermal processing. For example, thermal processing has standards like D- and Z- values, holding-times, and minimum temperatures developed for different foods; likewise, standards need to be set for High Pressure treatments.

Very few studies on the High Pressure inactivation of micro-organisms discuss effects of pressure in terms of D-value associated with each particular strain of organism [70; 95; 101; 12]. More research has to be done in order to obtain a database of D-values for different micro-organisms can be prepared.

Further works have to be achieved to define better and optimize the parameters of the High Pressure treatment of fish products, in particular caviar and other fish eggs.

It could be necessary to apply lower pressures associated to mild temperatures (room temperature or in any case lower that the pasteurization temperatures).

The use of pressure cycles permits to reduce the pressure levels and obtain best results about the inactivation rate of micro-organisms and preservation of organoleptic characteristics of fresh caviar.

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ANNEXES OF CHAPTER III

A.III-1 Preparation of bacterial suspensions

A.III-2 Description of culture broths and media composition

A.III-1

Preparation of bacterial suspensions
(*S.aureus* ATCC 6538 and *S.enteritidis* ATCC 13076)

1 - A lyophilized pellet of the cultures of *S.aureus* ATCC 6538 and *S.enteritidis* ATCC 13076 was resuspended in 5 mL of TSB (Tryptic Soy Broth, from Merck), followed by incubation at 37°C for 48 hours.

2 - After 3 ml of each cultured broth were inoculated in 100 ml of TSB and incubated at 37°C for 48 hours on a rotary shaker.

3 - A further dilution in TSB was necessary to achieve a suspension concentration of approximately 10^8-10^9 cfu/ml of *S.aureus* ATCC 6538 and of *S.enteritidis* ATCC 13076.

4 - A control of OD (Optical Density) at 600 nm by a spectrophotometer was made in order to verify that the microbial charge was in the same range for each experiment.

Description of culture broths and media composition

PCA (Plate Count Agar)

From AES Laboratoire

Approximate Formula per liter purified water:
Pancreatic digest of casein..... 5.0 g
Yeast extract..... 2.5 g
Dextrose..... 1.0 g
Agar..... 15 g
Final pH 7.0 ± 0.2

BP (Baird Parker Medium)

From AES Laboratoire

Approximate Formula per liter purified water:
Tryptone..... 10.0 g
"Lab-Lemco" powder (meat extract)..... 5.0 g
Yeast extract..... 1.0 g
Sodium pyruvate.....10.0 g
Glycine..... 12.0 g
Lithium chloride..... 5.0 g
Agar.....20.0 g
Final pH 6.8 ± 0.2

Hektoen Enteric agar

From AES Laboratoire

Approximate Formula per liter purified water:
Proteose peptone..... 12.0 g
Yeast extract.....3.0 g
Lactose.....12.0 g
Sucrose..... 12.0 g
Salicin.....2.0 g
Bile salts.....9.0 g
Sodium chloride…….5.0 g
Sodium thiosulphate……...5.0 g
Ammonium ferric citrate..... 1.5 g
Acid fuchsin.....0.1 g
Bromothymol blue.....0.065 g
Agar.....14.0 g

Final pH 7.5 ± 0.2

RP (Rappapport-Vassiliadis broth)
(from DIFCO,USA)

Approximate Formula per liter purified water:
Bacto tryptone..... 4.54 g
Sodium chloride..... 7.2 g
Potassium dihydrogen phosphate..... 1.45 g
Magnesium chloride anhydrous..... 13.4 g
Oxalate..... 0.036 g
Final pH 5.1 ± 0.2

TSB (Tryptone Soya Broth)
(*From Merck*)

Approximate Formula per liter purified water:
Pancreatic digest of casein..... 17.0 g
Papaic digest of soybean meal..... 3.0 g
Sodium chloride..... 5.0 g
di-basic potassium phosphate..... 2.5 g
Glucose.....2.5 g
Final pH 7.3 ± 0.2

TSA (Tryptone Soya Agar)
(*From DIFCO*)

Approximate Formula per liter purified water:
Tryptone.....15.0 g
Soya peptone.....5.0 g
Sodium chloride.....5.0 g
Agar.....15.0 g
Final pH 7.3 ± 0.2

Buffered Peptone Water
(*From OXOID, England*)

Approximate Formula per liter purified water:
Peptone..... 10.0 g
Sodium chloride..... 5.0 g
di-sodium phosphate..... 3.5 g
potassium dihydrogen phosphate..... 1.5 g
Final pH 7.2 ± 0.2

OGA (Oxitetracycline Glucose Agar)

(*From OXOID, England*)

Approximate Formula per liter purified water:

Yeast extract.....5.0 g
Destrose.....20.0 g
Biothina.....0.0001 g
Agar.....12.0 g
Final pH 7.0 ± 0.2

SS agar modified (Salmonella-Shigella Agar)

(*From OXOID, England*)

Approximate Formula per liter purified water:

"Lab-Lemco" powder (meat extract)..... 5.0 g
Peptone.....5.0 g
Lactose.....10.0 g
Bile salts.....5.5 g
Sodium thiosulphate.....8.5 g
Sodium citrate.....10.0 g
Ferric citate.....1.0 g
Brilliant green.....0.00033 g
Neutre red.....0.025 g
Agar.....12.0 g
Final pH 7.3 ± 0.2

Desox (Desoxycholate Agar)

(*From OXOID, England*)

Approximate Formula per liter purified water:

Peptone.....10.0 g
Lactose.....10.0 g
Sodium desoxycholate.....1.0 g
Sodium clorure.....5.0 g
Sodium phosphate.....2.0 g
Ferric citate.....1.0 g
Sodium citrate.....1.0 g
Neutre red.....0.03 g
Agar.....15.0 g
Final pH 7.3 ± 0.2

List of publications and comunications

1) F.Fioretto, T.Pepe, A.Santoro, T.A.Sarli
ISOLAMENTO DI YERSINIA ENTEROCOLITICA DA LINGUE DI SUINI MACELLATI IN CAMPANIA
Atti Giornate Scientifiche del Polo delle Scienze e delle Tecnologie per la Vita-Facoltà di Medicina e Chirurgia Farmacia Medicina Veterinaria e Agraria (Portici 6/7 giugno 2002*)*

2) A.Santoro, T.A.Sarli, L.Vallone, **F.Fioretto**, I.Dragoni
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3) Fioretto F.,Murru N.,Ferrante S.,Tozzi M.,Sarli T.A.
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ATTI XIII Convegno Nazionale AIVI, 6-7-8 Giugno 2003,Montesilvano (Pescara)

7) A.Santoro, N.Murru**,F.Fioretto,**C.Antonelli, T.A.Sarli
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Atti Giornate Scientifiche del Polo delle Scienze e delle Tecnologie per la Vita-Facoltà di Medicina e Chirurgia Farmacia Medicina Veterinaria e Agraria (Napoli,20-21 Maggio 2004)

10) A.M.Santoro, S.Ferrante, **F.Fioretto**, G.Pezone, T.A.Sarli
RICERCA DI IMPURITA' BIOTICHE ED ABIOTICHE IN PASTI DESTINATI ALLA RISTORAZIONE SCOLASTICA
ATTI XIV Convegno Nazionale AIVI, 3-6 Giugno 2004, Mondovì (Cuneo)

11) S.Ferrante, N.Costanzo, **F.Fioretto**, T.A.Sarli, A.M. Santoro
SOLID IMPURITIES DETECTED IN MEALS DESTINATED TO SCHOOL CATERING
Atti XXIII Congresso Nazionale Società Italiana di Parassitologia, 9-12 Giugno 2004, Vietri sul Mare (SA)

12) F.Fioretto, C.Cruz, A.Largeteau, T.A.Sarli, G.Demazeau, A.El Moueffak
INACTIVATION OF *Staphylococcus aureus* AND *Salmonella enteritidis* IN TSB AND CAVIAR SAMPLES BY HIGH PRESSURE PROCESSING
Book of Abstracts "3rd International Conference on High Pressure Bioscience and Biotechnology, Rio de Janeiro, Brazil, September 26-30, 2004".

STUDY ON THE INACTIVATION BY HIGH PRESSURE OF PATHOGENS IN FISH PRODUCTS: POTENTIAL APPLICATIONS.

ABSTRACT:

In this thesis a study concerning the action of High Hydrostatic Pressure on inactivation of two foodborne pathogens: as *Staphylococcus aureus* ATCC 6538 and *Salmonella enteritidis* ATCC 13076 - suspended in a culture medium and inoculated in caviar samples, and the behaviour under pressure of fish eggs -, was achieved.
Three important parts can be identified in the thesis.

1) The analysis of some changes in sensory properties (as colour, texture, structure, consistence) of pressurized caviar samples was performed at different temperatures and times of treatment in the pressure range 150 MPa – 350 MPa. No changes were observed in sturgeon, salmon and trout caviar samples treated respectively at 150 MPa, 250 MPa and 300 MPa during a 15 minutes-treatment in continuous and in cycles.
2) The baroresistance of *Staphylococcus aureus* ATCC 6538 and *Salmonella enteritidis* ATCC 13076 in TSB suspension at a concentration of 10^8-10^9 CFU/ml was tested for continuous and cycled pressurization in the range 150 MPa – 550 MPa and for 15 minutes-treatments at room temperature. The cycles of pressure used for the experiments were: 5 min x 3 cycles, 3 min x 5 cycles, 2 min x 7 cycles. The increase of cycles numbers resulted effective in total destruction of two micro-organisms.
3) The effect of High Hydrostatic Pressure on inactivation of *Staphylococcus aureus* ATCC 6538 and *Salmonella enteritidis* ATCC 13076, inoculated in sturgeon and trout caviar samples, and the influence of the matrix on barotolerance of the pathogens were studied.

The results of this work showed the effectiveness of High Pressure cycles on destruction of *Staphylococcus aureus* ATCC 6538 and *Salmonella enteritidis* ATCC 13076 and on preservation of organoleptic qualities of fish eggs, depending of the main characteristics of the treatment: temperature, pressure level and time.

KEY WORDS:

High Hydrostatic Pressure	pressure cycles	barotolerance
Staphylococcus aureus	*Salmonella enteritidis*	foodborne pathogens
TSB suspensions	caviar	organoleptic qualities

ETUDE SOUS HAUTES PRESSIONS DE L'INACTIVATION DE MICRO-ORGANISMES PATHOGÈNES DANS LES PRODUITS DE LA MER: APPLICATIONS POTENTIELLES.

RESUME :

Cette thèse présente l'effet des traitements sous hautes pressions sur la destruction de 2 souches pathogènes, *Staphylococcus aureus* et *Salmonella enteritidis*, dans un milieu modèle (TSB) et au sein d'échantillons d'oeufs de poisson (esturgeon et truite). L'influence des principaux paramètres caractérisant ces traitements: pression (150-550 MPa), temps (15 minutes), température (ambiante), sur la sensibilité des pathogènes à hautes pressions a été évalueé. En outre le comportement des oeufs de poisson aux conditions de pression et temps sélectionnés et à différentes températures de traitement a été étudié. Les résultats de ce travail ont, de plus, mis en évidence l'efficacité des cycles de pression dans la destruction des micro-organismes pathogènes aux niveaux de pression suffisants mais cependant optimisés de manière à garantir la préservation des propriétés sensorielles des oeufs de poisson.

MOTS CLES :

Hautes Pressions Hydrostatique
Cycles de pression
Caviar
Staphylococcus aureus
Salmonella enteritidis
Baroresistance
Micro-organismes pathogènes
Qualités organoleptiques