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Foods of Non-Animal Origin

Chemistry, Technology, Inspection Procedures

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Chapter 1
Regulating Risks in Imports of Foods of Non-animal Origin: The U.S. Food Industry Perspective

Arpan Bhagat

Abstract United States of America imports 50 and 20 % of fresh fruits and vegetables, respectively. This behaviour may create considerable opportunities for intentional and unintentional food safety risks for the nation's food supply. The Food and Drug Administration is responsible for inspecting all foods of non-animal origin being imported in the United States of America. One of the major changes following the terrorist attacks of 11 September 2001 was the promulgation of the 'Public Health Security and Bioterrorism Preparedness and Response Act' of 2002 (U.S. Congress 2002). This act has provided the Food and Drug Administration with more oversight over national food imports in cooperation with the Bureau of Customs and Border Protection. However, due to cost, time and logistics involved in the physical inspection of foods, less than 3 % of imported foods are inspected. Electronic screening and tools such as the 'Predictive Risk-based Evaluation for Dynamic Import Compliance Targeting' computer system have made the system less manual. However, this strategy cannot completely replace sampling and analyses for detection of microbiological hazards and other risks (including allergens, naturally occurring toxins, pesticides, drug residues and other physical, biological and chemical contaminants). Another milestone in overhauling the nation's food safety has been the introduction of the Food Safety Modernization Act in January 2011. This act requires food industry to follow risk-based approach with more emphasis towards self-regulating the food safety through proposed controls such as foreign supplier verification programs. The fresh vegetables and fruits industry is highly involved with public health.

Keywords FSMA · Food and drug administration · Foreign supplier verification program · Fresh fruits · Fresh vegetables · HACCP · HARCP · National food supply · On-site inspection · Public health

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Abbreviations

CDC	Centers for Disease Control and Prevention
CEC	Commission of the European Communities
CBP	Customs and Border Protection
DHS	Department for Home Security
HHS	Department of Health and Human Service
EMA	Economically Motivated Adulteration
EPA	Environmental Protection Agency
FBO	Food business operator
FDCA	Food, Drug, and Cosmetic Act
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service
FSMA	Food Safety Modernization Act
FSVP	Foreign Supplier Verification Program
GAO	General Accountability Office
HACCP	Hazard Analysis and Critical Control Points
HARCP	Hazard Analysis and Risk-Based Preventive Controls
PREDICT	Predictive Risk-based Evaluation for Dynamic Import Compliance Targeting
U.S.	United States of America
USDA	U.S. Department of Agriculture
WHO	World Health Organization

1.1 Imported Foods in the United States of America

1.1.1 The Current Scenario

United States of America (U.S.) imports approximately 20 and 50 % of its fresh vegetables and fruits, respectively (McGuire 2013; Keenan et al. 2015). In 2010 the consumption share of imported fresh fruits in the U.S. reached 48.8 % of the total quantity (Paggi et al. 2013) with a considerable increase between 2000 and 2010 (+6.4 %). U.S. has been a 'voracious' consumer of imported fresh foods of non-animal origin (Paggi et al. 2013) because of the remarkable growth of imported foods as compared to the domestic products (Huang and Huang 2007; Seale et al. 2013; Knutson et al. 2014). Certain foods have increased the perception of the import phenomenon: for example, the share of imported fruits between 2000 and 2010 rose to approximately 58.7 % in comparison to the original 20.1 % value, with the exclusion of vegetables and bananas (Paggi et al. 2013).

Statistically, majority of imported vegetable foods (and correlated food import refusals until 2013) can be mainly attributed to South American countries,

including Mexico, Dominican Republic, Guatemala and Chile (Paggi et al. 2013). This is dictated by the number of rejected vegetable products by the U.S. Food and Drug Administration (FDA) in relation to the food rejections ascribed to vegetables due to health alerts (Paggi et al. 2013; Pew Charitable Trust 2011). In addition, it has been reported that reputational spillovers can be an important factor when speaking of food safety precautions and rejections. In other words, the reputation of a certain exporting country seems to have a strong influence on the probability of the U.S. import rejections from the specific country and its neighbouring nations (Jouanjean et al. 2015). As a result, it could appear that the geographical position of Mexico and Latin American countries may have unsatisfactory and reciprocal influences on their related food export activities. On the other hand, the trade of agricultural products into the North America has been made easier by means of the North American Free Trade Agreement.

Another debated matter is the possible relationship between new and old restrictions in the legislative process as it relates to economic protection (Nakuja and Kerr 2013). Recent studies have highlighted only partial evidences of this hypothesis, as far as South American countries are concerned, as Asian nations such as China are not apparently impacted. For reasons mentioned, the above possibility of economic protectionism appears insufficient to support correlation (Zahniser et al. 2015).

Parallely, the importance of food safety inspections and audits on imported and domestic vegetables and fruits in the U.S. has constantly increased (Cheftel 2011; Hardesty and Kusunose 2009; Paggi et al. 2013). U.S. monitoring activity on vegetables with reference to pesticides involves the FDA, the Environmental Protection Agency (EPA) and the Food Safety Inspection Service of the U.S. Department of Agriculture (Liu et al. 1999).

The knowledge for the implementation of measures such as ‘Good Agricultural Practices’ and ‘Good Handling Practices’ is currently being provided though academic training. At the same time, the concomitant evolution and transformation of food safety standards and laws with reference to biological hazards, chemical contamination (detection of polycyclic aromatic hydrocarbons and pesticides) and adulteration has been also observed (Hatanaka et al. 2005; Humphrey 2012; Johnson 2014a; Josling et al. 2004; Ribera and Knutson 2011; Sanchez 2015a; Schneider et al. 2014; WHO 2000). However, the assessment of certain sanitary and phytosanitary conditions of imported foods and the correlated trade reduction may be difficult (Keiichiro et al. 2015; Josling et al. 2004; Peterson et al. 2013).

In addition, private standards are also employed for fresh fruits and vegetables (Humphrey 2012). Risk management measures are often taken on the basis of the well-known ‘precautionary principle’: the European position is generally coincidental to this approach (CEC 2000a, b). On the other side, a certain preference for regional initiatives (different but similar approaches to the problem of food security in agricultural industry and correlated risks) has been recently observed in the U.S. (Hinrichs 2013). In general, the higher the potential of food safety risk, the greater the number of industry standards with a common approach. This phenomenon is apparently the result when government laws and regulations do not keep

up to with new technological developments (Hatanaka et al. 2005; Reardon et al. 2001). As a result, the private regulation has increased its own role without detriments to national legislations (Dolan and Humphrey 2000; Farina and Reardon 2000; Sporleder and Goldsmith 2001).

The Food Safety Modernization Act (FSMA) is a reform that the FDA defines a selection of science-based requirements for the safe production of raw vegetables and fruits (Calvin 2013; Johnson 2014b). Interestingly, these requirements are substantially focused on microbiological hazards because of the low number of risks associated with contaminants in fresh products of vegetable origin (Matthews 2014).

A significant impact on the U.S. import activities is ascribed to organic agricultural products, tropical and subtropical crops. The economic value of tropical and subtropical crops in terms of the value of organic imported foods in the U.S. reached 66.5 % in the first 6 months of 2013 (Greene 2013). These products include non-animal products such as coffee and bananas (17.3 and 26.7 %, respectively). In addition, notable importance has to be recognised for 'Mediterranean-like' products such as olive oil (9.7 %). Evidently, the remarkable share of these imported foods is dependent on the 'natural' unavailability of the specific domestic products in the U.S. (Greene 2013).

Consequently, grains and oilseeds (rice, corns, soybeans, etc.) do not appear to exceed 8.5 % of the total economic value of organic imported foods. U.S. exports of wheat, soybean meal and soybean oils are expected to decline by 2022 because of a strong competition from Latin American (Westcott and Trostle 2012). In addition, amounts of imported wheat and soybean into the U.S. are expected to continue to come from China (accounting for approximately 25 % of agricultural imports), in contrast with economic protectionism hypotheses (Wang et al. 2015). The U.S. Department of Agriculture (USDA) projects definitive increase in the import of these agricultural products through 2023.

Organic agricultural produce such as apples, almonds, pears, etc. do not appear as significant from an important standpoint as compared with previous categories. It is also interesting to note that there is an increasing trend in the production of domestic fruits and vegetables in the category of the U.S. organic fresh exports category: especially for certain produce typologies, fresh apples and lettuces are the predominant organic export products (Greene 2013).

However, due to cost, time and logistics involved in the physical inspection of foods, less than 3 % of imported foods are currently being inspected (GAO 2005; McGuire 2013; Paggi et al. 2013). Imported foods are inspected by the U.S. Custom and Border Protection on behalf of the FDA with the aim of identifying possible safety threats. In addition, it has been reported that the theoretical inspection of registered foreign food facilities would be really unworkable: for example, only 0.05 % of these plants have been inspected by the FDA in 2008, with a predicted increase (0.32 %) of this activity in 2010 (GAO 2008; Paggi et al. 2013). In fact, the FDA has opened different offices in foreign countries with the aim of reducing times for the importation of certain low-risk products (Leavitt and Eurofins 2012; McGuire 2013). However, this activity is expected to increase

(Kelley Drye 2011), especially as the FSMA will raise safety levels through increased authority for record access (Neumann 2015).

1.2 The General Food Control System in the U.S.

The remarkable growth of imported foods in the U.S. in addition to domestic products and the general increasing trend of fruits and vegetables consumption from 2000 to 2010 (approximately 20 % increase) may create considerable intentional and unintentional food safety risks for the U.S. food supply (Huang and Huang 2007; Seale et al. 2013; Knutson et al. 2014).

The risk perception of consumers is highly connected with the so-called 'globalisation'. In other words, the increasing amount of information by means of conventional and new communication media has also influenced the current level of perceived danger of food. The possible presence of genetically modified organisms (GMO) in certain imported foods, the creation of new processing and preservation technologies with some 'non-natural' ingredient such as active scavengers (Parisi 2009) and the increasing number of cases of environmental contamination with potential influence on food safety have to be investigated and seriously considered (Anderson and Bokor 2012; Balzekiene et al. 2014; Ward et al. 2010).

On the other hand, the number of actual foodborne illnesses in the U.S. has demonstrated that a certain risk level should always be taken into account and possibly predicted. This monitoring activity of foods and beverages in the U.S. is carried out by the FDA since 1906 (Young 2003).

1.2.1 The Role of Food and Drug Administration and Other Agencies

FDA is responsible along with other agencies for the food safety and control system in the U.S. The monitoring activity and correlated functions are ascribed to two main institutions (Keenan et al. 2015):

- The FDA
- The Food Safety and Inspection Service (FSIS), within the USDA.

Food safety controls for interstate business and import activities involve other organisations (Keenan et al. 2015) including the Centers for Disease Control and Prevention (CDC), the Department for Home Security (DHS) and the EPA. In addition, each State of the Union uses its own institutions (State Departments of Health) at a 'regional' level, with the possibility of carrying out audits on behalf of the FDA.

With reference to imported foods, the FDA is the primary agency responsible for the food safety and control system. In addition, the FDA has undertaken the following objectives (Keenan et al. 2015):

(a) Evaluate safety and regulatory compliance of the U.S. food supply with some exceptions (most poultry and meat products)
(b) Carry out actions from the regulatory viewpoint to ensure compliance with the Food Drug and Cosmetic Act
(c) Create and implement guidance documentation and standards within the ambit of food safety. This critical area of responsibilities is accomplished through training and technical consultations in collaboration with academia and private industry
(d) Evaluate the level of food safety and compliance of manufacturers, processors and other players (transportation and warehouse) in the commercial chain of FDA-regulated products
(e) Act synergically with other Federals & state agencies official Institutions, as needed.

On the other hand, the FSIS is mainly responsible for most meat and poultry foods (Keenan et al. 2015). Technically, this agency exists within the USDA. Other institutions that are assigned to sustain and implement food safety of fruits and vegetables (domestic and imported foods) include the following:

- The DHS. An intergovernmental institution, in collaboration with the FDA and USDA, supports the Food and Agriculture Sector Coordinating Council. It is specifically created for promoting food security and food defense initiatives with the private industry
- The Customs & Border Protection (CBP) with competencies concerning entry of suspect commodities in the U.S. (e.g. harmful pests and diseases)
- The CDC. This Institution monitors foodborne outbreaks in the U.S.
- The EPA sets legal limits for pesticides in the environment, including food production facilities and all food.

1.2.2 The Public Health Security and Bioterrorism Preparedness and Response Act of 2002

Monitoring activity on imported foods has been dramatically bolstered with the 'Public Health Security and Bioterrorism Preparedness and Response Act' of 2002 (Khan 2011; Laux and Hurburgh 2012; Montalbano 2011; Sauer et al. 2009; U.S. Congress 2002; Woodlee 2012) following the terrorist attacks of 11 September 2001. This act has provided the FDA with more oversight over national food imports in cooperation with the Bureau of Customs and Border Protection (CBP). The Bioterrorism Act requires that all manufactured, processed and packaged foods industries be registered with the FDA (Keenan et al. 2015; Ramaswamy and

Mosher 2015). On these bases, CBP personnel can carry out special examinations under the Bioterrorism Act. In addition, suspect shipments can be detained by the CBP with the aim of performing more detailed sampling and analyses. Finally, the CDC reviews the list of biological agents and toxins with possible severe effects on public health and safety (CDC 2012).

1.2.3 Improvements in the Food Control System: The Role of Predictive Risk-Based Evaluation and Screening

Due to cost, time and logistics involved in the physical inspection of foods, less than 3 % of imported foods are inspected.

For this reason, the U.S. food controls have been progressively improved by means of electronic screening using tools such as the 'Predictive Risk-based Evaluation for Dynamic Import Compliance Targeting' (PREDICT) computer system (Cheftel 2011; Johnson 2014a; Riviere and Buckley 2012; Sanchez 2015a, b). In fact, the electronic transformation of predictive models for the evaluation of food safety risk into software applications may turn routine controls into a less manual system. Moreover, the use of similar predictive tools could be considered as a preliminary risk assessment system (Wallace and Oria 2010). Future FSMA improvements are expected to be facilitated by means of such softwares with the ability to rapidly identify outbreaks in electronic databases (DeWaal and Plunkett 2013).

With reference to the PREDICT system, the basic feature of the software is its ability to screen the enormous numbers of imported articles in the U.S. The database includes foods and medical products (drugs); as a result, PREDICT is able to find out (probabilistic model) the most remarkable dangers for public health (McGuire 2013).

Similar approaches can be used for other objectives such as the research on food fraud and 'Economically Motivated Adulteration' (EMA) cases. The EMA Incidents Database may be used with the aim of predicting and recognising most foods likely to be adulterated in the U.S. food supply (Johnson 2014a). Another interesting tool is the iRisk software, created by the Joint Institute for Food Safety & Applied Nutrition, Risk Sciences International and the FDA. iRisk deals with the analysis of both chemical and biological hazards in food products. This software provides semi-quantitative characterisations of different scenarios with the final aim of estimating risk of illness and thereby, the food safety measures. In addition, the following food safety risk models can be employed for general food safety assessments (Wallace and Oria 2010):

- The Foodborne Illness Risk Ranking Model (by the Food Safety Research Consortium, U.S.)
- The Risk Ranger, by the Australian Food Safety Center. This system is an interesting example of excel-based application
- The Food Safety Universe Database, by the Ontario Ministry of Agriculture and Food.

On the other side, the predictive strategy cannot completely replace sampling and analyse for the detection of microbiological hazards and other risks, including allergens, naturally occurring toxins, pesticides, drug residues and other chemical contaminants. In addition, electronic predictive systems are extremely complex. Moreover, the direct electronic dissemination of analytical data has to be necessarily improved both with relation to food safety predictive systems and networks of interconnected laboratories (Akingbade et al. 2014).

A milestone in overhauling the nation's food safety has been the introduction of the Food Safety Modernization Act in January 2011. This act requires food industry to follow risk-based approach with greater emphasis towards self-regulating the food safety through proposed controls such as foreign supplier verification programs.

1.3 The Food Safety Modernization Act

The FDA Food Safety Modernization Act was signed by the U.S. President Barack Obama on 4 January 2011. Basically, this act represents the first major modification of the national food safety system since 1938 by means of the Food, Drug, and Cosmetic Act (FDCA). Relevant modifications impact imported foods and ingredients on the one side and all interested (domestic and foreign) food business operators on the other side (Calvin 2013; Kelley Drye 2011; Leavitt and Eurofins 2012).

1.3.1 A General Introduction to FMSA

Basically, the FSMA has two main aims (Akingbade et al. 2014):

(a) The enhancement of the U.S. food safety system by means of continuous engagement by the FDA, and
(b) The ameliorated protection of public health.

Because of the previously mentioned number of foodborne illnesses caused by the consumption of domestic and foreign foods and correlated concerns (Coffman et al. 2014; CDC 2011), the FSMA strategy is based on the prevention of hazards caused by contaminated food and feed by means of the effective cooperation of different players, including federal, state and local authorities and foreign institutions (Akingbade et al. 2014; Keenan et al. 2015).

The FSMA is subdivided into four main titles (U.S. Congress 2011):

(1) Title I deals with the improvement of the capability of preventing food safety problems
(2) Title II aims towards the enhancement of the responsiveness when food safety problems are detected and an immediate action is required
(3) Title III addresses the safety of the U.S. imported foods
(4) Finally, Title IV follows through additional topics (funding, jurisdiction, etc.).

1.3.2 The FMSA: Prevention of Food Safety Concerns

The introduction of the FSMA has been of great interest to the entire food industry community because of the practical approach to food safety problems and the focus on preventive actions (Johnson 2014b). Briefly, the following sub-topics highlight the specific strategies (Fig. 1.1) to reinforce the food safety management (Johnson 2014b; U.S. Congress 2011):

(a) Inspection of records. Substantially, the Department of Health and Human Service (HHS) through FDA can inspect documents concerning 'suspect' foods. The documentation may not be directly related to food products (analytical reports): on the contrary, the attention is focussed on manufacturing, processing, packaging and subsequent pest processing steps (distribution, importation, etc.)
(b) Registration of food plants. The U.S. approved foods and feeds have to be produced and commercialised by registered food facilities (the registration

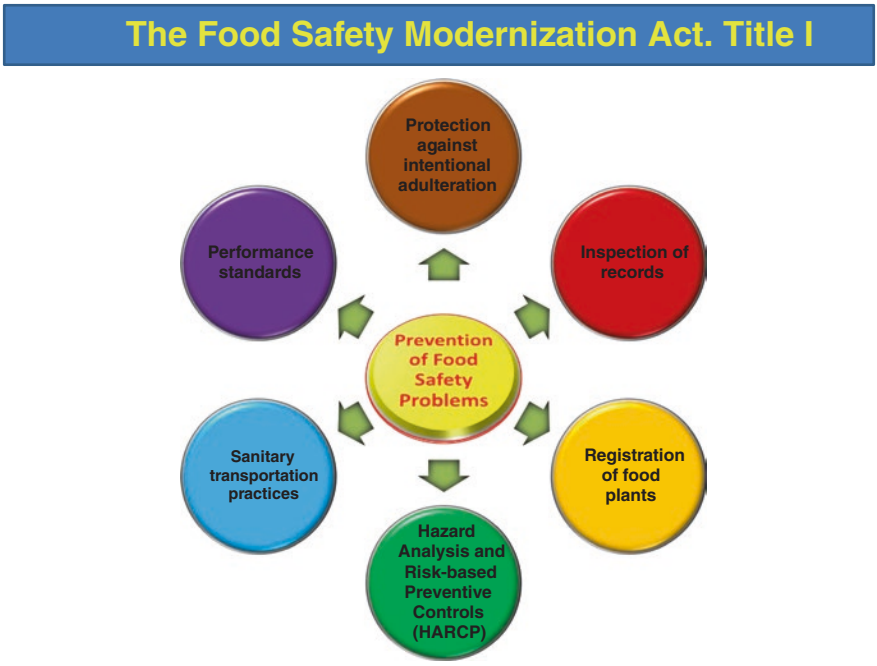

Fig. 1.1 Title I of the FSMA concerns the improvement of the capability of preventing food safety problems by means of different actions: the inspection of records concerning 'suspect' foods; the registration of the U.S. and foreign food plants; mandatory preventive controls for food plants on the basis of Hazard Analysis and Risk-based Preventive Controls (HARCP); the creation of performance standards (with reference to the most important foodborne contaminants); the definition of 'Standards for Produce Safety' (safe production and harvesting of fruits and vegetables); the enhancement of actions against intentional adulteration; the creation and implementation of detailed sanitary transportation practices

has to be renewed every 2 years). Should foods be imported, the role of the U.S. agent for the foreign facility would be considered as contact person. The registration can be suspended unless an adequate corrective action plan has to be taken and immediately implemented if a certain food linked with a registered facility may hypothetically cause serious adverse health consequences or death to humans or animals

(c) Hazard Analysis and Risk-based Preventive Controls (HARCP). This section concerns mandatory preventive controls for food plants and the new HARCP strategy, with the important exclusion of 'small' and 'very small business' activities. Certain 'on-farm' and operations defined as farms are also not subject to the preventive control rules
(d) Performance standards. This section focuses on gaining a greater understanding of foodborne contaminants and correlated guidelines. These documents will be issued every 2 years
(e) Standards for Produce Safety. The HHS is directly involved in the publication of mandatory minimum standards for the safe production and harvesting of fruits and vegetables with the important exclusion of 'small' and 'very small business' activities
(f) Protection against intentional adulteration. This section has been developed as a tool to provide effective actions against food frauds
(g) Sanitary Transport (detailed sanitary transportation practices).

1.3.3 The FMSA: Responsiveness to Food Safety Crises

The second title of the FSMA is focused on the enhancement of the responsiveness when food safety problems are detected and an immediate action is required (Johnson 2014b). The following sub-topics discussed below apply directly to the aim of improving responsiveness to food safety crises (U.S. Congress 2011):

(a) Inspection Resources. The FDA identified 'high-risk' plants with the concomitant need for augmenting the number of inspections on domestic and foreign facilities. In addition, ports of entry are crucial from the point of view of maintenance of the food safety chain, in addition to audits
(b) Tracking and tracing food records. With reference to high-risk foods and product tracing/tracking systems to improve the overall recall efficiency
(c) Surveillance. The assessment of the U.S. state and local food safety and defense capabilities are considered as one of the pillars of the FSMA
(d) Mandatory Recall Authority. The FDA has received new powers referring to its ability to order food recalls 'under certain circumstances'.
(e) Administrative Detention of Food. Under this criterium, FDA can commission detention of edible products that it identifies as being adulterated or misbranded

(f) Decontamination and Disposal Standards and Plans. This section highlights the role of EPA in spearheading the support and technical assistance in clearance and recovery activities following decontamination and disposal of specific threat agents and foreign animal diseases.

1.3.4 The FSMA and Imported Foods: Consequences for Foreign Suppliers

The third title of the FSMA deals directly with the safety of imported foods, including fresh fruits and vegetables (Johnson 2014b; Kelley Drye 2011; U.S. Congress 2011). Basically, the new approach requires that foreign food business operators (FBO) comply with the FDCA (amended by the FSMA) before the commercialisation of food products and the consequent arrival of edible commodities in the U.S. (Kelley Drye 2011). For this reason, each importer—the foreign FBO or the U.S. agent—has to consider certain measures with the aim of mitigating food safety risks and gather evidence of the required compliance. The following modules of FSMA are significant from the point of view of bolstering the objectives of Title III (Akingbade et al. 2014; Johnson 2014b; Keenan et al. 2015; Kelley Drye 2011; U.S. Congress 2011):

- Foreign supplier verification program and certification
- Third-party certification bodies and correlated accreditation
- Establishment of foreign FDA offices
- Agreements with foreign governments and other actions.

With relation to basic aims of this book, some of the above mentioned points need to be discussed carefully in the following sections because of the mandatory controls that have to be in place for fresh fruits and vegetables. Other arguments without mandatory requirements are not discussed here.

As an example, the expedited entry of certain foods is correlated with the creation and the implementation of a voluntary program for qualifying those specific U.S. food importers. In addition, the role of third-party certifications will be equally important, especially considering the fact that all importers may not be able to demonstrate maintaining a high level of controls over the safety and the security of their supply chains.

1.3.4.1 Foreign Supplier Verification Program and Certification

Basically, foreign food business operators involved in the exportation of their own products to the U.S. have to assure the legal compliance of every U.S. imported product in adherence to FDCA requirements. Dedicated guidance

documents are provided by the FDA with the aim of helping importers. In fact, the creation and the implementation of the FSMA rule for foreign supplier verification programs (FSVP) for importers of food for humans and animals are dedicated to this objective. However, seafood, low-acid canned foods, fruit juices and other edible products currently already subject to the 'Hazard Analysis and Critical Control Points' (HACCP) approach are exempted from the FSVP requirement (U.S. Congress 2011). Similarly, foods imported for research purposes in small amounts may be imported without being subject to FSVP (Kelley Drye 2011). All other foods have to be commercialised in the U.S. with an implemented supplier verification program in place. However, the deadline for these provisions is 2 years after the enactment of the FSMA. At present, general rules should be in force between the last part of 2016 and beginning of 2017[1] (Keenan et al. 2015).

The publication of the final FSMA rule for FSVP is set for 3 October 2015 (Keenan et al. 2015). In the meantime, the U.S. food importers should (Fortin 2015; U.S. Congress 2011):

- Consider the review of their food imports and corresponding suppliers with relation to compliance standards
- Define and implement a careful analysis of hazards
- Carry out audits and/or different surveillance actions on suppliers
- Define and perform corrective activities to ensure that their suppliers are 'approved'
- Create and/or implement and update records of performed actions as per to FDCA requirements.

1.3.4.2 The Role of Third-Party Certification Bodies

The FDA may be assisted by third-party certification bodies when particular food products need to be defined as 'compliant' with the requirements of the U.S. food safety legislation (Kelley Drye 2011). Moreover, these actions of the certification bodies can be specific for single shipments, or focused towards getting the 'source' (food facilities). Parallely, certifications issued by certain institutions may be accepted on condition that the U.S. established standards are observed. Should the FDA consider this option (the third-party certifications), the action of non-FDA entities would be restricted to high-risk foods only. In addition, certification and related documents must be scientifically reliable and the analysis of known food safety risks has to be considered (Keenan et al. 2015; Kelley Drye 2011). FDA has created and implemented a particular user fee-based program for the accreditation of domestic and foreign third-party auditors and certification bodies.

[1] 18 months after the final publication of the FSVP regulations.

1.3.4.3 Establishment of Foreign Inspectorates and Other Actions

The FDA plans to increase its own activity in foreign countries by means of the creation of FDA offices. This measure has two distinct aims:

(1) The enhancement of communication with the non-U.S. institutions and agencies
(2) The augmentation of a powerful transnational cooperation, in spite of known differences (language, cultural behaviour, regulations, etc.).

At the same time, the FDA can establish synergic agreements with foreign institutions with the aim of raising the number of 'actual' audits and inspections, with greater emphasis on high-risk foods (Kelley Drye 2011). In fact, the sheer number of foreign inspections is really challenging for FDA staff: the predicted number of these activities is anticipated to be 19,200 in 2016 (Fortin 2015).

In addition to the creation of a detailed plan in strong cooperation with foreign authorities, partnerships and working relationships with food industries and consumers associations will be equally valuable to ensure food safety of edible goods from foreign countries (Kelley Drye 2011).

1.4 Possible Consequences of the U.S. Imported Fresh Fruits and Vegetables on the Market

On the basis of a previous discussion of FSMA modules, it can be concluded that the U.S. as well as foreign food producers must take steps towards the upcoming requirements in a coherent manner. Briefly, the following points may concern fresh fruits and vegetables industries, with a specific focus on imported foods as a consequence of the strengthening of FSMA rules (Fig. 1.2):

(1) The attention of FDA auditors and other inspectors should be focused on high-risk foods (Calvin 2013) and adjacent factors impacting the safety of those high-risk foods
(2) The verification activities for the inspected and uninspected farms should include the amount or raw materials, the final destination of the agricultural commodities and the processing activity leading to the commercialisation from their non-inspected farms (Keenan et al. 2015)
(3) The application of hazards analysis is mandatory for foreign foods and facilities, if HACCP-based approach has not been used
(4) The differences in food labelling requirements could be an issue, especially in relation to allergens
(5) The definition of 'high-risk' fruits and vegetables has to be based on their association with previous outbreaks (Leavitt and Eurofins 2012). Consequently, the 'history' of certain foods will become a notable predictor for all produce within a correlated category.

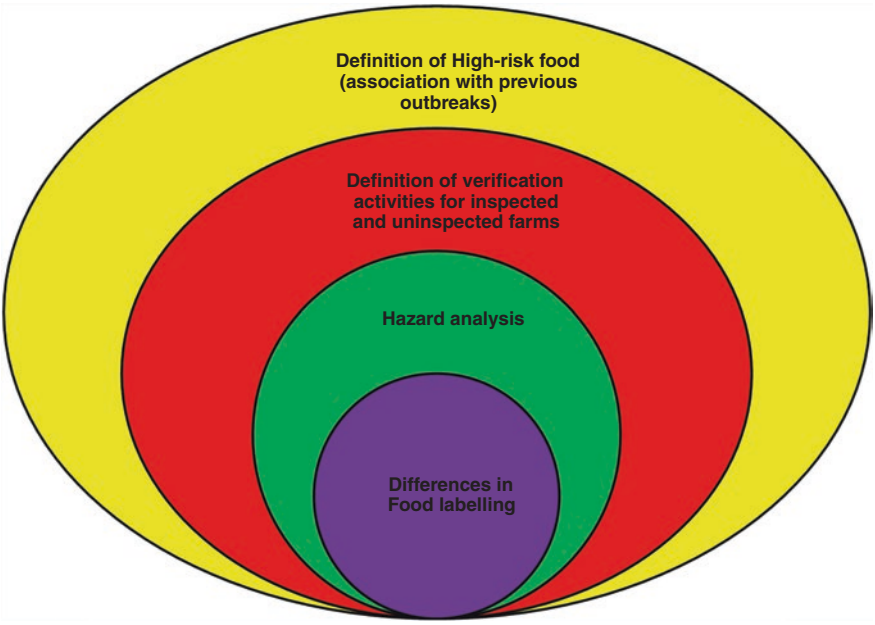

Fig. 1.2 U.S. as well as foreign food producers must take steps towards the upcoming requirements in a coherent manner. The following points may concern fresh fruits and vegetables industries, with a specific focus on imported foods as a consequence of the strengthening of FSMA rules: the definition of 'high risk' fruits and vegetables (this definition has to be associated on the basis of previous outbreaks); the definition of verification activities for inspected and uninspected farms; the mandatory application of hazards analysis; the different applications of food labelling requirements

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Chapter 2
Clostridium Botulinum and *C. perfringens* in Vegetable Foods: Chemistry of Related Toxins

Maria Micali

Abstract The problem of food-borne human diseases by *Clostridium botulinum* and *C. perfringens* remains one of the most interesting research arguments in the field of food technology. Actually, there are two different lines of research depending of the peculiar microorganism and related food-borne disease outbreaks. The clinical syndrome of Botulism is related to *C. botulinum* and a few other species of the same genus. Botulism is caused by botulinum toxin, a potent neurotoxin with the capability of attacking neuromuscular synapses. 90 % of the total number of episodes is correlated to vegetable foods and preparations. Because of the variability of *C. botulinum* (three different subtypes are reported at present), four different neurotoxins at least are known with concern to Botulism. Basically, these molecules are zinc-proteins with endopeptidasic activity; the general structure is functional with reference to the attacking mechanism to pre-synaptic receptors of human or animal cells. At the same way, *C. perfringens* should be considered: the detection in unwashed vegetables and soups by vegetable ingredients may not be excluded. *C. perfringens* is reported to have five different subtypes: one of these bacteria is particularly lethal because of the production of 10 different toxins, one enterotoxin and one neuraminidase. All these molecules are associated to a specified and maybe lethal pathological action.

Keywords Clostridium botulinum · Clostridium perfringens · Endotoxins · Exotoxins · Food additives · Food-borne botulism · Neurotoxins · Pasteurisation · Sterilisation

Abbreviations

HC	Carboxy-terminal
CDC	Centers for Disease Control and Prevention
ELISA	Enzyme-linked immunosorbent assay
H	Heavy

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MW	Molecular weight
HN	Nitrogen-terminal
NaCl	Sodium chloride
USA	United States of America
A_w	Water activity

2.1 Introduction to Biological Toxins

The action of pathogenic bacteria is mediated by the production of specific virulence factors such as toxins: exotoxins (produced mainly by Gram-positive agents) and endotoxins (produced only by Gram-negative bacteria).

Exotoxins are proteins with molecular weights (WM) ranging from 10 to 900 kDa. These molecules are often controlled by extrachromosomal genes (plasmids) excreted into the surrounding medium (Aoki 2003; Di Bonaventura 2011; Rackley and Abdelmalak 2004). They can also be released after cell lysis; subsequently, the binding to specific cellular receptors (cell specificity) can be observed, with toxic or lethal effects for host cells. Exotoxins have a high toxicity for animals; in addition, these proteins can become highly antigenic (immunogenic) stimulating the formation of neutralising antitoxins. Antitoxins are non-pyrogenic substances: these molecules can be converted to toxoids or anatoxins after treatment with formalin, acids or under heating conditions (Di Bonaventura 2011).

A further classification of exotoxins is made on the basis of two different criteria: the specificity of the target (cell tropism) and the mechanism of action (Middlebrook and Dorland 1984). With relation to targets and related specificity, the following classification can be proposed:

- Neurotropic toxins, if they act at the level of central or peripheral nervous system. Examples: tetanus and botulinum toxins
- Enterotoxins, if they act on the intestinal mucosa. Examples: Cholera toxin, enterotoxin of *Escherichia coli*
- Pantropic toxins, if the diffusion of cellular receptors is observed. Examples: diphtheria, pertussis, Shiga toxins.

With concern to observed mechanisms of action, the following types can be identified (Kotb 1995; Middlebrook and Dorland 1984):

(1) Superantigens (type I toxins). These proteins, produced by *Staphylococcus aureus* and *Streptococcus pyogenes*, are able to stimulate the immune response even with a concentration of 10–15 g/ml. In fact, superantigens can notably stimulate the immune response
(2) Exotoxins (type II toxins). These molecules can damage membranes of the host cell by means of the production of spores in the cell membrane. They can also digest cellular materials and alter the composition of membranes
(3) AB-toxins and other toxins (type III toxins). These substances can interfere with vital functions of the host cell.

With concern to *C. perfringens*, related exotoxins are reported to have haemolytic and necrotising effects. In addition, the relaxation of tissues, caused by the by gas produced by the fermentation of carbohydrates and the consequent production of gaseous substances, can favour the spread of gas gangrene (myonecrosis). Moreover, a mixed form of diarrhoea-dysentery may be ascribed to the action of these toxins. Anyway, *C. perfringens* exotoxins are usually subdivided in the following way (Bakker 2012; Carli 2009; McDonel 1980):

(1) α-toxin (lecithinase). This toxin is reported to increase the permeability of capillaries and muscle cells by destroying the lecithin present in the cytoplasmic membrane. This phenomenon may cause edema when myonecrosis is reported
(2) κ-toxin (collagenase). It is considered responsible for injuries of soft gas gangrene. The production of collagenase is due to the degradation of connective tissues and muscles
(3) μ-toxin (hyaluronidase). The production of this molecule is due to the breakage of intercellular bonds in the tissue.

Dimeric AB-toxins can be chemically subdivided in two parts: the active part A and the binding component B. These toxins are produced by both Gram- positive and Gram-negative bacteria and secreted in external environment; the component B is responsible for the target specificity because it can bind to the receptor surface of the host cell. Subsequently, the AB-toxin can be transferred across the membrane for endocytosis, whereby the component A is separated from B to migrate into the cytoplasm. However, certain AB-toxins can partially enter into host cells by means of the introduction of the component A only through a pore membrane.

The main host defence against toxins is represented by the production of specific toxin antibodies. After the connection to the antibody, the toxin can no longer bind to the cell surface receptor. Good examples of AB-toxins are the enterotoxin of *C. perfringens* and botulinum toxin of *C. botulinum*.

C. botulinum produces an endopeptidase: this substance is able to block the release of acetylcholine at the neuromuscular junction. Botulinum toxin cleaves proteolitically synaptobrevin interfering with the formation of synaptic vesicles. The result of this action is flaccid paralysis (bilateral descending weakness of peripheral muscles, paralysis of respiratory muscles with consequent death); the toxin is called 'neurotropic' because it interferes with the release of neurotransmitters (Karalewitz and Barbieri 2012; Carli 2009).

2.2 *Clostridium Botulinum* and Related Toxins

C. botulinum is a rod-shaped bacterium with Gram-positive features when speaking of young cultures. It produces a protein toxin that can cause flaccid paralysis of botulism in humans and animals, also defined neurotoxin (CDC 1998). The ability to synthesise

the botulinum neurotoxin is the only common feature that unifies all life forms of this species, even though these are very heterogeneous. Following the isolation of other strains of *C. botulinum* from clinical (human), food and environmental samples, researchers have deducted that botulinum toxins produced by these strains are not identical, in spite of the similarity of botulism diseases and related mechanisms of action. In fact, antisera produced against a specific toxin have been recognised unable to neutralise the toxicity of other types. At present, seven different antigenic variants of botulinum neurotoxins—named with capital letters from A to G—have been reported (CDC 1998). Botulinum A, B, E and rarely F toxic types cause botulism in humans, while animal botulism is caused by botulinum C and D toxins; in addition, botulism is not apparently associated with the remaining botulinum G toxin, until now.

In the most part of situations, *C. botulinum* X (where X = A, B, C and so on) is reported to produce one toxin type only: this substance is named with the same capital letter. For instance, the botulinum toxin F is produced by *C. botulinum* type F. However, there are also rare *C. botulinum* strains which can produce two toxins at the same time, including new types (Barash and Arnon 2014): generally, one of the two toxic molecules is produced in greater quantities than the other substance.

Another type of microbial classification can be considered with concern to the subdivision of life forms (species) according to their metabolic and physiological features (CDC 1998). In detail, four different groups have been considered: each category includes strains with similar properties, even if they produce neurotoxins of different types.

2.2.1 Proteolytic and Non-proteolytic Clostridia

A selected group of life forms—'proteolytic' strains—can obtain metabolic energy by means of the simple scission of proteins, while other 'non-proteolytic' life forms are accustomed to use sugars by means of different mechanisms. Just after the production of catabolites, foods contaminated with *C. botulinum* usually assume more or less unpleasant organoleptic characteristics, depending on the peculiar food matrix (CDC 1998; Lund and Peck 2013).

Life forms of these two groups also differ in their physiological features. All life forms are mesophilic but:

(a) Proteolytic clostridia grow at temperatures up to 40 °C (on the other hand, they are not able to live below 10 °C)
(b) Non-proteolytic life forms can survive and increase their number at 3.3 °C, although optimal temperatures are reported to be around 30 °C.

As a result, the environmental distribution of strains is different. The first group (proteolytic strains) is reported to be normally found in dry and temperate geographical areas, while the latter category non-proteolytic life forms—can be often observed in more humid and cold climates. Furthermore, spores of the first group

(proteolytic species) have a higher resistance to chemical (pH, water activity, salt concentration) and physical (temperature and oxidation-reduction potential) conditions, compared to those of the second group. In other words, the enhanced resistance of proteolytic strains can explain the survival of life forms in extreme environmental conditions, with some possible sanitary reflection when speaking of food safety and teÉchnology problems (food preservation).

2.2.2 Animal Botulism-Related Strains and Asaccharolytic Clostridia

Strains which cause botulism in animal species have features. In brief, they grow with difficulty only in conditions of strict anaerobiosis. In addition, these life forms produce few spores. Moreover, differently from other *C. botulinum* strains, these clostridia are strongly haemolytic if cultured on blood agar plates because of the known haemolysin activity. These strains are also capable of producing two botulinum toxins other than those defined as C2 and C3. This classification is needed because of the existence of another type C neurotoxin. Actually, the role of these strains in the pathogenicity has to be clarified yet.

Finally, *C. botulinum* type F remains to be considered. These strains are distinguished because they are completely asaccharolytic (José et al. 2014) and do not produce lipases. However, their physiological proÉperties are not completely known for two reasons: these asaccharolytic life forms have been isolated recently (in the 1970s); in addition, no episodes of botulism have been ascribed to toxin-type F until now.

2.3 Botulinum Neurotoxin

The main route of entry for toxins is generally correlated with the digestive system. For this reason, toxins should show notable resistance to proteolytic reactions and denaturation into the gastric apparatus. Otherwise, toxic molecules could not remain in the intestinal tract.

By the chemical viewpoint, the solution appears linked to the dimension of toxins: similar substances are produced as complex systems (MW: 300, 500 and even 900 kDa) with associated non-toxic proteins that allow them to resist in the gastric environment. Because of the instability of the multimeric complex at alkaline pH values (in the intestinal tract), the toxin is easily released and subsequently absorbed into the bloodstream.

Similarly to tetanus toxin, botulinum toxins are metal proteins with recognised endopeptidase activity: the metal is zinc (Schiavo et al. 1992). The general structure is shown as a double chain with MW of about 150 kDa (approximate value). The double chain can be subdivided (CDC 1998) in a heavy (H) structure of

100 kDa and a lighter (L) chain of 50 Kda; chemical structures are linked together by means of a disulfide bridge (non-covalent interactions are not reported). The heavy chain is in turn constituted by the nitrogen-terminal (HN) and the carboxy-terminal (HC) domains. The L chain performs the catalytic function of the toxin while HC domain (on the H structure) is responsible for binding action to pre-synaptic receptors for internalisation. Moreover, HN is also called 'translocation domain': in fact, the translocation of the L chain from the lumen to the endosomal cytoplasm of cell is ascribed to this domain (CDC 1998).

Botulinum toxins are produced in an inactive form as single polypeptide chains of about 150 kDa; later, a proteolytic cut generates the previously described active double-chain form. In many situations, the cut is carried out by proteases produced by clostridia themselves. However, some strains do not possess these enzymes: as a result, toxins may be released in the single-chain form; the subsequent activation is performed by other proteases in the host tissue.

H and L chains of various serotypes are composed of 840 and 430 amino acids, respectively (average estimation); in addition, these structures show homologous regions separated by other regions with little or no homology. The most preserved segments of the L chain are represented by one hundred of amino terminal residues; moreover, the central area contains the 'HExxH' zinc—protease consensus sequence (Schiavo et al. 1992). On the other side, the H chain is the less preserved structure, especially in the carboxy-terminal part. Finally, cysteine units are totally preserved: their role is related to the formation of the inter-chain disulfide bridge.

The determination of the crystallographic structure of botulinum neurotoxins, types A and B (Lacy et al. 1998; Swaminatan and Eswaramoorthy 2000), reveal that these structures are composed of three distinct domains. The presence of a peculiar handle in the HN domain should be considered in fact, the catalytic domain remains inaccessible to the active site because of the protection offered by this area around the perimeter.

2.3.1 Binding Domain of HC

The crystallographic structure of the HC domain reveals a structural subdivision in two subdomains. The first of them can be defined nitrogen-terminal subdomain (HCN) while the other may be named carboxy-terminal subdomain (HCC).

HCN is composed of two sheets with a peculiar 'jelly-roll' topology (Gallego del Sol et al. 2002), similarly to certain legume lectin crystal structures (carbohydrate-binding proteins). This subdomain is highly preserved in clostridial toxins.

The HCC subdomain is formed from a pattern called 'β-trefoil' structural motif. This feature is present in various proteins involved in 'recognition' and binding functions such as interleukin-1, fibroblast growth factors and Kunitz-type trypsin inhibitor. The amino acid sequence of this subdomain is poorly preserved between clostridial neurotoxins (Montecucco et al. 2004).

2.3.2 The HN Translocation Domain

This portion of the toxic molecule has a high homology in various serotypes; the same thing can be affirmed with relation to the expected secondary structure. The central part is formed by a pair of 10 nm-helices: the complete structure is similar to certain proteins that interact with membranes. Two similar examples are colicine and hemagglutinin of influenza virus: these proteins are able to show structural variations when placed in acidic environments. As previously mentioned, an important feature of HN domain is the existence of a loop wrapped around the catalytic domain with the consequence of preventing the catalytic activity (Lacy et al. 1998).

2.3.3 The Catalytic Domain

The light L chain is the part of the toxin with catalytic activity. This domain consists of a set of helices and sheets with the helix of the zinc binding motif in the central portion. The central helix consists of a peculiar amino acid sequence preserved in all zinc—peptidase systems: this structure is generally named 'histidine-glutamic-XX-histidine' (HExxH), where H stands for histidine, E means glutamic acid and X stands for a generic amino acid (Schiavo et al. 2000). Involved amino acids, whose chains appear to be closer to zinc, are: His 223, Glu 224, His 227, Glu 262 and Tyr 366. Imidazolic rings of histidine are intended to interact with zinc as well as the side chain of Glu 262. The amino acid Glu 224 is particularly important because of the coordination of a water molecule which is needed in the hydrolysis reaction of the peptide bond of the targeted protein (Rossetto et al. 2004). The organisation of the active site is similar to that of thermolysin (Morante et al. 1996). With relation to Tyr 366, the phenolic ring is not completely located behind the metal: the distance is about 5 Å. It may be supposed that this ring performs functions of coordination with the substrate: substitutions of this amino acid with glycine have demonstrated an inability of proteolysis of the toxin. In conclusion, we can say that the structural organisation of botulinum toxins collimate perfectly with their mechanism of action. This mechanism is based on four key events (Montecucco and Schiavo 1994):

(1) Binding to receptors
(2) Internalisation
(3) Translocation into the cytoplasm
(4) Enzymatic modification of the target.

Once entered into a body and introduced in the body fluids, toxins are able to arrive at the muscle junctions. Subsequently, after the internalisation step, they can perform the typical toxic activity on nervous impulses: the blockage of transmissions.

2.4 Different Forms of Botulism

Botulism is a serious neuroparalytic syndrome that affects both man and animals, caused by botulinum neurotoxin in neuromuscular synapses. The toxic activity is expressed by blocking the release of neurotrasmitter acetylcholine; the subsequent step is the blockage of nervous transmissions at the neuromuscular junction (flaccid paralysis). Signs and symptoms of the disease are those characteristic of bulbar palsy with typical symmetrical descending trend and possible respiratory paralysis, in severe situations (Montecucco and Schiavo 1994).

Actually, the diversity of clinical diseases is strictly correlated with the collection of epidemiological data in different Countries and the difficult estimation of the actual incidence. Basically, the availability of data on botulism (incidence and prevalence, clinical forms and causes) depends on the existence of a surveillance system. Botulism has to be obligatorily notified in many industrialised countries; however, the exact moment of notification is variable.

2.4.1 Wound Botulism

Wound botulism is an infectious form reported for the first time in the United States of America (USA) about 50 years ago, due to the germination of *C. botulinum* spores and toxin formation in wounds. Neurological symptoms of flaccid paralysis are not different when compared with food-borne botulism except for some unique features (CDC 1998):

(a) It is a very rare event, involving a single case per situation
(b) The incubation period is longer compared to food-borne disease (between four and 14 days)
(c) Gastrointestinal symptoms are not reported. This lacking feature is the indirect evidence that botulinum toxin causes essentially neurological symptoms, while gastrointestinal signs can be attributed to other toxic substances produced during the bacterial spreading in foods
(d) Fever is often reported in wound botulism as a result of wound infection.

2.4.2 Intestinal Toxaemia Botulism

Intestinal toxaemia botulism refers to two forms of botulism infection and the correlated intestinal colonisation of the infant and adults. Infant botulism was described for the first time in 1976 from Arnon in California: this Researcher has associated neurological symptoms of an infant subject with the colonisation, multiplication and production of toxins in the intestine by spores of *C. botulinum*.

However, infant botulism is not only due to *C. botulinum* (Anderson 2012): other strains of toxigenic clostridia may very rarely cause the same syndrome, including the toxin-type F *C. baratti* (USA 1979) and the toxin-type E *C. butyricum* (Italy 1984). Infant botulism is reported to affect children under 12 months of age (especially between 3 and 6 months).

Neurotoxigenic clostridia are ingested in the form of spores and can survive gastric acidity with the arrival to the intestine. Because of the 'immaturity' of intestinal flora of the host (the absence of a reliable microbial competition between clostridia and intestinal life forms), bacterial spores can germinate, multiply, colonise temporarily the intestinal lumen at the level of the colon and produce in situ the neurotoxin. It has to be noted that the intestinal mucosa is not affected by the infection. The symptomatology is similar to that of adults.

The botulism from intestinal colonisation of the adult subject is an extremely rare possibility worldwide. Generally, this syndrome is the result of the production of the toxin in the intestinal lumen of adults and children. Patients have typically some functional or anatomical abnormalities of the intestinal tract. Alternatively, prolonged antimicrobial therapies may allow the colonisation.

2.4.3 The Food-Borne Botulism

The food-borne botulism is an intoxication that follows the ingestion of aliments in which neurotoxigenic clostridia have spread and developed sufficient quantities of botulinum neurotoxin (Auricchio 2009; CDC 1998; Meucci and Muli 2006). Approximately 90 % of all reported situations in the world are related to the consumption of home-made preserves, especially canned vegetables; on the other hand, industrial meat and fish preparations are rarely associated with botulism. A very small quantity of toxin (30 ng) is sufficient to cause disease and even death: symptoms are reported between 2 h and 8 days after ingestion of the contaminated food. However, the most part of situations usually appears between 12 and 72 h.

Three types of *C. botulinum* are generally discussed when speaking of food-borne botulism (CDC 1998). *C. botulinum* type A is predominant in the western USA, China and Argentina: it is usually associated with vegetable products. *C. botulinum* type B is typically observed in Europe (meat, vegetables). Finally, *C. botulinum* type E is present in foods of marine and cold coastal regions.

At present, it can be noted that the number of botulism-related episodes reported annually during the last century have substantially been reduced in industrialised countries because of the implementation of appropriate control measures by manufacturers of canned foods. These procedures have been specifically created with the aim of preventing risks arising from the possible presence of *C. botulinum* spores in food ingredients. As a result, botulism is now often associated to home-made preserves.

In fact, *C. botulinum* may cause intoxication if:

(a) Related spores are not inactivated by technological procedures
(b) The food intermediate or the final product is re-contaminated after processing
(c) The food intermediate or the final product has a favourable chemical composition when speaking of possible spreading and toxin production by *C. botulinum* (storage temperatures have be favourable)
(d) The edible product is used without baking or heat processes with the aim of inactivating preformed toxin molecules.

When speaking of preserved foods, manufacturers have always considered *C. botulinum* and the production of related toxins as the main danger; in addition, foods have to be processed without unpleasant organoleptic variations. The degree of safety achieved in the production of preserved foods can be estimated on the basis of the estimation of residual lethality (or inhibition) of *C. botulinum*. Inhibition is directly dependent (Auricchio 2009) from:

- The preservation process or system, and
- The frequency (probability of detection) of this life form in food commodities.

C. botulinum is reported to be detectable in many foods (CDC 1998); in addition, it is certainly ubiquitous when speaking of spores. The soil is the main habitat, but spores can be also isolated from water, dust, sediment, feces, insects and various organic materials (Poda 1997). Consequently, *C. botulinum* can be easily found on many vegetables—red peppers, carrots, onions, potatoes, parsley, spinach, garlic, cabbage, cultivated mushrooms, etc. Moreover, spores may be detectable in connection with fertilisers.

Interestingly, the contamination of farmed fish and fishery products by *C. botulinum*—especially non-proteolytic types—can be correlated to the presence of sediments of terrestrial origin (Burns and Williams 1975). On the other side, meat preparations are reported to be contaminated with a notable frequency when speaking of pork meats instead of cattle and sheep meats or poultry for the same reason: the increased but accidental consumption of soil.

With concern to raw milk, derived products are rarely associated with botulism in spite of the probable and often observed contamination with clostridial spores because of the use of silage feedings for dairy cows.

The prevention of botulism is generally obtained by means of processing techniques capable of destroying spores or preventing the production of toxins. After packaging, a reduced number of canned foods could appear swollen or bruised: should this be the situation, the canned food would be surely contaminated and dangerous. However, certain foods are not exposed to risk of contamination by botulinum toxin: tomato puree (the typical acidity of tomatoes does not allow the multiplication of *C. botulinum* or toxin production), fruit jams (the amount of sugar prevents spreading) and pickles (pH values lower than 4.5 are not favourable for clostridia).

2.5 Inactivation of *C. botulinum*, Other Neurotoxigenic Clostridia and Related Toxins

2.5.1 Destruction of Spores in Foods

Clostridial spores remain viable for long periods of time even when environmental conditions are absolutely unfavourable to their development. Consequently, should intrinsic factors (pH, water activity, redox potential values, microbial antagonism, preservatives) and extrinsic variables (temperature, retention time) be insufficient, produced foods or edible intermediates would necessarily be thermally treated for a sufficient time. In fact, heat is currently the most common, practical and cheap treatment used for the sterilisation of foods.

The modern industry ensures the safety of low acidity foods (packed in hermetically sealed containers) by means of the use of a thermal process: generally, foods have to be treated at 121 °C for 3 minutes until the number of active *C. botulinum* spores arrives to 10–12 per serving size (Auricchio 2009).

Various factors can influence the thermal resistance of spores. Basically, spores can become more resistant if water activity (A_w) is low. On the other side, acidic or alkaline pH values can decrease thermal resistance. In addition, lipid or protein substrates may protect the *C. botulinum* spores under heating processes.

With relation to milk, the ultra high temperature (UHT) process—up to 137.8 °C for 2 seconds only—has shown excellent results. In fact, *C. botulinum* spores are reported to be destroyed at 125 °C for 5 s. Alternatively, the use of ionising radiation—gamma rays (produced by radioactive cobalt −60 isotopes) and X-rays—can destroy spores; however, these systems are not used because *C. botulinum* are resistant to allowed irradiation levels for stored foods.

Botulinum spores can also be present on equipment, packaging materials, in waters for washing or cooling purposes, etc. For these reasons, non-thermal systems such as chemical treatments can be preferred to avoid phenomena of recontamination.

Chlorine and chlorinated agents are among the most used sanitisers in the food industry (Gurnari 2015): they can show an effective action against bacterial spores when the substrate is apparently free from residues of organic materials. The resistance of *C. botulinum* spores to the action of free available chlorine may change depending on strains. In general, the best heat-resistant forms require more prolonged exposure times; consequently, the use of aqueous solutions with 100–200 ppm of hypochlorite (contact time $\geq$2 min) is needed and recommended for the sanitation of equipment. Moreover, *C. botulinum* spores can be inactivated by ozone and ethylene oxide, generally used for sterilisation treatments of dried foods, or hydrogen peroxide, used in the aseptic packaging of foods (milk, eggs).

The protein responsible for botulism is sensitive to heat; however, the thermal inactivation of this toxin cannot be linearly elaborated. In fact, toxin is more stable at pH 5 in comparison with other conditions. Moreover, the possible presence of

certain substances in foods (divalent cations, anions of organic acids) may protect toxins from heating consequences; anyway, botulinum toxins A, B, E and F are inactivated by heat treatment at 79 °C for 20 min or 85 °C for 5 min (Auricchio 2009).

2.5.2 Control of Growth and Toxin Production

Certain factors may be helpful in food productions when speaking of the possibility of managing the growth of *C. botulinum* and/or toxin production. These factors can act individually or in combination and their action is extremely important in foods with high moisture: these products cannot be thermally processed without the complete alteration of organoleptic features.

With relation to cold storage, it should be noted that normally accepted temperature values for food preservation by mass retailers can be an acceptable safety factor against botulism. In fact, the lowest temperature for the growth of proteolytic strains types A and B are reported to be 10 °C, while non-proteolytic clostridia appear to be inhibited below 3.3 °C. Anyway, growth and toxin production at low temperatures are long enough; consequently, non-proteolytic strains can become really dangerous when a long shelf life is assessed and labelled. On the other hand, proteolytic clostridia may grow notably if a moderate or severe abuse of storage temperatures is observed, e.g. after a thermal storage of 7 days at 15 °C and 2–3 days at 20 °C.

2.5.3 pH and Inhibition of Clostridia

The minimum pH value reported for the growth of clostridia proteolytic strains is 4.6; in addition, this value can be considered above 5.0 when speaking of non-proteolitic strains (CDC 1998; Sobel et al. 2004).

The protection of high moisturised foods with low protein content (vegetable products) can be obtained with the addition of acidulants. The aim is to assure a final pH value around 4.6: this value allows the easy management of spore germination and the consequent inhibition of toxin production at room temperature. On the other hand, the inhibitory action can be nullified by the concomitant spreading of competitors, such as yeasts, moulds and/or bacilli with consequent pH increase ('metabiosis' effect).

The pH-induced inhibition may be lowered in food with high protein contents: in fact, proteins may slow down acidification with the exception of fermented sausages where the production of botulinum toxin is difficult because of the concomitant action of fermenting (natural and/or added) starter cultures. However, fish products can show different situations because of prolonged fermentation times and low concentrations of carbohydrates with consequent 'high' pH values. As a result, the inhibition of *C. botulinum* can be obtained in fish and fisheries products

with the additional use of salt and the management of storage temperatures. pH can play an important role when speaking of *C. botulinum* inhibition in dairy products.

2.5.4 Water Activity, Sodium Chloride and the Inhibition of Clostridia

With concern to *C. botulinum*, growth and toxin production are influenced by the amount of available free water or A_w. Sodium chloride and other solutes such as potassium chloride, sucrose, lactose, etc., are able to lower A_w. In the industry of fish products, the use of sodium chloride (NaCl) can be helpful: refrigerated products can be easily protected with only 5 % of NaCl in the aqueous phase, while fish products at room temperature would require 10 %. Because of the influence of pH on the inhibitory effect of NaCl, the reduction of added salt can be easily justified when pH is lowered.

2.5.5 Inhibition of Clostridia in Foods: The Use of Allowed Additives

Nitrite is employed in the processing of meat and fish products because of its known inhibition properties against *C. botulinum* (Barbieri et al. 2014; CDC 1998). However, the inhibitory effect of nitrite can be fully enhanced in synergy with other factors (pH, A_w, temperature, etc.). In addition, the possible carcinogenicity and mutagenicity caused by the formation of nitrosamine (produced from the reaction of nitrite with amines) have recently been considered with the consequent research for alternative strategies and substances: the final aim should be the reduction or the total replacement of nitrite.

Sorbic acid and its salts appear able to delay the growth of *C. botulinum* and toxin production (Auricchio 2009; Lund et al. 1987): this action is enhanced when pH decreases; the inhibitory effect depends on the concentration of undissociated sorbic acid.

Other substances such as ascorbic acid, normally used to ‘speed up’ the maturation process in meats, and ‘liquid smoke’ (smoking aromas), especially used for hot processed fish products, allow to reduce the concentration of nitrite and NaCl, respectively. At the same time, the inhibitory effect on the germination and toxin production by *C. botulinum* remains unchanged.

Essential oils (garlic, onion, black pepper, clove, oregano) or alcoholic extracts (nutmeg, garlic, rosemary, thyme and sage) of many aromatic plants are reported to inhibit spore germination or growth of vegetative clostridia (De Wit et al. 1979). A good safety effect against *C. botulinum* can also be obtained by adding natural preservatives or biopreservatives to foods. Generally, the addition of certain lactic

acid bacteria or their purified metabolites (bacteriocins) such as nisin can be useful when speaking of sterilised vegetable preserves or thermally treated spreadable cheeses. Actually, these agents and substances play an indirect effect because of the observed reduction of thermal treatment times and the lowering of phosphate and NaCl at the same time. As an example, the aqueous content can be increased even in cheeses stored at room temperature.

2.6 Methods for Toxin Detection and *C. botulinum* Isolation

The analytical confirmation of suspected cases of botulism is performed by means of the search for botulinum toxins and spores of neurotoxigenic clostridia in biological samples (serum, feces, edema, gastric contents, wound exudate, animal organs, such as liver, spleen and intestines) and food products (CDC 1998).

With relation to the research of botulinum toxins, the only validated method remains the in vivo testing method on rats (mouse test). This examination requires the use of polyvalent and mono-specific botulinum antitoxins. Three days are also needed even if definitive results may be observed in 3–4 h in certain situations. Alternative in vitro methods based on the enzyme-linked immunosorbent assay (ELISA) technique have been developed (CDC 1998); however, these ELISA testing methods are not apparently able to show the required sensitivity and specificity, especially when applied to complex matrices such as food and fecal samples.

The search of *C. botulinum* spores in the stool of a patient with botulism is as important as the detection of toxins. The presence of prolonged spores in stools is an important demonstration of the intestinal colonisation by the organism and suggests, therefore, a toxinfective form of the disease.

Because of the prevailing chemistry-oriented discussion on toxins, the cultural search of neurotoxigenic clostridia is not discussed here. However, it may be highlighted here that the isolation of *C. botulinum* spores is not considered a good result when speaking of foods without botulinum toxin. Substantially, the importance of the cultural isolation and identification of clostridia is important when contaminated foods can be good ‘culture media’ and related features can really favour germination. With concern to the identification of contamination sources, the isolation of spores should be carried out in food or environmental samples.

2.6.1 C. perfringens

It is a short and wide bacillus, rarely arranged in chains, with a spore-forming polysaccharide capsule. It may rarely produce spores in normal culture media (Auricchio 2009): in fact, the production of spores needs specific conditions and special media such as the so-called ‘sporulation broth’.

C. perfringens includes five types designated by the letters A to E (McDonel 1980). Type A only is responsible for most infections in human beings: this strain can produce four exocellular lethal toxins, an enterotoxin, a neuraminidase and a number of biologically active proteins conventionally indicated with letters of the Greek alphabet. In particular, some of these proteins are toxic molecules. Other enzymes, especially the κ toxin (collagenase), cause the degradation of connective tissues and muscles, while the μ toxin (hyaluronidase) may break intercellular bonds in the tissue (Auricchio 2009; Poilane et al. 1998).

The toxigenicity of *C. perfringens* has to be necessarily evaluated on the basis of the evaluation of induced sporulation of vegetative cells: the enterotoxin production is observed during this step.

α toxin is produced by all *C. perfringens* strains (Tweten 2001): this phospholipase C (lecithinase) can lyses erythrocytes, leukocytes and endothelial cells with the consequent increase in vascular permeability, haemolysis, bleeding and tissue destruction. In other words, α toxin is able to damage lecithin in the cytoplasmic membrane; the alteration of membranes is responsible for gas gangrene and other situations.

β toxin is responsible for necrotic lesions (necrotising enteritis), while ε toxin is a protoxin activated by trypsin: it can cause the increase of vascular permeability in the gastrointestinal wall. Finally, the 'iota' or ι toxin is reported to be also responsible for necrotic activities (McDonel 1980).

With concern to food products, especially meat preparations, *C. perfringens* is often considered responsible for different poisoning episodes. Generally, the following symptoms and infections are ascribed to *C. perfringens* (Altemeier and Fullen 1971; Finsterer and Hess 2007; Fisher et al. 2004);

- Cellulitis (with formation of gas in the soft tissues)
- Suppurative fasciitis or myositis (accumulation of pus between the muscle bundles but without muscle necrosis and systemic symptoms)
- Myonecrosis or gas gangrene (extensive localised degeneration of muscles with rapid tissue necrosis accompanied by shock which occurs in 50 % of cases).

Anyway, the metabolic activity of *C. perfringens* is the cause of necrosis associated with gas production; related toxins are responsible for extensive bleeding and haemolysis. Systemic infections such as gas gangrene and suppurative myositis should be immediately treated surgically with penicillin in high doses; mortality is reported to range from 40 to 100 %.

2.6.2 C. perfringens and Food Contamination

With concern to food safety, the most important feature of *C. perfringens* is the ability of developing at high temperatures (Auricchio 2009; Loewenstein 1972). Substantially, this life form can show optimum growth between 43 and 45 °C (range: 15–50 °C). Moreover, *C. perfringens* can tolerate A_w values between

0.95 and 0.97, while pH can range from 5.0 to 8.0. Finally, NaCl has not negative (inhibitory) effects when the related addition does not exceed 5–6 %.

This clostridium is reported to be a strict anaerobe: it can grow in liquid media at low redox potential and in soil obtained by including reducing agents such as sodium thioglycolate and tank (some strains are inhibited by thioglycolate). A small amount of agar (0.1–0.3 %) is normally added to liquid media with the aim of decreasing oxygen diffusion and maintaining low redox potential values.

However, *C. perfringens* is reported to show a certain tolerance to aerobic conditions in foods: this attitude can be very notable in comparison with other anaerobes. It can grow not only in vacuum-sealed foods, but also in bulk food if reducing substances are present (Auricchio 2009).

Generally, food contamination causes are correlated with the intestinal tract of human beings and animals (Miwa et al. 1999). Food poisoning from *C. perfringens* has normally a short incubation period (8–24 h), clinical manifestations with abdominal cramps and watery diarrhoea without fever, nausea or vomiting, and a 24 h-clinical course (maximum estimation).

Food-borne diseases caused by *C. perfringens* are carried out through the action of a heat-fleeting enterotoxin produced during sporulation (Auricchio 2009; McClane 1996). This enterotoxin, able to change the permeability (cytotoxic and enterotoxic behaviour), is not produced by all known strains. Anyway, enterotoxic effects are due to ingestion of a large number of life forms (10^8–10^9 germs). The disease can be determined either by the consumption of massively polluted raw foods or, more frequently, from cooked foods containing spores before cooking (even low numbers). Storage temperatures have to be necessarily between 15 and 50 °C. After the release of toxins in the intestinal tract, the host perceives the following symptoms after 10–24 h after ingestion: severe abdominal cramps, gas formation and diarrhoea (nausea, vomiting, and fever are rare).

With relation to the possibility of toxin production in foods, a good safety advice is the use of refrigerated systems for storage after preparation (maximum temperature: 4 °C); alternatively, heating can destroy toxins (minimum recommended temperature: 65 °C). Another technological advice is correlated to the rapid cooling of meat preparations: rolled meat foods should be subdivided in small pieces with the aim of performing cooling in the fastest way (these meat products may be vulnerable in catering companies and hotels).

C. perfringens is responsible for two forms of enteritis, produced by distinct strains of the germ (Auricchio 2009). Type A produces a form of diarrhoea with abdominal pain within 8–20 h after ingestion of the contaminated food (24 h—clinical course). This situation has been often observed (catering services). On the other hand, type C is responsible for necrotising enteritis (bloody diarrhoea with abdominal pain, shock, peritonitis and obstruction of the intestinal mucosa). This situation occurs within 5 days after ingestion of the contaminated food; the mortality can reach 50 % of patients. Necrotising enteritis is a rare process of acute necrosis; at present, it has been reported in Papua New Guinea because of two main risk factors and causes: the exposure to large amount of germs and malnutrition. The analytical confirmation of enteritis is carried out in the laboratory by

means of cultural isolation and immunological tests on clinical samples. With relation to toxins, α and β types are generally reported with notable frequency.

In general, enteritis is associated with cooked meat (beef, pork or poultry), meat gravies, sauces and soups. Once more, the prevention of contamination by *C. perfringens* involves cooking procedures of foods, recommended storage at temperatures below 10 °C or above 70 °C immediately prior to heating in order to reach inner temperatures of 75 °C or more.

2.7 The Microbiological Contamination in Food Industries

The management of food plants can be differently carried out depending on dissimilar health and hygiene factors (Auricchio 2009; Dodds 1993): specific chemical properties (acidity, sugar content, water content), degree of ripeness, harvest time, physical features, type of cultivation (on-ground, underground, open field cultivations; with mulches, greenhouse systems, etc.).

Non-acidic vegetables with a pH > 5.1 (salads), are more easily attacked by microbial life forms; acidic vegetable products with pH ranging from 4.1 (tomatoes) to 5.1 are more resistant.

A special attention has to be considered when speaking of leafy vegetables, risky products with concern to contamination, for various reasons: the product is close to grounds; tissues are extremely exposed to microbial attacks; surface/volume ratios are remarkable. As an example, the rupture of tissues and the consequent leakage of juices (promotion of cell spreading and microbial growth) can easily create an environment rich in nutrients and water.

Many types of life forms can populate the surface of vegetables (Auricchio 2009): the variety of genera and species—especially mesophilic bacteria (25–30 °C), psychotropic germs (able to spread under refrigeration temperatures), coliforms and *enterobacteriaceae*—reflects growing conditions and nutrient media. However, the dominant population on soils and vegetables is given by *Pseudomonas spp* (50–90 %) (Villani 2007). On the other side, the following life forms are not often found:

- Lactic acid bacteria
- Anaerobic spore-forming organisms, occurring as natural microflora of the soil (up to 10^6/g), including clostridia
- Other pathogens such as *Salmonella spp*, *Campylobacter spp*, *Shigella spp*, *Listeria monocytogenes*, *Staphylococcus aureus*, Escherichia *coli*, *Bacillus cereus* (Gardini 2011).

These organisms can contaminate vegetables during the cultivation and the subsequent harvest: in fact, the soil is a reservoir of different pathogens. On the contrary, environmental conditions of industrial plants are really different; sources of microbial contamination can be (Gurnari 2015):

(1) The quality of irrigation water, if contaminated with high levels of fecal bacteria
(2) Compost and sewage sludge of wastewater.

The quantitative importance of contamination depends on the structure of the industrial plant and irrigation techniques: in the first case, high contamination are associated with large-leaved plants or fissures, with the consequent promotion of the adhesion and entrapment of life forms. In these conditions, the elimination of contamination agents is difficult even during washing; in addition, sprinklers can also increase contamination.

From a general viewpoint, spoilage of fresh-cut vegetables and fruits depend on the result of separate and synergic processes:

(a) The alteration of the physiological nature (of vegetables) determined by the destruction of natural protection
(b) The release of enzymes and the acceleration of metabolic processes in response to stress (consumption of oxygen and production of ethylene)
(c) Enhancement of drying processes.

For these reasons at least, the production of peculiar ready-to-eat foods can involves cooking: this step can reduce significantly the risk of microbial contamination when speaking of medium risk products. The use of this process is important especially for those foods that do not require further cooking before consumption (Auricchio 2009).

On the contrary, canned vegetables are very durable because of the use of standard heat treatments (sterilisation, etc.) and the strong inhibition of enzymes, on condition that metal packaging remains sealed. However, some very slow spoilage phenomena may persist; as a result, declared shelf life is not unlimited very long. Anyway, canned vegetables can be stored at room temperature.

2.7.1 Contamination of Industrial Plants by C. botulinum and C. perfringens

Toxins by *C. botulinum* are certainly the most serious microbiological risk for canned vegetables with pH > 4.5 and A_w > 0.93 (products in brine, unfermented and/or acidified canned foods in oil, non-fermented pickled vegetables, ready soups, and non-acidified creams). With relation to these products, the only stabilising and sanitising procedure remains the heat treatment sterilisation (Auricchio 2009).

Naturally acid or acidified creams are surely one the most monitored products when speaking of botulinum toxin: pH values ≤4.5 appears to be the only reliable strategy. In addition, low pH values are expected to ensure the stability of food products until the end of commercial life if the subsequent heat treatment is not sterilisation. Generally, pH can increase in these products for different causes, including the penetration of external air inside the packaging with consequent development of moulds or *Bacillus*-type microorganisms: these life forms can use organic acids and thus cause notable pH variations (Auricchio 2009; Dodds 1993).

As a result, pH measurements should be considered in the so-called 'hazard analysis and critical control points' approach as one of main controls (regular, ideally continuous inspections). Other countermeasures are certainly challenge (stability) tests on foods with the aim of validating shelf life periods, pH variations and the integrity of packages.

With relation to 'low risk' aqueous foods—canned vegetable oil (peppers, green beans, asparagus, beans, peas, etc.) and non-acidic sauces with notable oil content (like Italian *pesto* sauce), the low-risk classification depends on the formulation. A useful example concern jams: the overall quantity of sugar in the finished product, the evaporation effect during the long cooking time and consequent A_w values are able to inhibit the germination of botulinum spores. The natural acidity of fruits, sometimes augmented with the addition of lemon juice or additives, has to be also considered.

These jams and similar product show normally pH and A_w 'safe' values can be heat-treated (pasteurisation, sterilisation) with the aim of eliminating the presence of residual air in the so-called 'headspace'. Another strategy is the addition of sugar (sucrose) amounts around 60–65 % of the total weight of raw fruits before cooking (or 100 % of the total weight of cooked fruits). Sucrose can inhibit the development of spore-forming *C. botulinum* with good results (Dodds 1993).

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Chapter 3
Chemistry and Technology of Ready-to-Eat Vegetable Foods

Giorgia Caruso and Salvatore Parisi

Abstract Ready-to-eat vegetable food refers to minimally processed fruits and vegetables, which have undergone treatments of mild intensity, without the alteration sensorial characteristics such as freshness. This type of product is ready for consumption: in recent years, it has emerged as a growing reality as it responds to consumers' needs by offering new services (convenience food). Given the direct consumption, the producer must associate a high quality of the product. This food, normally fresh and without added preservatives, is exposed to chemical and microbiological alterations; as a result, it is surely associated with a reduced shelf life. Even if these products receive some degree of minimal technological processing before market distribution, the used processing technology may be not sufficient, in most cases, with reference to microbiological stability and the complete removal of pathogens. Numerous techniques are currently been used in order to reduce microbiological and chemical spoilage, including chlorine washing, irradiation and modified atmosphere packaging. This chapter concerns recent updates about correlated technologies, including new recyclable trays, and correlated chemical and physical modifications of ready-to-eat packed products: the 'respiration' of vegetables, colorimetric modifications and other sensorial alterations.

Keywords Enzymatic browning · Ethylene · Lactic acid bacteria · Microbial spoilage · Modified atmosphere packaging · pH · Ready-to-eat vegetable food · Respiration · Shelf life · Water activity

Abbreviations

AEW	Acidic electrolysed water
CO_2	Carbon dioxide
ClO_2	Chlorine dioxide
DNA	Deoxyribonucleic acid
EHEC	Enterohaemorrhagic
HPP	High pressure processing

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H_2O_2	Hydrogen peroxide
LAB	Lactic acid bacteria
MAP	Modified atmosphere packaging
N_2	Nitrogen
O_2	Oxygen
ppm	Parts per million
POD	Peroxidase
PAL	Phenylalanine ammonia lyase
PPO	Polyphenoloxidase
RTE	Ready-to-eat
RNA	Ribonucleic acid
UV	Ultraviolet light
A_w	Water activity

3.1 Introduction

In recent years, the demand for minimally processed and ready-to-eat (RTE) fresh food products has increased dramatically in developed countries. The main reason is substantially correlated with the offer of a suitable choice for contemporary lifestyles: RTE products provide incorporated services (convenience food) to consumers. Moreover, the awareness of benefits of a diet rich in fruits and vegetables has simultaneously risen with clinical investigations and the epidemiological research. In particular, recent studies have associated the consumption of vegetable foods to a reduced risk of cardiovascular, chronic and neurological diseases, as well as some kinds of cancer (Ragaert et al. 2004; Su and Arab 2006). As a matter of fact, RTE foods contain high levels of micronutrients, fibres and antioxidants, including carotenoids and flavonoids.

Minimally processed vegetable foods are fruits and vegetables which have undergone treatments of mild intensity with the aim of increasing their functionality. On the other hand, these processing techniques do not alter sensorial features, such as freshness, and the expected nutritional quality (Allende et al. 2006). The initial quality of produce before processing has high relevance when speaking of the final RTE product. In fact, vegetables are in a raw state and ready for consumption. Consequently, these foods require very special attention because of their peculiar physiological, enzymatic and respiratory features. In addition, the problem of microbiological risks for consumers' health has to be considered.

3.2 Shelf Life and Processing

Generally, vegetable foods are known to be among the most perishable edible products. In fact, they display a high water activity (A_w) together with a neutral to slightly acidic pH value and higher carbohydrate contents with respect to proteins (Ramos et al. 2013).

In addition, minimally processed vegetable foods differ from traditional and intact products both for their physiology and their handling and storage requirements. As a matter of fact, processing procedures include very often cutting, slicing, shredding, dicing, peeling, washing and other procedures; these steps can affect the final storage life (Siddiqui et al. 2011). Washing water serves to reduce microbial contaminations because of the presence of sanitising agents such as chlorine (Sect. 3.5.1) and other chemicals (Gurnari 2015a, b).

First of all, some fruits and vegetables require the peeling step because of the necessity of removing inedible parts. Subsequently, chopping operations are required with the aim of facilitating prompt consumption. The disruption of tissues and cells integrity caused by processing can decrease shelf life.

In fact, wounded tissues undergo enhanced deterioration; as a result, derived products have a very short shelf life: 4–7 days, depending on the initial quality, the initial microbial load and the used processing technology (Watada and Qi 1999). However, various factors can influence the extent of disruption and senescence during cutting process: in particular, the size of vegetable pieces, the sharpness of cutting blades and mechanical properties of the product have to be carefully studied (Siddiqui et al. 2011).

3.3 Chemical and Biochemical Mechanisms of Spoilage

Minimally processed fruits and vegetables have different physiological rates if compared with intact products: their metabolism is accelerated similarly to the observed situation of stressed plant tissues. Even minimal processing can lead to an increase in respiration, ethylene production, water loss, microbiological replication, as well as enzymatic browning, formation of volatiles, loss of chlorophyll and lipid oxidation (Toivonen and DeEll 2002). These modifications influence directly the appearance of the final product; unfortunately, the consumer' approach is first focused on the estimation of appearance, colour and texture (Wismer 2009).

Ethylene has been reported to increase in minimally processed vegetable foods even if this phenomenon is dependent on intrinsic factors (i.e. climacteric vs. non-climacteric produce). Temperature has also an effect on the induction of ethylene production: for instance, it has been found in cantaloupes stored at very low temperatures. In this situation, the suppression wound-induced ethylene has been recognised (Madrid and Cantwell 1993). Generally, ethylene increases ripening, senescence and textural modifications by means of the stimulation of enzymatic activity; enzymes can be peroxidase (POD) and polyphenoloxidase (PPO) as well as phenolic compounds (Saltveit 1999). The initiation of wound ethylene response starts usually within 1 hour; the maximum rate is achieved between 6 to 12 h (Abeles et al. 1992).

In turn, ethylene stimulates the respiration rate: consequently, a notable enhancement of the tricarboxylic acid cycle, the electron transport chain and starch breakdown can be observed. In fact, post-harvest vegetables are living

tissues similar to normal vegetables; therefore, these tissues utilise reserve energy during ageing. For instance, respiration rates have been reported to increase in baby carrots by two–threefolds after peeling and slicing (Simõ et al. 2011). In agreement, tissues with high respiration rates and low energy reserve have a shorter shelf life (Eskin 1990). However, the augmented respiration is not only due to the enhancement of aerobic respiration: the role of α-oxidation of long-chain fatty acids with the production of carbon dioxide (CO_2) has been also proposed as synergic cause (Rolle and Chism 1987).

Moreover, minimally processed products are more susceptible to water loss because peeling and cutting operations expose interior tissues. As a consequence, the peridermal tissue—which acts as a protection against excessive transpiration—is removed and surface-to-volume ratios are forced to increase (Toivonen and DeEll 2002). The decrease in water leads to a loss of turgor, reducing the firmness of the products and hence the consumer's acceptance.

Another factor correlated with the consumer's evaluation of vegetable foods is enzymatic browning. This phenomenon is primarily caused by

(a) Cell disruption, which activates metabolic pathways, ultimately leading to the synthesis of enzymes and substrates, and by
(b) Loss of cellular compartmentation, which brings cell units together.

Phenylalanine ammonia lyase (PAL) is one of the key enzymes in phenylpropanoid metabolism and is wound induced. As a matter of fact, PAL produces various phenolic compounds, which are then oxidised in reactions involving POD and PPO (Barry-Ryan and O'Beirne 1998). POD, widespread in plant cells, is iron-porphyrin organic catalyst with a notable role in browning through two possible routes. The first of these mechanisms involves the formation of hydrogen peroxide (H_2O_2) during the oxidation of phenolic compounds, whereas the second reaction route utilises quinonic forms as substrates (Richard-Forget and Gauillard 1997).

PPO is a tetramer that contains four atoms of copper per molecule and catalyses the hydroxylation of monophenols to *o*-diphenols. PPO can also further catalyse the oxidation of *o*-diphenols with the consequent production of *o*-quinones. As a result, quinones can react with non-enzymatic reactions with other quinones, amino acids or proteins. The result is a melanin pigment, responsible for the well-known black to brown colour. Another enzyme involved in senescence is lipoxygenase, an iron-containing enzyme that catalyses the oxidation of polyunsaturated fatty acids in lipids containing a *cis*-*cis*-1,4-pentadiene structure (Lamikanra 2002). Therefore, lipoxygenase generates free radicals with the ability of provoking further membrane rupture; the structural lipidic membrane is degraded. In addition, lipoxygenase is responsible for production of certain volatiles: involved biochemical pathways are usually triggered by cell damage.

As a matter of fact, plants produce secondary metabolites in response to wounding: these secondary compounds may affect dramatically the perceived odour. Each vegetable species is believed to synthesise its own characteristic volatile pattern (Pichersky et al. 2006), even if phenylpropanoid and polyketide phenolics, aldehydes, alcohols and terpenoids are the main compounds.

Sulphur-containing compounds may also accumulate during time as a result of the loss of cellular compartmentation. Enzymes such as cysteine sulfoxide lyase can oxidise various substrates and convert these compounds into sulphur-containing molecules which may be responsible for off-odours. Peculiar examples can be methanethiol, dimethyl disulfide and allyl isothiocyanate in cut cabbage tissues (Chin and Lindsay 1993; Dan et al. 1997).

Furthermore, discoloration can also occur with a general loss of green colour, due to chlorophyll degradation. Two enzymes are considered responsible for chlorophyll breakdown: chlorophyllase and magnesium dechelatase. Two alternative alternative pathways have been reported at present, both resulting in the formation of a common product: pheophorbide *a*, an olive-brown compound, which is the precursor of the colourless product in a reaction mediated by pheophorbide *a* oxygenase (Toivonen and Brummell 2008).

Finally, the residential microbial flora also affects the quality of vegetable products through spoilage and/or with possible risks for consumers' health. Processing operations can provide further opportunities for microbial contaminations; in addition, they can also cause leaking of small molecular weight compounds and cellular fluids from damaged tissues. In fact, microbial growth is usually higher in fresh-cut products with respect to the whole product. As a result, spoilage may occur: peculiar signs are loss of texture, brown colours, production of off-odours and soft rot.

3.4 Microbiological Quality

Vegetable food possess a natural saprophyte microflora deriving from soil, water, insects and consisting of bacteria, yeasts, moulds that find favourable pH and A_w conditions. As a consequence, microbial flora tends to increase during all post-harvesting stages.

The number and species of microorganisms can vary depending on the type of produce and growing conditions; however, normal counts usually range from 10^3 to 10^9 colony forming units/g, with a general predominance of Gram-negative bacteria in vegetables, and of yeasts and moulds in fruits (Oliveira et al. 2010). Even biofilms may occur in vegetable leaves, mainly composed of environmental species which may act either preventing adhesion to plant surfaces by other bacteria. Alternatively, pathogens may be embedded in their matrix, hence decreasing the efficacy of sanitising treatments.

The dominant microflora in vegetables is composed of *Pseudomonas*, generally up to 50–90 % (Arvanitoyannis and Stratakos 2010). The most abundant species appear to be *Pseudomonas fluorescens*, *P. putida* and *P. cepacia*, whose role as spoilage microorganisms is notable. As a matter of fact, they can synthetise enzymes—also under refrigeration conditions—such as pectinases, cellulases, glycoside hydrolases and lipoxygenase, in addition to well-recognised proteolytic and lipolytic activities (Heard 2002). Pectic substances are very abundant in vegetable

cell walls. Chemically, these compounds are linear chains of α-(1–4)-linked D-galacturonic acid, with carboxyl groups either esterified (pectin) or non-esterified (pectic acid) with methanol.

Pectic substances are used by many microorganisms as energy source, resulting in enzymatic liquefaction of these compounds and consequently in tissue softening (Chen 2002). Involved enzymes are pectinases: these compounds exist in a wide variety of forms and are classified according to the reaction. In detail, should the mechanism of action involve β-elimination or hydrolysis, two categories would be considered: pectinesterases and depolymerising enzymes. Pectinesterases catalyse a de-esterification reaction of pectin resulting in pectate and methanol, whereas the second type of enzymes is able to cleave the pectinic chain, thereby releasing shorter portions (Sakai et al. 1993).

A peculiar enzyme, cellulose, catalyses the decomposition of cellulose, specifically by hydrolysis of the 1, 4-β-D-glucosidic bond. Basically, cellulases break down the cellulose molecule into monosaccharides such as glucose, or shorter chain of oligosaccharides. These enzymes are used by bacteria with the aim of obtaining short soluble sugars as food resources: they are divided into three general major types, based on the type of catalysed reaction:

- Endocellulases, which cleave internal bonds at random sites, thus creating new chain ends
- Exocellulases or cellobiohydrolases, which cleave two to four monomers from one end of the chain, producing cellobiose and/or glucose
- Cellobiases or β–glucosidases, which can hydrolyse exocellulase products into single monosaccharides (Singh and Hayashi 1995).

Enterobacteriaceae are well represented: generally, the most reported life forms are *Enterobacter*, *Pantoea* and *Serratia*. With the notable exception of *Erwinia carotovora*, a well-known plant pathogen, these bacteria are environmental microbes, encompassing a wide variety of ecological niches (Caponigro et al. 2010). Their role in the spoilage process has not been so well examined until now: consequently, more research would be needed at present.

Lactic acid bacteria (LAB) such as *Lactobacillus*, *Leuconostoc* and *Pediococcus* are also commonly found. LAB may affect the observed shelf life of fresh-cut products during storage (Stiles and Holzapfel 1997) through their fermentative metabolism (souring of products and gas production in anaerobic conditions). Finally, fermentative yeasts like *Kloeckera*, *Saccharomyces* and *Hanseniaspora* may cause spoilage in damaged fruits and salads, growing at low temperatures (Barnett et al. 2000).

Beside environmental microflora, human pathogens may also be conveyed by fresh produce. In fact, these products have been increasingly involved in foodborne outbreaks by bacterial, viral and parasitic pathogens. Among most common bacterial infectious agents, *Salmonella* spp. is a main concern with respect to the number of reported situations; on the other side, other species can be a major concern with concern to the severity of caused diseases.

For instance, the Gram-positive psychrophilic bacterium *Listeria monocytogenes* can determine listeriosis in pregnant women, elderly and immunosuppressed subjects. Consequences include gastroenteritis, meningitis, septicemia, abortion and death also.

Another dangerous bacterium with food safety and public health implications, *Escherichia coli*, has to be considered. In fact, aside from commensal strains, many different enteropathogenic strains are reported: enterotoxigenic, enteropathogenic and enterohaemorrhagic *E. coli* are the ones involved in foodborne outbreaks (Caruso and Parisi 2015). In particular, enterohaemorrhagic (EHEC) *E. coli* have been increasingly linked to the consumption of fresh vegetable foods. The main symptom of EHEC infections is hemorrhagic colitis; hemolytic uremic syndrome and other potentially lethal complications may also arise.

Viruses such as Norovirus and Hepatitis A virus and parasites, as *Cyclospora*, *Cryptosporidium* and *Toxoplasma*, can be a notable concern (Heard 2002) because of their involvement in foodborne outbreaks (contamination of foods from water and sewage). Moreover, RTE salads may be also a vehicle for the dissemination of antibiotic-resistant bacteria with clinical interest and genes that can be acquired by other opportunistic pathogens (Campos et al. 2013).

The multiplicity of bacteria and pathogens found in these products suggests that washing and disinfection procedures may be not sufficient to ensure a good microbiological quality, highlighting the necessity of implementing more efficient post-harvesting decontamination methods.

3.5 Methodologies to Improve Quality

Physiological and microbial-induced modifications in appearance and quality of minimally processed vegetable foods can be slowed down and minimised through a multi-phase approach, combining pre-harvest, pre- and post-processing treatments and management procedures. Obviously, the primary objective is to prevent microbial contamination and extend shelf life of food products; because of the intrinsic difficulty, various techniques, above all chemical and physical ones, are available at present.

3.5.1 Chemical Methods

Among sanitising agents, chloride-based rinses are the most widely used in the produce industry. Chlorine compounds are usually utilised in a concentration range between 50 and 200 parts per million (ppm) for less than 5 min (Rico et al. 2007). Theoretically, chlorine is more efficient at acidic pH levels, but usually it is used at pH between 6.0 and 7.5; the reason is the necessity of minimising machinery corrosion (Beuchat 2000). Although observed the advantages (reduction of

microbial counts), chlorine can lead to the formation of chlorine vapours or chlorinated by-products, that may have potential harmful health effects (Parish et al. 2003). Therefore, chlorine dioxide (ClO_2) has been introduced as an alternative to chlorine, as it does not form noxious chloramine compounds. Moreover, this chemical has a higher oxidation capacity: about 2.5 times greater than normal chlorine. ClO_2 has also shown (Ramos et al. 2013):

(a) A higher level of penetration with respect to the liquid agent
(b) A high efficacy against pathogens, acting on cellular aminoacids and ribonucleic acid (RNA).

H_2O_2 has a strong oxidising power leading to the generation of cytotoxic reactive oxygen species, hydroxyl radicals above all. H_2O_2 has hence a notable bactericidal activity: it is used up to 80 ppm in washing water (Alexandre et al. 2012).

Organic acids (e.g. ascorbic, lactic, citric and tartaric acid) are also frequently used as antimicrobial agents, as they have a role in environmental and intracellular pH reduction, anion accumulation and damage of membrane permeability and transport (Beuchat 2000). Ascorbic acid is frequently used as antioxidant in fruits and vegetables because of its antioxidant activity which prevents browning and inhibits polyphenol oxidase reactions.

Ozone is a potential method for extending shelf life of fresh commodities, due to its high reactivity and penetrability. It can be used both in water and in gas form where higher concentrations—around 20,000 ppm—can be reached even if gaseous ozone is considered to be more effective (Klockow and Keener 2009). Ozone has shown various advantages, including decomposition in non-toxic products, reduction in enzyme activity, decomposition of some pesticides and reduction in the oxygen demand. On the other hand, this agent has also some side effects, as it rapidly disappears; moreover, ozone is reported to be associated with lower crispiness and colour degradation (Guzel-Seydim et al. 2004; Rico et al. 2006).

Calcium-based additives (e.g. calcium lactate) are also used for products with a high senescence index. In fact, calcium helps in maintaining firmness by interacting with cell walls and middle lamella pectins to form calcium pectate. Furthermore, calcium-based solutions have been shown to reduce chlorophyll and protein loss, as well as inhibit tissue senescence (Smout et al. 2005).

Lastly, electrolysed water is utilised for its bactericidal effect. Generally, it is generated by the electrolysis of water containing dissolved sodium chloride. This process leads essentially to the production of gaseous hydrogen and hydroxide ions at the cathode, hence forming an alkaline solution consisting of sodium hydroxide. At the anode, chloride and hydroxide ions are oxidised to gaseous chlorine, hypochlorous acid, hydrochloric acid and hypochlorite ions. Should the formation of these compounds be allowed, acidic electrolysed water (AEW) would be obtained with a pH value between 2.1 and 4.5. Despite its strong bactericidal activity, AEW has shown adverse effects on produce quality because of pH values and high oxidation-reduction potentials (Rico et al. 2007; Wang et al. 2004). On the contrary, pH can be raised to neutral values (i.e. neutral electrolysed water): this solution does not affect colours and the general appearance of products (Izumi 1999).

3.5.2 Physical Methods

In recent years, different physical technologies are emerging as processing applications in the food industry.

Modified atmosphere packaging (MAP) is still commonly used in the food industry as preservation technique and consists in the alteration of the normal air composition, usually by lowering oxygen (O_2) percentage and replacing it with CO_2 or nitrogen (N_2). The gas modification is reached either actively, by flushing a gas mixture before sealing, or passively. However, gas composition will inevitably be modified in both cases during the commercial life of MAP products, due to respiration and film permeability to gases (Sivertsvik et al. 2002).

CO_2 and N_2 concentrations vary depending on the type of product and on processing methods. MAP extends storage life of both whole and processed commodities of about 50–400 % by reducing ethylene production, respiration rates and other metabolic activities (Ramos et al. 2013). Moreover, MAP delays enzymatic browning and growth of aerobic bacteria, even if excessively reduced O_2 concentrations may lead to the overgrowth of anaerobes with fermentative metabolism and consequent off-odours. The increase of microbial counts ascribed to potential pathogens has to be also considered.

Irradiation is an innovative and very effective method of decontamination: the application of this technology is gradually increasing at a global level. Irradiation is a physical treatment that consists in exposing foods to an energy source such as gamma rays and X-rays. It is effective against microorganisms because it ionises atoms, removing electrons from their orbits with the generation of free radicals. This process destabilises essential cellular macromolecules such as proteins, deoxyribonucleic acid (DNA) and RNA (Kundu et al. 2014), hence delaying also senescence. On the other side, irradiation treatments are essentially safe from the toxicological point of view when speaking of consumers' health. Moreover, adequate doses do not compromise organoleptic and nutritional quality of irradiated foods (Ahn et al. 2004).

The irradiation of foodstuffs can be performed by means of gamma rays, emitted by sources of caesium-137, cobalt-60 or, alternatively, by electron beams. The formation of radicals and their spread depend on A_w values of the irradiated food: the treatment is reported to be less effective in anhydrous and frozen products. On the other hand, irradiation is not widely accepted by consumers; in addition, it may produce some textural alteration.

Ultraviolet light (UV) is used as antimicrobial agent because of its direct damage to DNA: in fact, UV rays cause the production of pyrimidine dimers, a disruption in the genetic sequence. UV light is subdivided into three different types according to wavelengths:

- UV-A rays (range: from 315 to 400 nm)
- UV-B light (range from 280 to 315 nm)
- UV-C, also named 'far UV' rays (range from 100 to 280 nm).

UV light, especially UV-C rays, is commonly used because of the inexpensiveness of equipment and the induction of the synthesis of health-promoting molecules such as anthocyanins and stillbenoids (Cantos et al. 2001). However, it has to be noted that the application of UV light has various limitations. For instance, this technology can increase respiration rate of the produce and induce lignification-like processes (Ramos et al. 2013).

High pressure processing (HPP) is another method for the inactivation of microorganisms and enzymes: the technology is based essentially on the application of elevated pressures (100–1000 MPa) on foods. Although a high pressure is achieved, flavours and the general nutritional quality appear to remain unchanged, even if this method shows some adverse effects on vegetables. In fact, high pressures can damage the integrity of porous products due to the intrinsic compression and expansion cycle of the process (Palou et al. 2000). In addition, another limitation that can become a notable concern for traders is the expensiveness of the technological system.

Ultrasound technology has also been studied because of its application in food science: it is environmentally sustainable and considered one of the new 'green' technologies (Chemat et al. 2011). In detail, high-intensity ultrasound (low frequencies from 20 to 100 kHz) is used in order to inactivate bacteria and enzymes. Basically, ultrasounds in a liquid medium can determine the production of high energy amounts through the compression and expansion of particles of the treated medium (Butz and Tauscher 2002). Its bactericidal activity depends on the cavitation phenomenon: in other words, the formation, growth and subsequent collapse of bubbles are observed during the treatment. The result is the creation of a localised mechanical energy that causes disruption of cellular walls and membranes. In addition, free radicals and highly reactive molecules such as protons, hydroxide ions and H_2O_2 are generated by means of a peculiar reaction, water sonolysis, thus targeting DNA and lipid membranes (Bermúdez-Aguirre et al. 2011; Rastogi 2011).

Enzyme inactivation could be due to the breakage of hydrogen bonds and van der Waals interactions through polypeptide chains, with the consequent disruption of secondary and tertiary enzyme structures and hence of biological functions (São Jose et al. 2014). Ultrasound technology has proved to be more effective in disaggregating microbial biofilms and accessing surfaces that are difficult to reach with respect to other disinfection methods. On the other hand, a large-scale usage is still being discussed: at present, best results for disinfection are provided by utilising ultrasound in combination with other technologies and agents such as peracetic acid (Gao et al. 2014; São José and Vanetti 2012).

Lastly, another innovative option is the realisation of innovative packaging materials (active or 'smart' packaging) by means of the use of active agents of various types: ions, enzymes, fungicides, organic acids, ethanol, etc. The final aim is to improve food safety and quality.

As a matter of fact, active packaging strategies include principally

- Addition of volatile antimicrobial agents as ethanol generators, oxygen and moisture absorbers into packages
- Incorporation of bioactive agents into packaging polymers. Examples: silver ions into polyethylene, polypropylene and butadiene styrene
- Use of antimicrobial polymers such as chitosan and polylysine (cationic polymers) that interact directly with cell membranes (Appendini and Hotchkiss 2002).

3.5.3 Biological and 'Generally Recognized as Safe' Methods

Because of the increased consumer concern about the toxicological safety of chemicals and synthetic additives, the request for 'Generally Recognized As Safe' (GRAS) substances or natural food preservative is rising.

Research has been carried out on biocontrol agents, specific species of bacteria which are known for their antagonistic potential on pathogens: LAB. Biocontrol bacteria are strong competitors for physical space and nutrients; they may generate diverse antimicrobial metabolites such as bacteriocins (Sagong et al. 2011).

Bacteriocins are proteinaceous toxic compounds with either a broad or a narrow spectrum of inhibition. Numerous bacteriocins have been tested for their application as food preservatives: some of these compounds are already commercially available such as nisin. They can be added in concentrated preparations or produced in situ by LAB starter cultures. In addition, bacteriocins have been used in polymers (bioactive food packaging materials).

Another promising strategy concerns the use of essential oils because of their own antioxidant properties. These organic substances, derived from spices and other plants, are attracting interest for their potential in enhancing storage life as antimicrobial agents. For instance, oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) oils contain two strong antibacterial compounds, carvacrol and thymol, respectively. Many essential oils have been recognised as GRAS at present; however, their practical application is still limited because of the altering effect on food organoleptic properties (Oussalah et al. 2006).

Edible coating films are now being recognised for their potential applications. First of all, these coatings can remarkably delay sensorial modifications (appearance and aroma) during storage. Moreover, these materials can act as carriers of active compounds as antimicrobials, nutrients and anti-browning agents. A wide variety of substances can be used in edible films including lipids, resins, polysaccharides and proteins, either individually or combined. Some of the most used compounds are vegetable starch, fruit wax, gum, pectin, carboxymethyl cellulose, chitosan, alginates and carrageenan.

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