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# Development of the packaging and overview of the literature

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# Use of olive leaf extract (OLE) to inhibit the growth of *Campylobacter* spp. in an active packaging for fresh chicken preservation. Development of the packaging and overview of the literature

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in

Food Engineering

by

Ismael Chahed el Ouazzani

Supervisor: Dr. Fátima Poças

Co-supervisor: Prof. Paula Teixeira

March 2021

# Abstract

This work is composed of a bibliographic survey and an experimental part. The bibliographic part consists of a study on the epidemiological situation of *Campylobacter* spp. in Europe, advances in research on natural solutions for the conservation of chicken, studies aimed at reducing contamination by *Campylobacter* spp., the use of olive leaf extract (OLE) for the conservation of food, and other new alternatives for food preservation. The experimental part consists of a laboratory study on the effectiveness of OLE against *Campylobacter* spp. and its application in an active packaging for the conservation of chicken.

The epidemiological situation related to *Campylobacter* infections in the EU is not homogeneous between countries and its related to the consumption of foods contaminated with these bacteria. Some seasonality has also been observed for infections.

In recent years many studies have been carried out to develop new natural solutions for the conservation of chicken, due to the need to increase the shelf life of food. In these studies, new natural plants have been found as well as very effective minerals to lengthen the shelf life of food.

OLE is a by-product of the olive industry that is widely used in phytotherapy, but in recent years it has been shown that it has a potential to increase the shelf life of food mainly due to its antioxidant and antibacterial effects. In microbiological studies it has been demonstrated its effectiveness against *Campylobacte*r spp., however, there are no studies in the literature that have the objective of reducing or eliminating *Campylobacter* spp. in chicken with the use of OLE. For this reason, OLE was chosen to carry out an experimental study.

In the experimental work, results from *in vitro* and *in situ* tests were in agreement. OLE has been effective as a growth inhibitor at the optimal growth conditions of *Campylobacter* spp., that is, at 41.5 ºC and in microaerophilia. Nevertheless, once samples were removed from contact with OLE growth was observed,this means that the extract has not killed all the bacteria. The experimental design was not able to prove the efficacy of OLE in reducing the viable cell count at low temperatures.



# Key words

Olive leaf extract; *Campylobacter*; Chicken; Poultry; Natural extracts; Food safety; Campylobacteriosis; active packaging; oleuropein; hydroxytyrosol

# Resumo

Este trabalho é composto por uma parte de pesquisa bibliográfica e uma parte experimental. A parte bibliográfica consiste num estudo sobre a situação epidemiológica de *Campylobacter* spp. na UE, avanços na investigação de soluções naturais para a conservação de frangos, estudos destinados a reduzir a contaminação por *Campylobacter* spp., a utilização do extrato de folha de Oliveira (OLE) para a conservação de alimentos, e outras novas alternativas para a conservação de alimentos. A parte experimental consiste num estudo laboratorial sobre a eficácia da OLE contra *Campylobacter* spp. e a sua aplicação numa embalagem ativa para a conservação de frango.

A situação epidemiológica relacionada com as infeções por *Campylobacter* spp. na UE não é homogénea entre os países da união e está relacionada com o consumo de alimentos contaminados com estas bactérias. Também tem sido observada alguma sazonalidade nas infeções.

Nos últimos anos têm sido realizados muitos estudos para desenvolver novas soluções naturais para a conservação de frango, devido à necessidade de aumentar o tempo de conservação dos alimentos, nestes estudos foram abordadas novas soluções naturais, bem como minerais eficazes para prolongar o tempo de conservação dos alimentos.

O OLE é um subproduto da indústria do azeite amplamente utilizado na fitoterapia, mas nos últimos anos tem sido demonstrado que tem potencial para aumentar o prazo de validade dos alimentos, principalmente devido aos seus efeitos antioxidante e antibacteriano. Em estudos microbiológicos foi demonstrada a sua eficácia contra *Campylobacter* spp., contudo, não existem estudos na literatura que tenham como objetivo reduzir ou eliminar *Campylobacter* spp. em frangos com o uso de OLE. Por esta razão, OLE foi escolhido para realizar um estudo experimental.

No trabalho experimental, os resultados dos testes *in vitro* e *in situ* foram concordantes. O OLE revelou-se eficaz como inibidor de crescimento nas condições ótimas de crescimento de *Campylobacter* spp., ou seja, a 41.5 °C e em microaerofilia. Contudo, uma vez que após remoção das amostras do contacto com o OLE, observou-se crescimento, indicando que haviam ainda células estavam viáveis, o que significa que o extracto não matou todas as bactérias. Não foi possível, com o design experimental seguido, provar a eficácia do OLE na redução do número de células viáveis a baixas temperaturas.



# Palavras-chave

Extrato de folha de oliveira; *Campylobacter*; Frango; Extratos naturais; Segurança alimentar; Campilobacteriose; Embalagem ativa; Oleuropeína; Hidroxitirosol

# Acknowledgement

I would like to thank professors **Fátima Poças** and **Paula Teixeira** for having agreed to be my supervisors in the master's thesis in food engineering after having also been my professors during the master's.

I would like to especially thank Professor **Cristina Mena** for her dedication and help to carry out the research work in the CINATE microbiology laboratory.

During this work in the facilities of the ESB and its associated centers CINATE and CBQF, many people have helped me to carry out the laboratory tasks, especially: Nanci Ferreira, Isabel Santos, Luisa Carneiro, and Joel Pereira.

In general, I am grateful to all the wonderful people of the ESB, who are an example of professionalism and responsibility.

I also thank the company NUTEXA for having provided us the OLE used in the experimental work.

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# Abbreviations

**AIMPLAS:** Instituto Tecnológico del Plástico
**AITC:** Allyl isothiocyanate
**ASLT:** Accelerated shelf life testing.
**CBQF:** Centro de Biotecnologia e Química Fina
**CFU:** Colony Forming Units
**NC:** Cellulose Nitrate
**CINATE:** Center for Innovation and Technological Support
**EDTA:** Ethylenediaminetetraacetic acid
**EFSA:** European Food Safety Authority
**EO:** Essential Oil
**ESB**: Escola superior de Biotecnologia
**EU:** European Union
**FDA:** Food and Drug Administration
**FDE:** Freeze-dried extract
**GPE:** Grape pomace extract
**GRAS:** Generally Recognised As Safe
**HPP:** High Pressure Processing
**MAP:** Modified Atmosphere Packaging
**MC:** methylcellulose
**MBC:** Minimum bactericidal concentration
**MIC:** Minimum inhibitory concentration
**LAB:** Lactic Acid Bacteria
**LDL:** Low-density lipoprotein
**OLE:** Olive Leaf Extract
**PET:** Polyethylene terephthalate
**PP:** Polypropylene
**PLA:** Polylactic acid
**PVA:** Poly(vinyl alcohol)
**PVOH:** Poly(vinyl alcohol)
**SSI:** Supercritical solvent impregnation
**TBARS:** thiobarbituric acid reactive substances
**TS:** tensile strength
**TCPN:** thyme essential oil/β-cyclodextrin ε-polylysine nanoparticles
**UK:** United Kingdom
**VBNC:** Viable But Non Culturable
**WVP:** Water Vapour Permeability
**YOPI:** Young, Old, Pregnant and ill.

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# Introduction

Preserving food is a necessity due to the seasonality and scarcity of some raw material, and poorly developed production techniques. Furthermore, the intensive production, the population increase, the remoteness of the production and consumption areas, the long supply chains, the busy pace of life, the new nutritional needs and food tastes, microbiological risks and the need for long shelf life make food conservation an increasingly important area which main objectives are to guarantee the food safety and the sensorial and nutritional properties for long periods of time. Food safety is the first concern because safe food support national economies, trade, tourism, contribute to food and nutrition security, and underpin sustainable development (Mehlhorn, 2015).

Food can become contaminated at any point of production and distribution, and the primary responsibility lies with food producers. Yet a large proportion of foodborne disease incidents are caused by foods improperly prepared or mishandled at home, in food establishments or markets (Byrd-Bredbenner et al., 2013). Not all food handlers and consumers understand the roles they must play, such as adopting basic hygienic practices when buying, selling, and preparing food to protect everybody's health.

Preservatives are used in foods to kill or to prevent growth of undesirable bacteria present in the raw materials or environmental contaminants. In healthy adults, the immune systems can deal with small numbers of bacteria and viruses but high levels can cause illness. Vulnerable people, such as pregnant women, elderly or people with poor immune systems (YOPI), can get very ill or even die from foodborne microbial agents.

In addition to all this, competitiveness and high consumer demands due to urbanization and changes in consumer habits, including travel, have increased the number of people buying and eating food prepared in public places. Globalization has triggered growing consumer demand for a wider variety of foods, resulting in an increasingly complex and longer global food chain which makes that the food industry has to meet the needs of the final consumer, which are mainly to have a product with high sensory quality, safe for health and with a long shelf life, aspects that heat treatments, artificial preservatives, salt or sugar cannot satisfy at all.

These challenges put great responsibility on food producers and handlers to ensure food safety. Local incidents can quickly evolve into international emergencies due to the speed and range of product distribution. Serious foodborne disease outbreaks have occurred on every continent in the past decade, often amplified by globalized trade.

In recent years, societies have been suspicious about the use of artificial preservatives (Bearth et al., 2014), plastic, high amounts of salt and sugar to preserve food because many studies have established a direct relationship between their use and health problems such as cancer, obesity, hypertension and many other diseases (WCRF et AICR, 2018). The use of natural products such as plants to treat diseases, pests, and preserve food is booming because society perceives them as safe because they are not related to secondary health problems, their use dates back from immemorial times, which gives

the perception of more security. Additionally, many of the new synthetic components have proven to be harmful such as phthalates and parabens. As the world's population grows, the intensification and industrialization of agriculture and animal production to meet the increasing demand for food create both opportunities and challenges for food safety. Climate change is also predicted to impact food safety, where temperature changes modify food safety risks associated with food production, storage, and distribution (WHO, 2018).

Historically humans used natural products such vinegar, oil, and salt or the combination of all (ex. Escabeche) to preserve food during long periods, which were very effective food preservatives and actually, these conservation techniques are still used more for the taste that they give to food rather for preserve food.


**Figure 1.** *Escabeche* and salting are examples of old techniques to preserve food with natural products such vinegar and salt, nowadays they are more used for taste purposes *(Source: Web of Mercadona and https://www.clubevinhosportugueses.pt)*.

Because of the great pressure on the food industry to reduce the amount or eliminate preservatives on food, the use of plant extracts can be a good solution. Some big companies are responding to the demands of the consumers to reduce or eliminate synthetic substances that are currently used to preserve food like EDTA, sodium benzoate, sulphites, etc. and replace them with natural substances like celery extract (Figure 2).

One of the big companies that took the initiative is the French supermarket group CARREFOUR which made an initiative called ACT FOR FOOD, which consists of commitments to the consumer, and one of this commitment is to ban the use of controversial substances in its own-brand products, as it is written in one of the last press releases: "Carrefour has anticipated regulatory and legislative changes: in 2018, it banned 100 controversial additives from the ingredients of these 8,000 Carrefour food products. Starting in 2019, Carrefour ended the use of more than thirty substances in its own-brand products" (Leclerc, 2018).


**Figure 2.** Celery extract and vinegar are natural alternatives to the use of nitrites to preserve charcuterie *(Source: www.grocerygateway.com )*.

In response to these needs, we have found it convenient to investigate new methods for preserving food, such as the use of olive leave extract (OLE). The OLE is a by-product of the olive oil industry, which is a very important industry in Portugal and in other Mediterranean countries such as Italy, Greece, and Spain. These countries account for the majority of olive oil world production. The use of OLE is useful for the implementation of a circular economy, focused on reducing food industry waste by transforming them into a product with high value for the industry and ultimately for society. Reducing waste is also reducing pollution, which is undoubtedly one of the biggest problems we face today as humanity. Portugal's relationship with the olive tree is millenary and is fully integrated into the history and culinary of the Portuguese people (Mota, 2006). The olive tree is spread throughout almost the entire geography of the continental country and has an important economic impact on people's lives, so valuing by-products could increase economic benefits for farmers.

Another of the economic advantages of this project because households are primarily responsible for the waste of perishable food (Evans, 2012; Gonzalez Vaque, 2015), increasing the useful life can have a positive economic and environmental impact. Also increasing the useful life increases the capacities of producers to sell their products in more remote geographical areas, because transport time becomes a less relevant factor.

In addition to all this, OLE has proven to be a healthy product because numerous scientific research have proven its effectiveness in fighting diseases and infections, this is why it is widely used in herbal medicine (Sabry, 2014).

The use of natural extracts is not a novelty, because it is currently used in charcuterie products (Figure 2) to increase its shelf life (Salehi et al., 2019), its use is not fully expanded because many natural products alter the sensory properties of foods in which they are applied, which is an important limitation, taking into account consumer sensory requirements.

Besides, the low homogeneity of the extracts of which their effectiveness depends on many factors (Chibueze, 2018), make the industry continue to opt for artificial preservatives because its effectiveness is widely demonstrated, they don't suffer risk shortages, they are homogeneous and easily transportable, and above all they are very cheap.

Portugal is in line with the most advanced countries in terms of consumer preference for organic products (Pacheco, 2018). It is a growing market that increasingly has adherents that reject products that have been produced and preserved using artificial products and OLE could be a potential product to respond to the demands of this type of consumer.

Another important advantage of OLE is that it may look good for society, which is why many brands use it as a gimmick to attract the consumer (Figure 3), adding it in few quantities to the products and hoping that the consumer associates it with health, making it a powerful marketing tool.


**Figure 3.** Brands use olive products as a gimmick to attract the consumer.

In this research project, the effect of OLE against *Campylobacter* spp. in chicken was investigated. Chicken meat which is one of the most consumed meats by the Portuguese population. OLE, besides possibly inhibiting the growth of *Campylobacter* spp. or killing it, has antioxidant properties (Lins et al., 2018). These combined effects can promote an increase in shelf life and in safety. The antioxidant effect is attributed to substances, namely to hydroxytyrosol, which is a polyphenol with antioxidant capacity ahead of resveratrol, and that, according to some studies (Ferran Font, 2015), may have a positive impact on combating aging.

OLE is an important by-product or waste from olive industry. *Campylobacter* spp. is one of the main pathogenic bacteria that causes infection and these bacteria were found to be sensitive to OLE (Sudjana et al., 2009). Nevertheless, there are few studies for the application in poultry conservation (Djenane et al., 2012). Taking into account the preference in Europe for chilled chicken instead of frozen chicken

(preferred in countries like Brazil), the development of an active packaging with OLE for chilled chicken would be a convenient option.

Medina et al. (2013) demonstrated that olive compounds are more effective food preservatives than vinegar, which confirm that it's a product of great interest in food preservation. In addition to that, OLE has great health benefits due to the presence of phenolic compounds (Talhaoui et al., 2015).

It is clear that there are other ways to inhibit and/or kill pathogens in food, but most use means, like heat treatments, that have a negative effect in some important quality aspects in food, mainly sensory aspects like freshness and texture. Therefore, food research has to find new ways to bring to the consumer a delicious food, with a healthy amount of salt and fat, without synthetic preservatives and an extended shelf life. Natural preservatives can complement heat treatment and refrigeration if their addition can contribute to increase the shelf life or reduce the intensity of the heat treatment.

The present work includes an extensive bibliographic search on the use of OLE in active packaging for chicken and a laboratorial work for the evaluation of OLE against *Campylobacter* spp.

# Objectives

This work is a bibliographic and laboratory research work targeting mitigation approaches through packaging for controlling *Campylobacter* spp. in chicken.

For the bibliographic search, information has been obtained from various sources, mainly scientific articles from prestigious journals and scientific books. Laboratory research has been carried out in the facilities of the CINATE microbiology laboratory. This laboratory is certified by IPAC - Instituto Português de Acreditação, I.P. under EN ISO 17025. After having obtained the results of a first bibliographical research on natural substances that can be effective against *Campylobacter* spp. and natural substances used for chicken preservation, it was decided to focus the second phase of the bibliographical research on the potential use of OLE as a useful substance for food preservation. The second part of the thesis consists of an experimental work to test OLE on chicken and its effectiveness in eliminating or reducing *Campylobacter* spp.

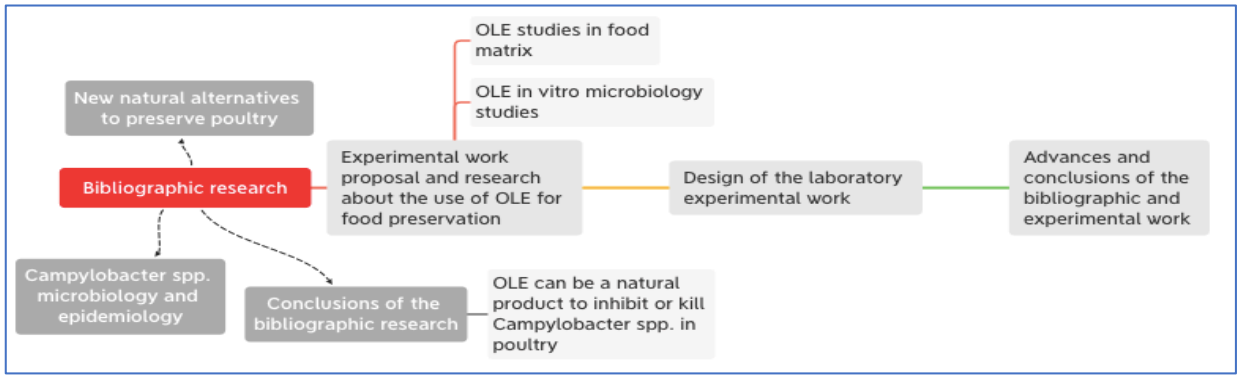


**Figure 4.** Diagram of the methodology.

The general objectives of this work were to review the advances in antimicrobial packaging for poultry and specifically for *Campylobacter* spp., focusing on the use of OLE as active packaging.

The specific objectives of this work were:

- Assess the epidemiological situation of *Campylobacter* spp. in the EU and in Portugal.
- Know the European legislation on the requirements that producers have to meet in terms of food safety in relation to *Campylobacter* spp.
- Identify the new trends related to active food packaging using natural products.
- Identify the new natural alternatives to reduce foodborne illness caused by poultry consumption and *Campylobacter* spp. by reviewing the most important studies of the last years.
- To develop scientific knowledge about olive leaf extract, its types, properties and composition.
- Collect information on the applications of OLE in active packaging, in order to know in which foods the best results are obtained.
- Conclude on the potential of OLE as a solution for *Campylobacter* spp. contamination in an active packaging for chicken preservation.

# 1. *Campylobacter* spp.: Microbiology, associated foods, and EU regulation.

## 1.1 *Campylobacter jejuni* microbiology

*C. jejuni* is a bacterial enteric pathogen that is associated with enterocolitis and diarrhea in humans and many other animal species like sheep, cats, calves and dogs (Hoefer et al., 2012). *Campylobacter* spp. are the most common cause of bacterial gastroenteritis in most developed countries and usually it's transmitted through food. Among the genus *Campylobacter, C. jejuni* is the main specie associated with human disease, although there are other species that affect humans including, among other, *C. coli* and *C. lari* (Kopecko e Hu, 2003).

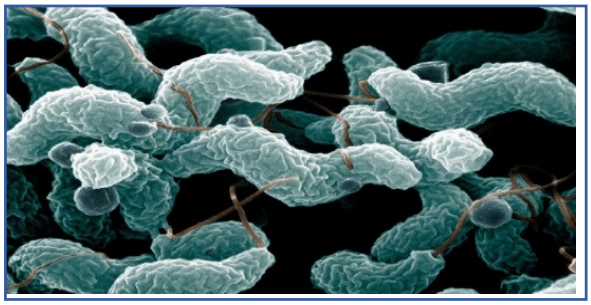

**Figure 5.** Microscopical view of *Campylobacter* spp. *(Source*: Web of the univ. of Leicester*).*

*Campylobacter* spp. are microaerophilic, non-sporeforming, oxidase-positive and Gram-negative spiral shaped cells (Figure 5) with corkscrew-like motility. During the log-phase cells have a characteristic slender, curved and spiral shapes. *Campylobacter* spp. cannot ferment or oxidize sugars and are oxygen-sensitive microaerophiles, growing best in an atmosphere containing 5–10% carbon dioxide and 3–5% oxygen (Kopecko e Hu, 2003). Table 1 presents the conditions for *Campylobacter* spp. growth.

**Table 1.** Limits for growth of *Campylobacter* spp. (ICMSF, 1996; Forsythe, 2000).

| Factor | Range | Optimum |
|---|---|---|
| **Temperature (ºC)** | 32-45 | 42-43 |
| **pH** | 4.9-9 | 6.5-7.5 |
| **NaCl (%)** | 0-1.5 | 0.5 |
| **Water activity ($a_w$)** | >0.987 | 0.997 |
| **Atmosphere** | | 5% $O_2$ & 10% $CO_2$ |

*Campylobacter* species are also sensitive to other adverse conditions such as drying and reduced pH (Martin et al., 2008)**.** *Campylobacter* spp. when is in unfavorable conditions has the ability to enter the viable but non-culturable state (VNBC), just observed under laboratory conditions (Silva et al., 2011).

## 1.2 Reservoirs of *Campylobacter jejuni*

The main reservoir of *C. jejuni* is the intestine of poultry. They do not cause disease to poultry as they live in a mutual relationship. When the bacteria get into the humans stomachs it can cause gastroenteritis. *Campylobacter* species have also as reservoir the alimentary tract of wild and domesticated animals like birds. This is can be due to the high body temperature of birds, which is close to the optimum growth temperature of *Campylobacter* species (Martin et al., 2008).

## 1.3 Optimum growth temperature for *Campylobacter* spp. and prevalence in chicken

All *Campylobacter* species grow at 37 ºC, *C. jejuni* and *C. coli* have an optimum growth temperature of 42-45 ºC (Table 1), but they cannot survive pasteurization or cooking temperatures. They don't grow below 30 ºC and survive poorly at room temperature (around 20 ºC). Their viability declines during chill and frozen storage, and they may not persist under these conditions for prolonged period of time (Martin et al., 2008). Freezing-thawing reduces the population of *Campylobacter* species; frozen storage at -15 ºC reduces the population of *Campylobacter* spp., but doesn't eliminate when present at high concentrations (Martin et al., 2008).

In a study conducted in the UK on the prevalence of *Campylobacter* spp. in chicken carcasses it was found that in all supermarkets more than 64% (Figure 6) of chickens have tested positive for *Campylobacter* spp.

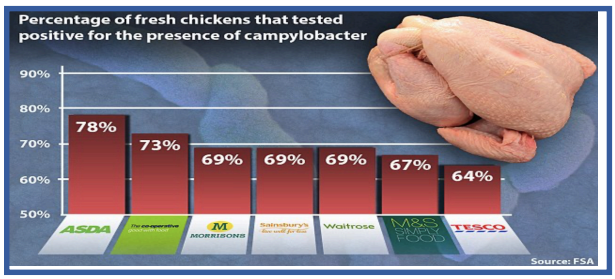


**Figure 6.** Percentage of fresh chickens that tested positive for the presence of *Campylobacter* spp. in the main UK supermarket chains *(source: Poulter, 2014*).

## 1.4 Other foods associated with *Campylobacter* spp.

Although *Campylobacter* spp. is often associated to poultry, eggs are not a source of *Campylobacter* spp. because prolonged survival on the dry egg surface is unlikely and egg albumin has been shown to be strongly bactericidal. In the case of unpasteurized milk, it can contain *Campylobacter* spp. as a result of faecal contamination or Campylobacter mastitis (Martin et al., 2008). Other foods associated with *Campylobacter* species include contaminated drinking water and bivalve mollusks (Source: Kopecko and Hu, 2003).

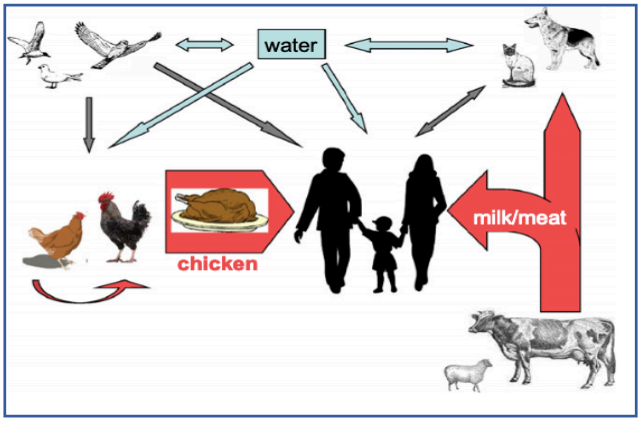


**Figure 7.** Most important routes for human infection by *C. jejuni* (Dasti et al., 2010).

A study (Figure 8) done in France confirmed that although chicken is the major source of *C. jejuni h*uman infection, ruminants, pets and environmental sources were also cause of infections (Figure 7), which suggest further research about the possible ways of contamination from other sources (Thepault et al., 2018).

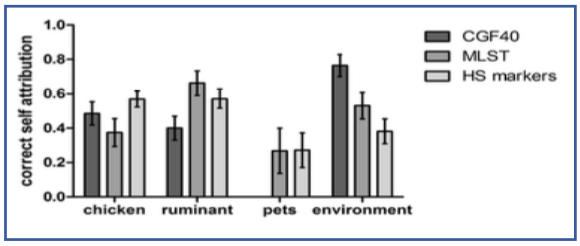


**Figure 8.** Correct self-attribution rates of *C. jejuni* isolates from four putative contamination sources based on genomic data *(Source: Thepault et al., 2018).*

## 1.5 Campylobacteriosis symptoms and treatment

Campylobacteriosis symptoms include severe diarrhea, abdominal pain, fever and sometimes vomiting. Recovery can sometimes take up to 10 days. It can also lead to irritable bowel syndrome, reactive arthritis and Guillain-Barré syndrome (Rees et al., 1995). At its worst, it can kill; the death rate from *Campylobacter* spp. is low, 24 deaths per 10,000 confirmed cases (CDC, 2006). Campylobacteriosis is the third leading cause of food-borne deaths.

**Risk groups:** Anyone who is exposed to the bacteria can get ill from it, but young children under the age of five, young adults (15-24 years) and those over 60 are at a greater risk.

**Campylobacter treatment:** Most people recover without treatment within two to five days. A rehydration solution to prevent dehydration can help. Severe infections are treated with antibiotics (García Peña et al., 2006).

## 1.6 New EU regulation about *Campylobacter* spp.

The EU has implemented new specific limits to control human campilobacterioris. This has been done after a cost-benefit analysis making the application of the criterion cheaper than the economic consequences of the human campilobacterioris (ICF GHK, 2012).

Previous European Union regulations such as those of 1989 (EC, 1989), 1992 (EC, 1992) and 2003 (EC, 2003) did not provide specific limits. The new regulations specify limits, which allow to guarantee the safest consumption of food.

Table 2 describes the microbiological limits that must be respected by the new regulation. This table specifies the limits allowed from 2020 and 2025, as well as the methodology for analysing the samples and the improvement actions in case of unsatisfactory results.

**Table 2.** Microbiological limits for *Campylobacter* spp. permitted in EU. (Source: European Commission, 2017)

| Food Category | Carcases of broilers |
|---|---|
| Microorganism | *Campylobacter* spp. |
| Sampling plan [1] | |
| n | 50 |
| c | c=20; From 2020: c=15; From 2025 : c=10 |
| Limits [2] | |
| m/M | 1000 CFU $g^{-1}$ (In this case m=M) |
| Analytical reference method | EN ISO10272-2 |
| Stage where the criterion applies | Carcase after chilling |
| Action in case of unsatisfactory results | Improvements in slaughter hygiene, review of process controls, of animals origin and of the biosecurity in the farms of origin. |

(1) n = number of units comprising the sample; c = number of sample units giving values between m and M.
(2) m= Threshold value for the number of bacteria.; M= Limit value for the number of bacteria.

According to recent changes in EU regulation that is becoming more severe and will be even more severe in the next years, as we can see (Table 2) from 2020 it is allowed to exceed the limit of 1000 CFU $g^{-1}$ in 15 carcasses out of 50, 5 carcasses less than before and in 2025 it'll be allowed to exceed the limit in just 10 carcasses, which at the end the regulation reduce in 50% the precedent (2019) limit.

# 2. Epidemiology of *Campylobacter* spp. in the EU and Portugal

The EFSA has published the results of a survey (EFSA, 2010) on *Campylobacter* spp. and *Salmonella* spp. in chicken at slaughterhouses in the EU in 2008. In most EU Member States, a high prevalence of *Campylobacter* spp. was found in chickens, whereas *Salmonella* spp. was less frequently detected. These zoonotic agents are the cause of the two most commonly reported foodborne diseases in humans in the EU: campylobacteriosis and salmonellosis. It was the first study (EFSA, 2010) to directly investigate the presence of *Campylobacter* spp. and *Salmonella* spp. in chickens at slaughter at the EU level.

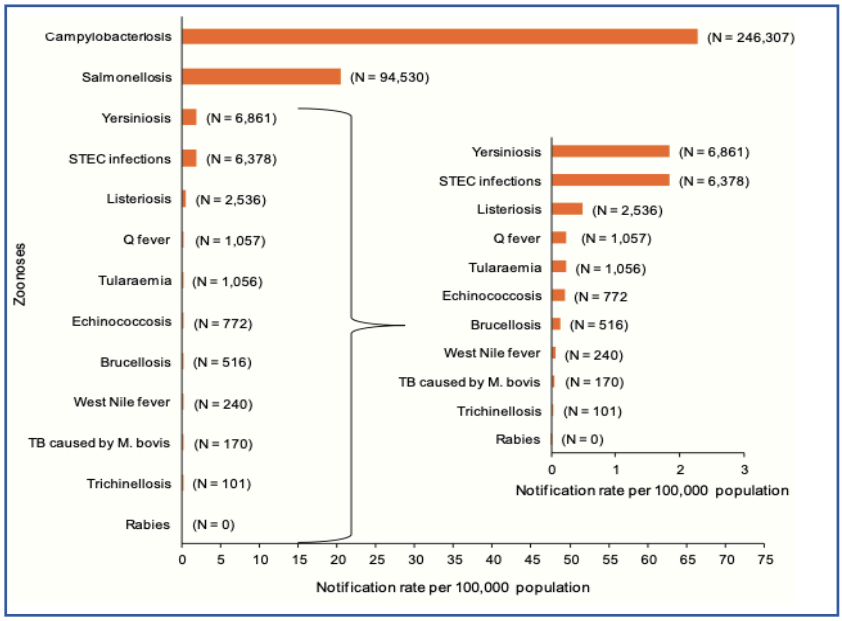


**Figure 9.** Reported numbers and notification rates of confirmed human zoonoses in the EU in 2017 (*Source: EFSA, 2018*).

Campylobacteriosis is the first food zoonotic disease with the highest number of cases in the EU since the surveillance of campylobacteriosis cases begun in 2000 and it still remain nowadays the first according to the report published by the EFSA in 2019. The reports of EFSA of 2018 and 2019 on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks present similar results from previous years (EFSA, 2018, 2019). EFSA declares: “Campylobacteriosis was the commonest reported zoonosis and its EU trend for confirmed human cases increasing since 2008 stabilized during 2013–2017.”; “In 2017, campylobacteriosis was the most commonly reported zoonosis as it has been since 2005, representing alone almost 70% of all the reported cases.” Even being the first zoonoses campylobacteriosis has a low hospitalization rate. Campylobacteriosis numbers evolution remains similar without significant changes even with last year efforts (2020) of improvement and modernization of prevention measures, safety requirements and legislation. Portugal has one of the lowest campylobacteriosis rates, 5.8 cases of campylobacteriosis per 100.000 inhabitants (EFSA, 2018).

**Table 3.** Distribution of strong-evidence outbreaks caused by *Campylobacter* spp., by food vehicle in the EU between 2010-2017 *(Source: EFSA, 2019).*

| Food vehicle | Number of strong-evidence FBO | % of total |
|---|---|---|
| **Broiler meat (Gallus gallus) and their products** | 106 | 44.4 |
| **Milk** | 61 | 25.5 |
| **Other, mixed or unspecified poultry meat and their products** | 19 | 7.9 |
| **Dairy products (other than cheeses)** | 5 | 2.1 |
| **Other or mixed red meat and their products** | 5 | 2.1 |
| **Meat and meat products** | 1 | 1.7 |
| **Total** | 33 | 100 |

Most of the outbreaks (Table 3) which occurred in the EU in the period 2010-2017 are related with broiler meat. The second cause of outbreaks is milk being responsible for 25.5% of the outbreaks, which if added dairy products, both sum 27.6% of the outbreaks, which is almost 1/3 of the outbreaks being related with milk and dairy products, more of them happened in Germany and the UK. There is a peak (Figure 10) of reported confirmed cases during summer, probably is due to higher temperatures during this season, which suggest that there is a seasonality in the outbreaks

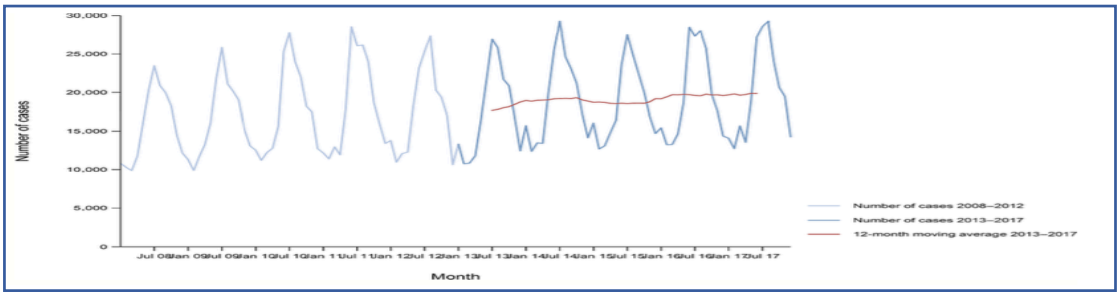


**Figure 10.** Trend in reported confirmed human cases per month of campylobacteriosis in the EU/EEA between 2008 and 2017 (*Source: EFSA, 2018).*

In the survey carried out in 2008 (EFSA, 2010) on average, the bacterium was found in the intestines of 71% of chickens, indicating that they were already infected when alive, and on 76% of sampled carcasses, which suggests some further contamination during slaughtering.

# 3. Active packaging

## 3.1 Concept and types of active packaging

The primary purpose of food packaging is to protect the food from oxygen, water vapor, ultraviolet light, and both chemical and microbiological contamination" (Prasad et Kochhar, 2014). This definition suits very well with what we know as traditional packaging. However, in recent years there has been a great technological development in food packaging (Figure 11) to try to meet the demands of consumers in terms of more natural methods of preservation to ensure the quality and safety of food.

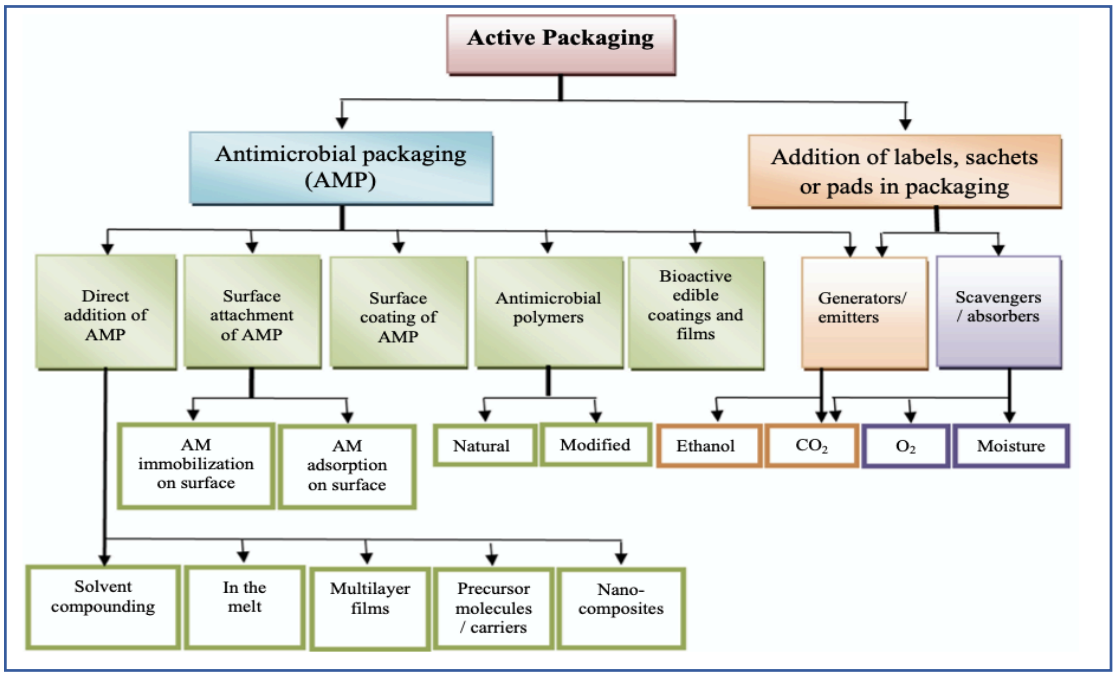


**Figure 11.** Classification of different AP systems *(Source : Ahmed et al.,2017).*

Advances were done in intelligent and active packaging. Intelligent packaging systems provide the user with information on the conditions of the food and should not release their constituents into the food (European Commission, 2009) and active packaging is defined as "packaging in which subsidiary constituents have been deliberately included in or on either the packaging material or the package headspace to enhance the performance of the package system" (Robertson, 2013). The main difference between active and intelligent packaging is the purpose: The purpose of the "active packaging" is the extension of the shelf life of the food and the maintenance or even improvement of its quality. While the purpose of "intelligent packaging" is to give indication on, and to monitor, the freshness of the food (Dainelli et al., 2008).

## 3.2 Overview of new natural active packaging for poultry preservation

Figure 12 shows the main approaches followed on antimicrobial packaging for poultry. A brief description of studies reported in the literature for each of the approaches are following presented.

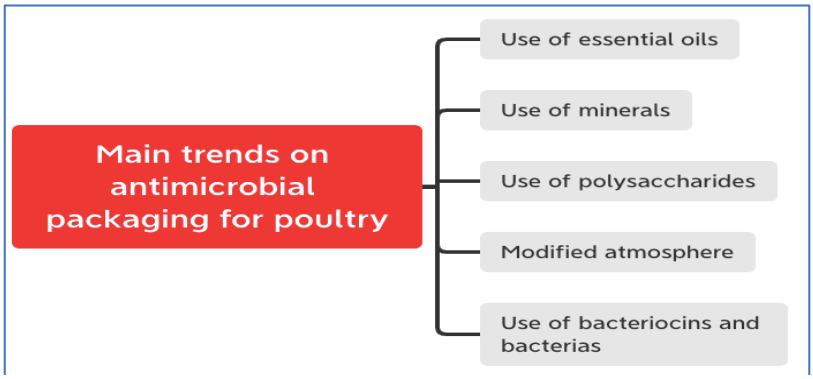


**Figure 12.** Main trends on antimicrobial packaging for poultry.

### 3.2.1. Use of EO's

- Souza et al. (2019) used rosemary EO and chitosan-montmorillonite in bioplastic chitosan film and found that fresh poultry wrapped in these films stored under refrigeration for 15 days showed a reduction of 1.2–2.1 log CFU $g^{-1}$ on the total microorganisms count, compared to control (meat without film). The active films also succeeded in retarding poultry lipid peroxidation and discoloration.
- Pires et al. (2018) found that the activity of Ginger and Rosemary EO's is similar, which suggests that the activity of the EO's depends more on the main components rather in the specific ones of each type of EO.
- Ginger EO proved to be effective to decrease the total aerobic psychrophilic bacteria of refrigerated chicken breast fillets during 12 days (Noori et al., 2018). This study was made with nanoemulsion based edible coatings with 6% of ginger EO nanoemulsion.
- In the study of Hamedi et al. (2017) they tested a novel bioactive edible coating based on sodium alginate (Alg), galbanum oleo-resin gum (GG) and the biocomposite of alginate and galbanum (CAG) containing different concentration of ziziphora EO (ZEO). The chicken fillets coated with Alg, GG, and CAG incorporated with ZEO had a good microbiological and chemical quality compared to uncoated samples. Gram-positive bacteria were more susceptible to GG and ZEO during 12 days of cold storage. The coating with only sodium alginate did not show an interesting antimicrobial activity.
- Citral and quercetin were tested by Giteru et al. (2017) in an active packaging made by kafirin (vegetable protein). Compared to the plain kafirin films and quercetin films, citral containing films showed significant antimicrobial activity against the total viable count (TVC) on chicken fillets.

### 3.2.2. Use of minerals

- Mathew et al. (2019) found that a complex packaging made by PVA-montmorillonite clay nanocomposite blend film with *in situ* generated ginger extract mediated silver nanoparticles is highly efficient to reduce *Staphylococcus aureus* and *S. Typhimurium* in chicken sausages.
- Montmorillonite proved also to be effective in the experiment of Souza et al. (2019) to increase the antimicrobial effect of EO's applied to fresh poultry meat.
- Souza et al. (2018) found that samples (fresh poultry) wrapped with this packaging composition (chitosan/montmorillonite incorporated with ginger EO) showed a reduction in microorganisms count of 1.2–2.6 log CFU $g^{-1}$. It also maintained color and pH values, and TBARS index increased at a lower rate compared to control, thus extending fresh poultry meat shelf life.
- Azlin-Hasim et al. (2016) tested silver nanoparticules (Ag/PVC nanocomposite films) in chicken breast fillets to reduce microbial spoilage, and they found it highly effective as antimicrobial packaging.
- ZnO increases the antimicrobial activity of chitosan (Mujeeb Rahman et al., 2018) in a concentration-dependent manner. This study confirms the results obtained by Akbar et al. (2014) where they tested ZnO *in vitro* and in poultry. Ag and ZnO proved to be both effective as antimicrobial against some foodborne bacteria, when used alone or in combination (Panea et al, 2014).

### 3.2.3. Use of polysacharides

- Ruiz Cruz et al. (2019) found that the combination of chitosan and tomato plant extract increases chicken shelf life and reduces microbial growth during 16 days at 4 ºC.
- Xu et al. (2018) developed a packaging with tapioca starch and grape pomace, they found to be highly effective in reducing *S. aureus* on ready-to-eat chicken meat. In this same study, they found that starch/cellulose nanocrystal/Viognier films are effective against *L. monocytogenes* when inoculated on the meat samples during a 10 days storage period at 4 °C.
- Tumeric extract increases the antimicrobial activity of chitosan as it was proven in the study of Kalaycıoglu et al. (2017). They tested the antimicrobial activity *in* vitro of this extract against *S. aureus* and *Salmonella* spp. and found high antimicrobial activity against theses bacteria.
- Carrageenan/chitosan coatings containing either AITC or Oriental mustard extract plus EDTA have the potential to reduce *Salmonella* spp. on raw chicken (Olaimat et al., 2015) and to control *Campylobacter* spp. (Olaimat et al., 2014).
- Pullulan is a polysacharide used for edible packaging that demonstrated to be effective as a carrier of EO´s and ZnO for antimicrobial packaging (Morsy et al., 2014) against poultry. In this same study they made additional experiments that demonstrated that these antimicrobial films "inhibited pathogens associated with fresh or ready-to-eat meat and poultry products."
- Higueras et al. (2013) developed a novel antimicrobial film based on chitosan with LAE (ethyl-Nα-dodecanoyl-l-arginate) for food packaging applications. Films were active against bacteria, yeasts

and fungi. Chitosans films had antimicrobial activity in the range 0.47-2.96 log reductions. Latou et al. (2014) and Petrou et al. (2012) found a significative effect on chicken combining only chitosan and MAP.

### 3.2.4. Use of MAP

Modified atmosphere packaging (MAP), in particular the mixture 70% $CO_2$ / 30%$N_2$ demonstrated to be effective in increasing chicken shelf life and to be more effective than oxygen scavengers, reducing microbiological count and oxidation in chicken thigh meats, with a 9 day shelf life extension (Dermihan and Cardohan, 2016).

### 3.2.5. Use of bacteriocins

Nisin is very effective against *S.* Typhiumurium when added in polysacharides films as it was demonstrated in the study of Natrajan et Sheldon (2000) in a contaminated broiler drumstick skin sample. With nisin they obtained reductions ranging between 1.8 to 4.6 log cycles after 72 to 96 hours of exposure at 4 °C.

### 3.2.6. Conclusions on new natural active packaging

Natural substances give good effects when associated with synthetic traditional packaging materials such as plastics. Combining EO's can lead to synergistic effects rather than the individual use of a single EO. EO's like rosemary EO are clearly bactericidal substances and their effect has been demonstrated when tested in food packaging. Also, the combination of different types of substances can boost the effect such as the combination of chitosan or pullulan with EO's, or minerals with EO's. The most cited substance with a demonstrated effect is chitosan, which in addition to being a by-product of the fishing industry and a substance widely used in health products, is a substance with great potential to be used in food packaging for the conservation of poultry. Montmorillonite clay and zinc are the most often reported mineral systems for active packaging.

## 3.3 Overview of active packaging targeting *Campylobacter* spp.

Studies on novel active packaging specifically targeting *Campylobacter* spp. are following described:

- Nouri Ala et al. (2019) tested different coating formulations based on carboxymethyl celulose (CMC) with EO's (Ziziphora clinopodioides EO and Mentha spicata EO) in fresh and sauced chicken breast fillets. The result was that the combination of oils with CMC inhibited completely the growth of *C. jejuni* after 10 days (with 0.25% of EO) and 12 days (with 0.5% of EO) in refrigerated conditions.

- Alkan et al. (2011) tested *in vitro* a material based on zein or zein-wax composite with gallic acid which have shown antimicrobial activity against *Campylobacter* spp. in a dependent concentration manner, confirming the results from a previous study by Arcan et al. (2011).
- Gañan et al. (2009) tested *in vitro* the antimicrobial activity of chitosan against *Campylobacter* spp. and found that the zone of inhibition is concentration related. In this study they tested chitosan against six bacteria and found that *Campylobacter* spp. is the more susceptible bacteria to chitosan. Chitosan caused a loss in the membrane integrity of *Campylobacter* spp. and have a MIC ranging from 0.005 to 0.05.
- Lin et al. (2018) tested thyme essential oil/β-cyclodextrin and ε-polylysine nanoparticles (TCPNs) against *C. jejuni* in chicken. They found that TCPN's are the most effective to reduce *Campylobacter* spp. population as showed in *in vitro* tests.
- Metals can be an interesting component of active antimicrobial packaging. Duffy et al. (2018) tested *in vitro* the effectiveness of Ag, ZnO and CuO nanoparticles against *Campylobacter* spp*.,* with MIC results ranging in this order: Ag > CuO > ZnO .
- Nair et al. (2015) combined natural substances with MAP and found interesting results to reduce *Campylobacter* spp. population with Carvacrol, which is the major component (~80%) present in oregano EO, extracted from the leaves of the Origanum vulgare plant. The antimicrobial activity of carvacrol against *Campylobacter* spp*.* is concentration related. However, the difference between the use of carvacrol with and without in MAP was not tested. Fernandez-Pan et al. (2014) found that oregano EO is also effective against other foodborne pathogens that contaminate chicken.
- In another study with carvacrol they tested (Mild et al., 2011) apple-based edible films containing carvacrol and cinnamaldehyde against *C. jejun*i strains on chicken. Cinnamaldehyde showed greater antimicrobial activity than carvacrol and the most interesting fact is that reductions at 23 ºC were greater than those at 4 ºC.
- In a study (Olaimat et al., 2014) for the development of an antimicrobial packaging, the incorporation of AITC or deodorized oriental mustard extract in κ-carrageenan/chitosan solutions as an edible coating significantly reduced viable numbers of *C. jejuni* on vacuum-packed chicken breasts. κ-Carrageenan/chitosan coatings containing 50 or 100 μL $g^{-1}$ AITC reduced numbers of *C. jejuni* on chicken breasts to an undetectable level (<1.0 log10 CFU $g^{-1}$) after 5 days storage at 4 °C.
- Another method to fight *Campylobacter* spp. is with protective cultures. Melero et al. (2013) inoculated C. jejuni with *Bifidobacterium longum*. A reduction of 1.16 log CFU $g^{-1}$ in *C. jejuni* together with a delay in the growth of LAB was obtained in chicken legs inoculated with *B. longum* and packaged under MAP after 9 days. This study also tested the difference with the addition of MAP, which shows to double the shelf life.
- MAP can be efficient in controlling *C. jejuni* depending on the composition of the atmosphere as shown in the study of Boysen et al. (2007). They reported that *C. jejuni* inoculated onto chicken fillets survived significantly longer in the presence of 100% $N_2$ and 70/30% $N_2/CO_2$ than in the presence of 70/30% $O_2/CO_2$ at 4 ºC.

# 4. Olive leaf extract (OLE). Concept, composition and quality parameters

OLE is the extract obtained from the olive (*Olea Europea L.*) leaves. The olive tree is one of the oldest cultivated species. It is a tree native to the eastern Mediterranean region, but it was exported by many civilizations around the world. Currently, it is grown mainly in the Mediterranean (Figure 13), although there are cultivars in America, Asia, and Oceania (Cimato et al., 2015).

**Figure 13**. World geographical distribution of olive tree growing areas *(Source: Cimato et al., 2015).*

There are many products (Figure 14) and by-products of the olive tree from which molecules of interest for health and other applications can be obtained. The richest product in bioactive molecules is OLE, but also other olive tree by-products are rich in molecules of interest. The by-products of interest are olive oil mill wastewater, olive preservation solutions, olive leaf juice, olive leaf powder, wash-waters and the olive pomace.

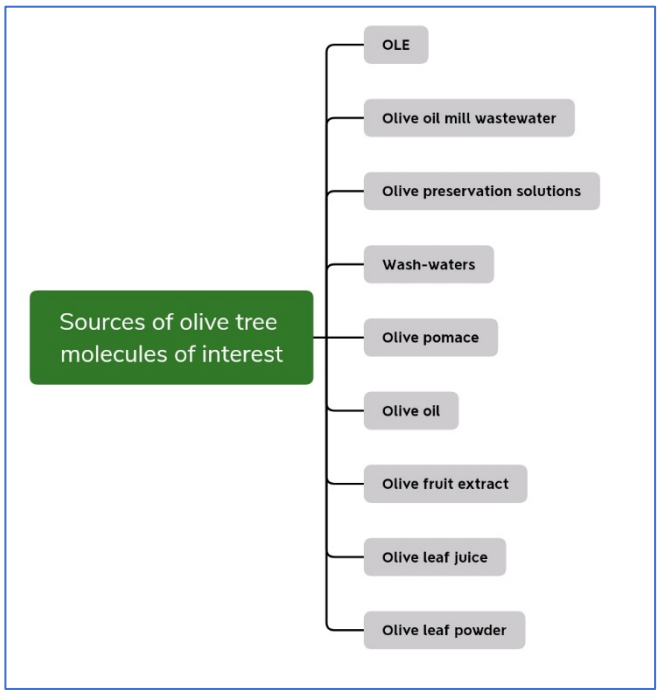


**Figure 14.** Sources of olive tree molecules of interest.

## 4.1 Concept of extraction and extraction methods to obtain OLE

Extraction methods used pharmaceutically involves the separation of medicinally active portions of plant tissues from the inactive/inert components by using selective solvents. During extraction, solvents diffuse into the solid plant material and extract different compounds with similar polarity (Pandey and Tripathi, 2014).

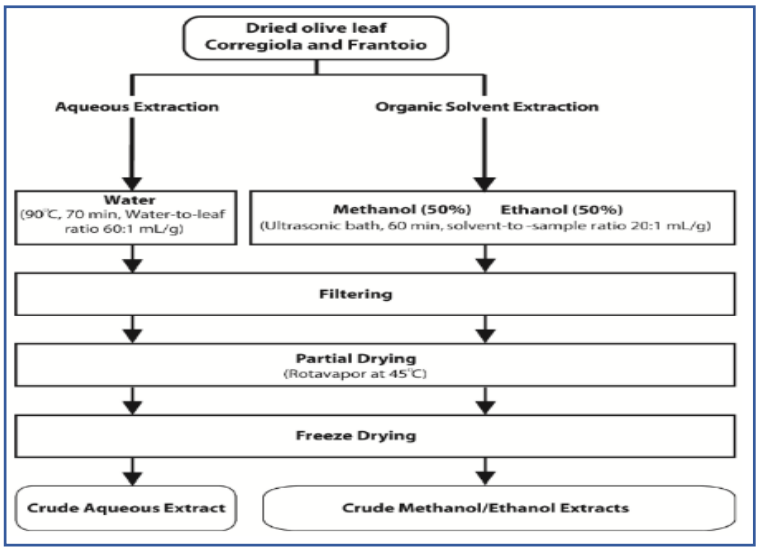


**Figure 15.** Methods for the preparation of olive leaf extracts *(Source: Goldsmith et al., 2015).*

The objective is to obtain an extract with the maximum concentration of active components to have an effective action to preserve food against oxidation and pathogenic bacteria. In the Figure 15 the main extraction methods to obtain OLE are depicted. Yateem et al. (2014) compared different methods and solvents to extract the maximum concentration of oleuropein (the most important active component of OLE). They tested the green extraction method, extraction with organic solvents, and Soxhlet extraction. They found that the best extract is the one obtained with 80% ethanol, but we will see that there is not a clear consensus about which is the best extract.

## 4.2 Olive leaf extract composition

OLE is mainly composed of phenols and flavonoids. Table 4 presents an example of the typical composition of OLE. Phenols are secondary metabolites of plants and some studies reveal that they are the most important group. The structure of phenols consists of a hydroxyl group (—OH) bonded directly to an aromatic hydrocarbon group. Phenols are widely distributed in plant tissues, and they are responsible for the color, flavor, and astringency of fruits. They are mainly found in the fruit and leaves. The quantitative composition of these substances depends on the climate, the degree of maturation, the crop, and the drying of the plant among others. The most abundant compound in OLE is oleuropein, followed by hydroxytyrosol, the flavone-7-glucosides of luteolin and apigenin and verbascoside.

**Table 4.** Abundance of the main phenolic compounds present in olive leaf extract (Benavente-Garcia et al., 2000).

| Phenolics | % Absolute |
|---|---|
| Hydroxytyrosol | 1.46 |
| Tyrosol | 0.71 |
| Catechin | 0.04 |
| Caffeic acid | 0.34 |
| Vanillic acid | 0.63 |
| Vanillin | 0.05 |
| Rutin | 0.05 |
| Luteolin-7-glucoside | 1.38 |
| Verbascoside | 1.11 |
| Apigenin-7-glucoside | 1.37 |
| Diosmetin-7-glucoside | 0.54 |
| Oleuropein | 24.54 |
| Luteolin | 0.21 |
| Diosmetin | 0.05 |

Oleuropein does not have a strong antioxidant effect, while hydroxytyrosol, despite not being the molecule with the highest antioxidant potency, is the one that gives the extract the highest antioxidant power. Rutin is the molecule with the highest antioxidant effect, but its concentration is low in the extract. It is not well known exactly which components are responsible for the antibacterial action. Oleuropein was discovered in 1908 by Bourquelot and Vintilesco (Sedef et Karakaya, 2009), and according to some studies, it is the most important compound for antimicrobial action, apart from being also the compound that gives health benefits of the extract. Oleuropein is also responsible for the intense bitter taste of the olives in which it is also present. Hydroxytyrosol is the principal degradation product of oleuropein as shown in Figure 16 (Sedef et Karakaya, 2009). The content of hydroxytirosol in free and combined form is higher in the leaves than in the fruits of the olive tree. Therefore, leaves provide extracts with higher yields.

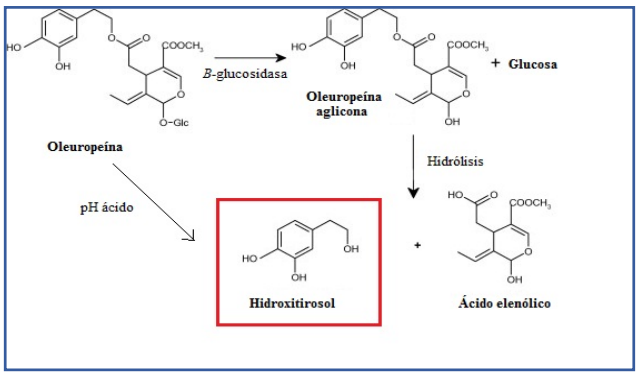


**Figure 16.** Hydroxytirosol natural Chemical and enzymatic reaction (Source: fitoquimicos.com).

## 4.3 Quality parameters to evaluate OLE effectivity and factors that affect it quality

The best extract is considered the one with the maximum concentration of compounds responsible for the antimicrobial and antioxidant activity of the OLE, in particular the oleuropein (Guinda et al., 2015). Although it may seem obvious that the best extract is the one which has a higher concentration of oleuropein, some studies focus more on evaluating the *in vitro* antimicrobial activity rather than measuring the oleuropein concentration. In addition to the type of solvents, other factors affect the effectiveness of the OLE, like the cultivars, crop origin, harvesting time and climate that affect the leaf composition, which could influence the antibacterial activity of extracts (Korukluoglu et al., 2008).

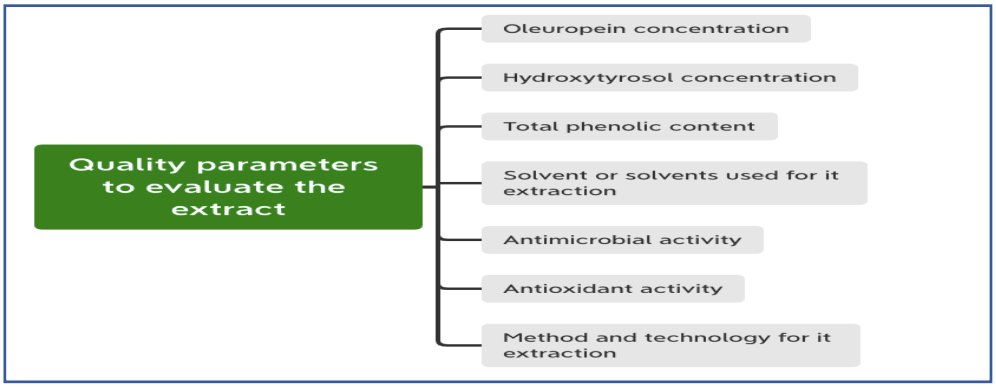


**Figure 17.** Quality parameters to evaluate the extract.

For example, in the study of Erdohan et Turhan (2012) the water extract showed a much lower concentration in oleuropein and a higher antimicrobial activity against *S. aureus* than the ethanol and methanol extract. However, results on the effect against one type of bacteria are not enough to support solid conclusions about the antimicrobial activity, but it proves that the antimicrobial activity doesn't depend only on the oleuropein concentration

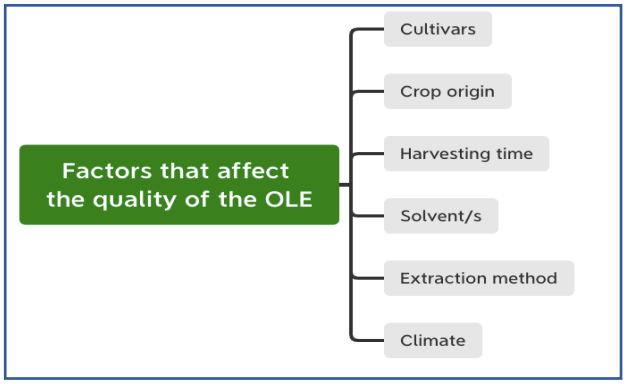


**Figure 18.** Factors that affect the quality of the OLE.

Besides the oleuropein content, the total phenolic content is also important for the quality of the extract, because other phenols also have an important antimicrobial activity (Galanakis, 2018; Rimawi et Salim, 2016; Tuck et Haybal. 2002; Brahmi et al., 2013; Medina et al., 2013; Hayes et al., 2011; Moudache et al., 2016; Lee et Lee., 2010; Benavente-Garcia et al., 1999).

## 4.4 OLE health properties

Olive tree products and by-products are a great source of substances with proven health benefits (Vogel et al., 2015; Nicoli et al., 2019; Sudjana et al., 2009). Molecules responsible for these effects are found in all products of olive tree such olives (Pereira et al., 2006), olive oil (Medina et al., 2013), OLE, (Sudjana et al., 2009) and also other by-products like olive oil mill wastewater (Vagelas et al., 2014), olive preservation solutions, wash-waters and olive pomace (Medina et al., 2013). The main therapeutic properties of phenols consist of antiseptic, anti-infectious, bactericidal, stimulant to the immune system, and stimulant to the nervous system (Dyakov T. et al., 2007). Tyrosol, hydroxytyrosol, and oleuropein are the polyphenols derived from the olive tree that have the greatest interest. Pereira et al. (2007) concluded that the addition of OLE can work as nutraceutical to lower the risk of microbial infections, particularly in the intestinal and respiratory tract, mainly due to the protective action of its phenolic compounds that also explains how the olive tree is resistant to adverse conditions compared to other trees (Kubo et al., 1995). The EFSA considers that olive oil polyphenols offer protection against oxidative damage and LDL (cholesterol indicator) and recommends a minimum consumption of 5 mg $day^{-1}$ (EFSA, 2011).

Numerous studies attribute health benefits to hydroxytyrosol, such as being protective of the cardiovascular system (Gonzalez Santiago, 2006), protective against neurodegenerative processes (Shaffer et al., 2010), muscle and joint protector, anti-inflammatory, protective against cancer (Warleta, et al., 2011) and AIDS (Lee-Huang, S. et al., 2007). It also boosts the immune system (Fistonic, et al., 2012) and prevents osteoporosis among others.

# 5. Potential use of OLE for food preservation

## 5.1 Antimicrobial activity of OLE

The antimicrobial activity of OLE is widely documented in various studies against various bacteria and fungi. OLE has a strong inhibitory effect against *C. jejuni, H. pylo*ri, and *S. aureus* (Sudjana et al., 2009; Markin et al., 2003; Friedman et al., 2002). OLE can be used to fight foodborne pathogens that represent a problem of food safety particularly in meat products, which need substances to inhibit the growth of all potential and dangerous food pathogens (Erbay et al., 2010). Liu et al. (2017) demonstrated that OLE inhibits completely the growth of *Listeria monocytogenes, Escherichia coli*, and *S.* enteriditis.

OLE is effective as antibacterial and compared to other extracts, like tea and oregano extract that can also be effective against *C. jejuni*, OLE is reported to be effective at lower concentrations (Sudjana et al., 2009), which is important from the point of view of sensorial properties, toxicological concerns and also economical.

Knowing that some specific compounds are responsible for antimicrobial activity and antioxidant activity, one could think that isolating that active principle could avoid the use of extracts and the inherent problems of purity and variability in properties. However, the study of Lee and Lee (2010) demonstrated that these compounds have a synergic antimicrobial action and are more effective when they are in association than when they are isolated, this was demonstrated by testing the main phenolic compounds individually and the whole extract, this latter showed better performance against *S. enteritidis* (Lee e Lee, 2010). Pereira et al. (2007) had similar a conclusion, that the extracts may be more beneficial than isolated constituents since a bioactive component can change its properties in the presence of other compounds. Serra et al. (2008) studied the olive extract (similar to OLE) and grape extract (both important by-products of the wine and olive portuguese industry), and they also concluded that the natural extracts showed more antimicrobial activity than the selected antioxidants alone against the tested microorganisms (*E. coli, S. Poona, Bacillus cereus, Saccharomyces cerevisiae and Candida albicans*).

Different studies evaluating *in vitro* antimicrobial activity of OLE have been reported:

- Karygianni et al. (2014) reported OLE to have great antimicrobial activity against *S. aureus*, *Enterococcus faecalis* and some *Streptococcus* species.
- Erdohan et al. (2013) tested the antimicrobial activity of OLE against S. aureus at different concentrations when incorporated in PLA. Like in the study of Ayana and Turham (2009) the antimicrobial activity was not proportional to the concentration in the film, but the study confirmed the antimicrobial effect against *S. aureus.*
- To find the best OLE concentration Ayana and Turhan (2009) tested the OLE in an impregnated disc at different concentrations obtaining different inhibition zones: 3% w/v of OLE inhibits 26.6 mm and 1% inhibit 21.4 mm. The concentration of 1.5% w/v was selected to be tested in Kasar cheese. In this study they found that OLE is effective against *S. aureus.*

- Sudjana et al. (2009) demonstrated that OLE is effective in an experiment with agar dilution and broth microdilution techniques against *C. jejuni*, *Helicabacter pylori* and *S. aureus* with MIC's as low as 0.31–0.78% (v/v).
- Pereira et al. (2007) focused on phenolic compounds against microorganisms related to human diseases (like *B. cereus, S. aureus, E. coli,* and *fungi)* and found that OLE at low concentrations showed an unusual combined antibacterial and antifungal action suggesting a great potential as nutraceutical and antibacterial, particularly as a source of phenolic compounds.

**Conclusions:**

OLE has a reported antibacterial effect against several bacteria (mainly *C. jejuni* and *S. aureus*) and fungi, that apparently is not proportional to the concentration. The antibacterial effect depends on the amount of polyphenols in the extract and the solvent, although there are discrepancies regarding which is the best solvent and the best technology to extract OLE.

It is not clear which molecule has the antibacterial effect, whether hydroxytyrosol or oleuropein, despite the fact that one is the metabolite of the other. In the evaluation of the OLE performance is important to consider the total content of polyphenols and not only the concentration of hydroxytyrosol or oleuropein.

## 5.2 Studies that evaluate OLE activity in foods

In this section, reported studies on the effect of OLE (and other olive tree derivatives with similar molecular composition) when added directly into food are discussed. The main uses tested in these studies are as antibacterial, antioxidant and for food fortification (Figure 19). Specific studies on the application of OLE in poultry are scarce and only one study was found testing OLE in turkey meat against *S. aureus*, *S. enteritidis* and *P. aeruginosa* (Djenane et al. (2012). The antimicrobial effect *in vitro* via diffusion method in agar and in turkey meat was studied. Results indicated a good antimicrobial activity without affecting sensory properties. In the following Tables (5 to 10) the main published works are summarized, respectively for the application of OLE in minced meats and fish, food recipes, dairy products, bakery products, fruit and oils.

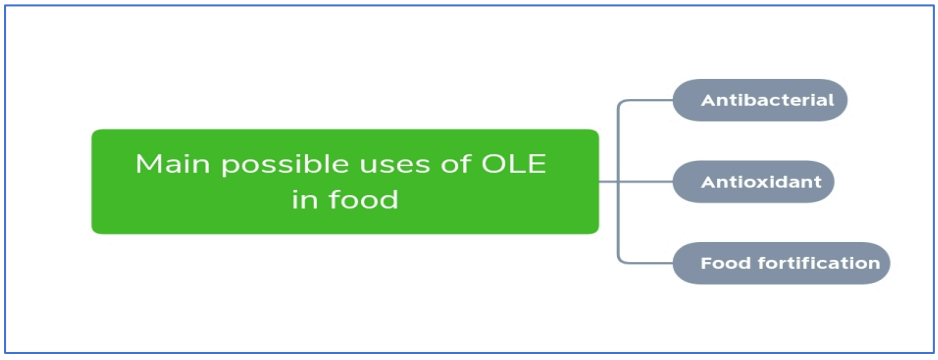


**Figure 19.** Main possible uses of OLE in food found in the literature.

**Table 5.** Main studies of OLE applied in minced meats and fish

| Reference | Food | Active ingredient | Observations |
|---|---|---|---|
| Khemakhem et al. (2019) | Salmon burgers | Hydrolized OLE and OLE | OLE decreased lipids oxidation in salmon. Salmon mince treated with hydrolyzed OLE showed lower microbial counts than OLE during the whole study, which extended the shelf life of the fish product at 4 ºC and vacuum-packed. |
| Shalaby et al. (2018) | Minced beef | Irradiated OLE | Test of OLE in minced beef focused more in the antibacterial activity with important activity from the microbiological against *B.cereus, S.aureus, E. coli, K. pneumonia, P. aeruginosa, and S. Typhimurium* "The results indicated that when 2 and 3 ml OLE were added to 100 mg of minced beef could improve quality attributes and extend shelf life of minced beef from 1 week to 3 and 4 weeks under cold storage." |
| Albertos et al. (2017) | Smoked salmon | Olive leaf powder (OLP) and water ethanol extract (OLE) | OLE was tested against *L. monocytogenes* in a food matrix well known as being a food that can be infected by *L. monocytogenes* which is the cold-smoked salmon. Edible fish gelatin films with OLE showed antibacterial activity against *L. monocytogenes* in agar diffusion tests and in the fish over storage. They found that de optimal film formulation is 5.63% OLE to be effective against L. monocytogenes. |
| Elama et al. (2017) | Frozen hamburger | Pure oleuropein | In this study they added a specific concentration of oleuropein to frozen burger and compared it to the food additive sodium erythorbate to compare their antioxidant activity. In this test both antioxidants had similar results suggesting that oleuropein is a potential substitute of synthetic antioxidants in food like sodium erythorbate. |

| Reference | Food | Active ingredient | Observations |
|---|---|---|---|
| Al-Rimawi et al., 2017). | Fresh hamburger | OLE and oleuropein | In this study they found similarly to Elama et al. (2017), that 0.5% of oleuropein and 1.5% of OLE was considered adequate to preserve fresh hamburger. |
| Alpizar Bermudez (2016) | Minced meat | OLE | In this test OLE had a higher antioxidant effect than rosemary extract for the preservation of ground meat, which suggest that more studies comparing both extracts should be done because nowadays rosemary extract is a widely used extract for food preservation in the industry. |
| Alirezalu et al. (2016) | Frankfurt sausages | OLE (Ethanol extract) | OLE in frankfurter type sausages showed that OLE has antioxidant activity and has a significant effect reducing the total count bacteria in a 45 days study at 4 ºC. |
| Ahmed et al. (2014) | Shrimp | OLE (Ethanol extract) | OLE reduced the count of the aerobic and coliforms bacteria at least 1 log cycle CFU $g^{-1}$ in comparison to the non-treated control group by immersing shrimp in a solution containing OLE at concentration of 1% (w/v). |
| Farhi, Hana (2009) | Minced meat | Olive leaf powder | Olive leaf powder added to minced beef found a significant reduction of bacteria (mesophilic aerobic bacteria) in meat products. In this study they found that olive leaf powder has an instantaneous antimicrobial effect and control the microbial count during 6 days at 4 ºC. |

**Table 6.** Main studies of OLE and other olive derivatives added to food recipes.

| Reference | Food | Active ingredient | Observations |
|---|---|---|---|
| Caponio et al. (2019) | Table olives | OLE | OLE added to table olives to increases their nutritional value, and they found that is not only useful to increase the nutritional value but also to improve sensorial properties (reduce bitterness) and reduce spoilage bacteria. In this study they also found a synergistic effect between *L. plantarum* and OLE against foodborne pathogens. |
| Garcia moreno, (2019) | Black olives | Water extract | OLE improves sensorial properties and reduces the production of acrylamide, a substance related to cancer. |
| Testa et al. (2019) | Anchovies | Liophilized metanol extract | OLE doesn't change organoleptic properties and it's effective against spoilage bacteria during 22 days. |
| Muiño et al. (2016) | Lamb meat patties | Olive waste extract | Similar results as other studies concluding that "This application reduced lipid and protein oxidation while maintaining an acceptable colour for a longer time period, representing a 3-day in the shelf life extension compared to patties without the added extract." |
| Ganje et al. (2016) | Tomato paste | Encapsulated OLE. Methanol/water extract | Encapsulated OLE in tomato paste compared to non-encapsulated one have better results. "Samples having microencapsulated OLE could maintain the original quality of the tomato paste very well, samples with non-encapsulated OLE rated the worst performance (among all specimens) in terms of maintaining this determinant index for long-time period." This study was done at high temperatures in an accelerated shelf life test (ASLT) and no microbial analysis was done. |

| Reference | Food | Active ingredient | Observations |
|---|---|---|---|
| Botsouglou et al. (2013) | Cooked pork meat patties enriched with n-3 fatty acids | OLE (etanol extract) | OLE proved to be a good antioxidant by delaying "lipid oxidation and reducing both primary and secondary lipid oxidation products ... It also inhibited protein oxidation in a concentration-dependent manner ... In addition, OLE improved the sensory attributes of the enriched patties." |
| Lalas et al. (2011) | Table olives | Water OLE extract | Similarities and some results that are contradictory to the study of Caponio et al. (2019) mainly regarding sensorial properties like bitterness where they found that "Sensory evaluation of treated table olives showed an increase in bitterness. However, treated and untreated table olives showed equal overall acceptability and overall preference." |
| Aytul et al. (2008) | Beef cubes | OLE | Study done immersing beef cubes in a OLE solution and they found that "using 2% and 3 % OLE had the beneficial effect in controlling the microbial load of beef cubes during 9 days of storage at 4 ºC". In this study they also confirmed the potential antioxidant effect of OLE on beef cubes. |
| De Leonardis et al., (2007) | Lard | Olive-oil mill wastewater | Olive mill wastewater is highly effective for oxidative stabilization of lard also at low temperatures. |

**Table 7.** Main studies of OLE and other olive derivatives to preserve and enrich dairy products

| Reference | Food | Active ingredient | Observations |
|---|---|---|---|
| Ribeiro et al., (2020) | Yoghurt | Olive pomace | Olive pomace made the yoghurt fulfil the nutritional claim "source of fiber", improved the nutritional value of the yoghurt and made it more stable from the nutritional point of view. |
| Palmeri et al. (2019) | Milk | OLE Water extract | Addition of OLE to pasteurized milk. The results have been excellent, as OLE was effective against *B. cereus* and inhibits the enzyme α-glucosidase which is related to the degradation of milk. "The addition of 5% OLE (v/v) to whole pasteurized milk increased its shelf life by 60%, which would lead to significant benefits in terms of costs linked to transport and to product returns to the dairy industry." |
| Alfonso et al. (2014) | Butter and margarine | n-hexane extract | OLE has antioxidant activity in dairy products. In this study they tested the addition of OLE to butter and margarine and obtained better results than the control regarding the antioxidant activity, although the effect was much more significant in margarine than in butter, which suggest that more studies have to be carried out to clarify the reason of this different of behavior. |

**Table 8.** Main studies of OLE and other olive derivatives applied to bakery products

| Reference | Food | Active ingredient | Observations |
|---|---|---|---|
| Faccioli et al., (2020) | Crackers | Olive leaf flour | The addition of Olive leaf flour improved sensorial properties and presented greater purchase intention. |
| Moghadam et al. (2020) | Gluten-free bread | Water extract | The addition of OLE and LAB in gluten-free bread improved sensorial properties and reduced the fungi count, increasing consequently it shelf life |
| Difonzo et al. (2018) | Backed snacks like breadsticks | Water extract | OLE reduced lipid oxidation increasing consequently its shelf life. The addition of OLE is highly recommended when a low-quality olive oil is used. “OLE effectively acted also in normal storage conditions, improving sensory data, induction times, antioxidant activity, and volatile compounds compared to control” |

**Table 9.** Main studies of OLE to preserve fruit

| Reference | Food | Active ingredient | Observations |
|---|---|---|---|
| Khalifa et al. (2017) | Apple | OLE (Ethanol extract) | They sprayed a chitosan coating solution with OLE in apple and they found that "the addition of OLE to chitosan coating films meaningfully reduced the gradual decline in total phenolic, flavonoids and antioxidants." and concluded "olive wastes extracts, incorporated into chitosan fruit coatings; relatively improve the nutritional quality of apple fruits during post-harvest." |
| Khalifa et al. (2016) | Strawberry | OLE (Ethanol extract) | The experiment was done spraying different coating formulas. They found that chitosan coating with OLE "led to keep the bioactive substances of cold-stored strawberry fruits.". "the addition of OLE into chitosan coating reduced the gradual decline in total phenolics, flavonoids, antioxidants, ascorbic acid and malondialdehyde." |

**Table 10.** Main studies of OLE and other olive derivatives applied to oils.

| Reference | Food | Active ingredient | Observations |
|---|---|---|---|
| Tarchoune et al. (2019) | Extra virgin olive oil | OLE Methanol extract | The addition of OLE to olive oil improves it nutritional value and its antioxidant stability. That proved that de addition of OLE to a standard olive oil make it similar to a high-quality olive oil. |
| Jimenez et al. (2017) | Sunflower and oil | Hydroalcoholic OLE | The addition of OLE to sunflower oil and canola oil reduces the formation of polar compounds during fresh potato frying and it is also proved that OLE reduces oxidation in sunflower oil which suggest that can be recommended as a potential source of antioxidants for maintaining stabilization, especially unsaturated vegetable oils. |
| Mohammadi et al. (2016). | Soybean oil | Nano-encapsulated OLE | Soybean oils have more oxidative stability when nano encapsulated OLE is added. |
| Malheiro et al. (2013) | Olive oil | Olive leaves | When olive leaves are added during the extraction process there is an increase in 30% of vitamin E compared to control besides the increase of other healthy substances in the olive oil like carotenoids, chlorophyl, etc. |
| Rafiee et al., (2012) | Sunflower oil | Microwave OLE and methanol extract | The OLE blocked the oxidation process during the heating of the oil at 70 ºC. Methanol extract gave better results. |
| Sanchez de Medina et al. (2012) | Various refined oils | OLE and Olive pomace | Enrichment of refined oils with olive industry derivatives like OLE and olive pomace give to these oils a similar (or better) profile to that of extra-virgin olive oil. In this study they compared the activity of the different olive industry derivatives. |
| Sanchez Medina et al. (2011) | Various refined oils | Microwave OLE | In this test all of the oils improved their quality and stability parameters in comparison with their nonenriched counterparts. In some parameters enriched refined oils had better healthy profile than extra-virgin olive oil. |

| Reference | Food | Active ingredient | Observations |
|---|---|---|---|
| Orozco-Solano et al., (2011) | Olive and sunfower oil. | OLE and olive pomace | Olive pomace is al also effective as antioxidant and an alternative to OLE to enrich and add antioxidants to oils. "Extra-virgin olive oil and high oleic sunflower enriched with hydrophilic phenols presented a similar fatty acid profile, but also a similar composition in hydrophilic antioxidants as a result of the enrichment process." In conclusion, the addition of olive derivatives to sunflower oil give to sunflower oil a similar profile to that of olive oil. |
| Andrikopoulos et al. (2007). | Various refined oils | Methanol OLE extract | OLE addition improved substantially antioxidant capacity and oxidative stability for all the oils studied after supplementation. |
| Farag et al. (2007) | Sunflower oil | Olive leaf juice | The addition of olive leaf juice to sunflower oil heated at 180°C improved remarkably the antioxidant activity. |
| Paiva-Martins et al. (2007) | Refined olive oil | Ethanol OLE extract | The addition of OLE to refined olive oil made some properties similar to extra virgin olive oil. |
| Artajo et al. (2006) | Refined olive oil | Hydroxitirosol and other antioxidants | The addition of antioxidants (OLE) prevents formation of toxic products like cholesterol oxides and increases it nutritional value. |

## 5.3 OLE integrated in food packaging materials

In this section studies reporting the use of OLE in traditional plastics and biomaterials are briefly discussed.

- Cejudo Bastante et al. (2019) tested OLE by SSI (supercritical solvent impregnation) in a film made by PET/PP to extend the shelf life of tomato cherry and study its antimicrobial effect in this food matrix. Their results showed that the films extended the shelf life of cherry tomato by 20 days.

- Amaro-Blanco et al. (2018) added OLE to a packaging made by polyamide and polyethylene to preserve dry-cured shoulders of Iberian pigs. They didn't find significant differences to the control sample. This study was carried out using additional treatment to food, such as refrigeration, HPP and vacuum which suggest that maybe these treatments had a greater effect and the addition to OLE did not add any additional value to the preservation.

- Moraes Crizel et al. (2018) tested films of chitosan with olive pomace (flour and microparticles). Olive pomace showed great antioxidant activity in packaged walnuts, which was proportional to the concentration of olive pomace. Films with 30% of flour or microparticles added to the film were effective as protective packaging against oxidation of nuts for 31 days.

- Moudache et al. (2017) tested OLE with pork meat. They added OLE to a film of multilayer polyethylene at different concentrations and the result showed an important antioxidant activity and increases in shelf life by two days of the pork meat packaged in OLE polyethylene film.

- Moudache et al. (2016) proved in *in vitro* studies that the addition of OLE and olive cake to polyethylene/polyethylene (PE/PE) film and polyethylene/paper (PE/P) exhibited high scavenging activity (method to test antioxidant activity) of free radicals.

- In a study with a film (Cryovac 100 µm) impregnated with OLE and FDE applied in pork loins (Delgado-Adamez et al., 2016) did not get positive results *in situ* but the antimicrobial activity *in vitro* against *E. coli0157, E. coli*, *S. enterica* and *L. innocua* was significative.

- The effectiveness of OLE in antioxidant active packaging was also proved by Licciardello et al. (2015) in a study with OLE and GPE in a food simulant. In this study film samples coated with Shellac and NC (cellulose nitrate), containing OLE and GPE at different concentrations were put in contact with three food simulants and the ATBS test showed great antioxidant activity.

- Marcos et al. (2014) incorporated α-tocopherol and OLE at different concentrations in Ecoflex and Ecoflex-polylacticacid (PLA) by blown film extrusion. The films demonstrated a good antioxidant activity. The films containing tocopherol exhibited higher antioxidant activity than the OLE films. It was also highlighted that this is an interesting eco-friendly packaging because OLE is a sub-product and PLA is a compostable plastic.

- Ayana and Turhan (2009) tested OLE impregnated in MC with glycerol to assess the antimicrobial activity of the packaging, besides water barrier and mechanical properties. The films were effective against *S. aureus.*

## 5.4 Legal status of OLE in the EU

The OLE is not authorised as food additive but the EU authorized the use of synthetic hydroxytorosol as a food ingredient in 2017 (European commission, 2017; Turck et al., 2017). But it specifies that it should be used only to fortify food due to its beneficial health effects, and in no case for food that is going to be cooked. It is also specified that products containing hydroxytyrosol must indicate on the label that they should not be consumed by pregnant women or children under 3 years of age. These restrictions are due to the fact that sufficient toxicity studies have not yet been carried out on OLE, especially genotoxicity, as well as studies of the possible effects on infants and pregnant women. When OLE is heated, it can give rise to metabolites that can be harmful, so it is important to carry out stability studies of OLE under different conditions and over time.

**Conclusions:**

-The effect of OLE may be similar to that of artificial synthetic preservatives, but it has a sensory effect that can be perceived as negative. More studies are needed to develop flavors masking strategies that can reduce the sensory quality of food. Sometimes it can give a taste that can be perceived as positive in certain foods.

-OLE has a greater antibacterial effect than other plant extracts, but there are no studies that differentiate the influence that each extract has on the sensory properties of food or studies on strategies to mask the flavors of extracts.

-Toxicological studies have to be carried out in order to authorize OLE as a food additive in foods that are to be cooked.

-OLE has proven its effectiveness as an antioxidant and antibacterial in different types of food. Therefore, its combined action against oxidation, bacteria, and fungi make it suitable for extending the useful life of food.

-It is advisable to combine OLE with other types of extracts, to obtain synergistic effects to reinforce the antibacterial and antioxidant activity.

-When OLE is mixed in polymers, there is a greater antioxidant than antibacterial activity.

-The addition of OLE in oils allows extending the shelf life and the quality of the oils, especially those that are most sensitive to oxidation.

-OLE has been shown to be effective in baking by reducing fungal growth and improve the sensorial properties of bread.

-OLE combined with chitosan has proven to be effective, taking advantage of reinforcing the positive qualities of chitosan in food preservation.

-Fruits and vegetables are foods that are eaten fresh and tend to degrade by oxidation and also by fungal contamination. Studies have shown that the addition of OLE increases its shelf life. Because fresh juices are pasteurized or made by HPP technology are widely consumed today, the addition of OLE can be a solution to extend even more their shelf life.

-The addition of OLE or other olive derivatives to oils improves their nutritional profile and their stability.

# 6. Experimental laboratory work

## 6.1. Introduction

The objective of this study was to develop an active OLE-based packaging against *Campylobacter* spp. in chicken. The optimal growth conditions for *Campylobacter* spp. are microaerophilia and a temperature of 41.5 ºC. In certain cold conditions, the bacteria can survive although does not grow. Campilobacteriosis is a real public health problem (see epidemiology of *Campylobacter* spp. in the EU and Portugal). Finding a solution to this problem is a real challenge for researchers and this study aims to provide scientific information of interest.

In the literature, there is only one study that tested OLE for poultry preservation (Djenane et al.,2012). They tested the effect of OLE in turkey meat against *S. aureus*, S. enteritidis and *P. aeruginosa.* The antimicrobial effect *in vitro* via diffusion method in agar and in turkey meat was tested. It was found a good antimicrobial activity without affecting sensory properties. Sudjana et al. (2009) found in an *in vitro* study that OLE is effective against *Campylobacter* spp, but this study was not specific for meat.

The experimental laboratory work was carried out in two parts, a first part *in vitro*, to have the most controlled conditions possible and a second part *in situ* (using chicken meat) to evaluate if that solution is feasible in real conditions.

Purpose of the study:

The objective of this study is to know if OLE has an effect on *Campylobacter* spp. under certain temperature and atmospheric conditions. The effect that a substance can have against a bacteria under certain conditions and at a certain concentration can be classified as bacteriostatic or bactericidal. A substance can be classified as bactericidal or bacteriostatic depending on the concentration used, it can for example be bacteriostatic at low concentrations and bactericidal at high concentrations.

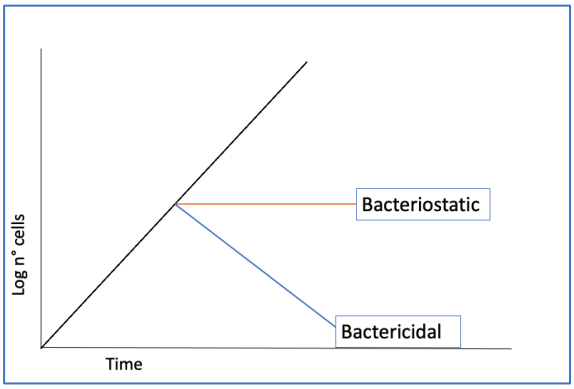


**Figure 20.** Concepts of bactericidal and bacteriostatic substances. Impact in level of cells *(Source: Al-nawas, and Ziegler, 2011).*

**Bactericidal** substances are able to destroy bacteria by targeting the cell wall or cell membrane of the bacteria. **Bacteriostatic** substances are those that slow or inhibit the growth of bacteria (Hamid and Saqib, 2017).

## 6.2. Material and methods

OLE extract:

The olive leaf extract (OLE) used is a standardized powder extract with a content of 20% oleuropein. Oleuropein is the active principle of OLE and the main substance used as criteria for the quality of the extract. The supplier of this extract is the Spanish company Nutexa, a laboratory specialized in food additives for the food industry. This extract is not sterilized, therefore, a control group was included to avoid bias in case of contamination of the powder extract, and also for this reason measures have been taken to avoid its contamination. Sterilization was not performed because the extract degrades at high temperatures.

Optimization of the OLE extract application for *in vitro* tests:

In order to design an experiment that provides with optimal, reliable, and conclusive results, preliminary work was carried out to define the required laboratory practices, namely to know the behavior of the extract, its solubility, and the feasibility for using *in vitro* and *in situ* tests.

To perform inoculation tests in agar plates (agar diffusion method), the application of OLE was optimized. The first approach was to solubilize OLE in water or in oil and to apply on surface of the agar plate. These approaches did not succeed. It was observed that the solution was not homogeneous in the first case, and in the second case, the oil spread totally in the surface of the agar. Additionally, the application of the OLE solution in a small well created in the agar by perforation was tested, but without success.

The solubilization of OLE in ethanol was not considered because of the ethanol bactericidal action which could jeopardize the results of the OLE. For this reason, the use in the study of any other substance with a bactericidal effect other than OLE was not performed.

Given the results above, the method selected was to dilute the extract powder in the agar, using modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA, VWR, Darmstadt, Germany). A more transparent culture medium was finally selected in order to control visually the solubility of the extract - Columbia agar culture medium (Merck, Darmstadt, Germany).

Agar dilution is a widely used method in clinical microbiology for the evaluation of the antibiotic susceptibility. The method was adapted to the OLE. Unlike many studies in which OLE is mixed with food, this study aimed at evaluating the OLE effect when incorporated in a simulating packaging surface to contact the food. Another advantage of this method is that by not mixing the OLE with the food, the organoleptic changes imparted by OLE are limited. At high concentrations, OLE has a strong odor that, although not being unpleasant since it is a particular aromatic smell of the olive tree, can affect the organoleptic properties of the food product. These sensorial issues limit the concentration at which OLE can be used the packaging material.

*In vitro* test:

The method of agar diffusion was followed. The suspension of *Campylobacter* spp. *(DSM 4688; DSM 4689; ATC33560)* was prepared transferring colonies from the plates with fresh pure culture of the bacteria into a tube containing 10 mL of Ringer's solution to obtained 0.5 in Mcfarland scale. The enumeration of *Campylobacter* spp. in the suspension was performed in accordance to ISO 10272-2:2017 (plate spreading method on Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA, VWR, Darmstadt, Germany).

One hundred microliters of the suspension *of Campylobacter* spp. have been inoculated in plates with Columbia agar culture medium (Merck, Darmstadt, Germany) with concentrations of 2%, 1%, 0.5% and 0.25% of OLE mass per volume of culture medium. The culture media were prepared following the manufacturer's instructions, they were sterilized, then the proportional amount of OLE was added and mixed with the medium.

The plates have been incubated in a microaerophilic incubator at 41.5 ºC in microaerophilia and in aerobiosis in a fridge at 4 ºC for 48 hours.

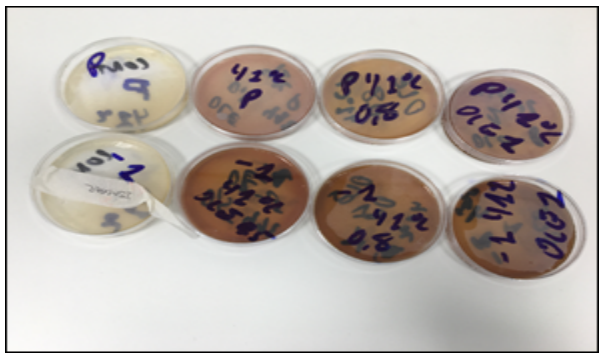

**Figure 21.** *In vitro* test of *Campylobacter* spp. in OLE (petri dish).

To confirm the results obtained by the agar diffusion method (Figure 21), a test with the microdilution method (Figure 22) was also performed. The microdilution plate method allows many replicates to be made very easily, in addition to testing many concentrations of OLE. The microdilution method does not simulate an active antibacterial contact packaging but allows greater contact between the bacteria and the OLE, since the bacteria are immersed in a medium rich in OLE and not only superficially in a solid medium with OLE. In addition, by subsequently inoculating this medium on a plate, it confirms whether the results of the microdilution to have more accurate conclusions.

This test was carried out according to Aumeeruddy-Elalfi et al. (2016): 100 µL of Mueller-Hinton broth (MH broth Biokar Diagnostics, Beauvais, France) was placed in each well of the microplates. Following 50 µL of the medium with OLE mixed was added to the first wells. From these serial dilutions were performed. The excess volume was removed from the last wells (50 µL). 50 µL of suspension of Campylobacter spp. was added to each well and a negative control wells series was also prepared without inoculation. The microplates were incubated at 41.5 ºC and 4 °C for 48 hours in microaerophilic conditions.

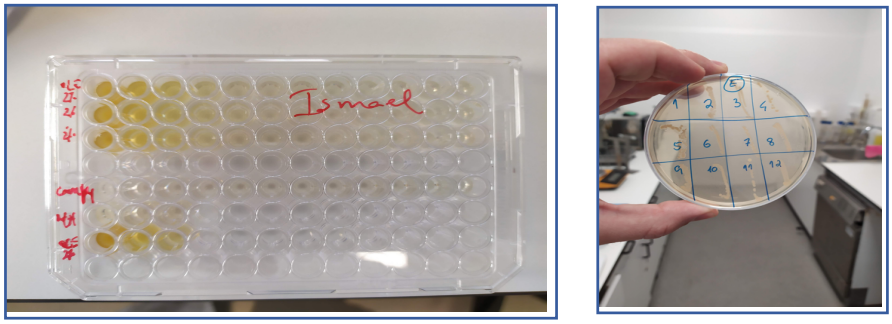


**Figure 22.** I*n vitro* test of *Campylobacter* spp. in OLE (microdilution).

## *In situ* test:

In the *in situ* test, sterile agar with OLE at 2% concentration was brought in contact with inoculated chicken pieces. Sterile agar was chosen to simulate the packaging material because is a non-nutritive, neutral culture medium. The OLE was homogeneously solubilized in this transparent medium and poured into plates.

The concentration of 2% OLE was selected because it is four times higher than the minimum obtained in microbiological tests and still does not impart a strong olive smell.

The small pieces of chicken (Figure 23) were surface sterilized with ethanol before inoculation. Pieces were immersed in ethanol and allow to dry for 5 minutes. Subsequently, they were put in contact for 1 min (only one face) with a Ringer suspension of *Campylobacter* spp. *(ATCC 33560).* This suspension was prepared transferring colonies from a fresh pure culture of the bacteria into a tube containing 10 mL of Ringer's solution to obtain 0.5 Mcfarland scale. *Campylobacter* spp. counts in the suspension were determined according to ISO 10272-2:2017 (plate spreading method on mCCDA medium).

**Figure 23.** Sterilized chicken pieces.

Three pieces of chicken were placed on each plate with 2% OLE. A negative control was also prepared with two pieces in an agar plate without OLE. The plates were stored in microaerophilia at 41.5 ºC and 4 ºC for 24 hours to 4 days.

*Campylobacter* spp. counts in the meat pieces were determined according to ISO 10272-2:2017 at the end of the incubation period and also at the beginning of the experiment in a control.

## 6.3. Results

### *In vitro* test:

The number of *Campylobacter* spp. in the suspension was 1.1 x$10^8$ CFU $mL^{-1}$ and the number in the inoculated in plates (Columbia agar culture medium + OLE) was 1x$10^7$ CFU $mL^{-1}$. Table 11 indicates if growth occurred or not in the plates with different concentrations of OLE and stored at refrigeration and optimum growth temperature.

In the tests at 41.5 ºC no growth has occurred in plates with 0.5% OLE and higher concentrations. However, when the inoculum was removed from the contact with the medium containing OLE, *Campylobacter* spp. growth occurred, indicating that *Campylobacter* spp. was inhibited but not eliminated at a 0.5% of OLE concentration. The test at 4 ºC did not give conclusive results because the bacteria did not growth in either the plates with OLE and in the control group. However, growth was observed when placing the plates in microaerophilia and at 41.5 ºC.

**Table 11.** Results of the test in plates.

| | **OLE %** | | | |
|---|---|---|---|---|
| **Temperature** | 0.25 | 0.5 | 1 | 2 |
| **4 ºC** | ? | ? | ? | ? |
| **41.5 ºC** | Growth | No growth | No growth | No growth |

The microdilution method test in MH broth at 41.5 °C has resulted in a MIC of 0.5% OLE in Mueller-Hinton medium. These results are similar to those of the testing in plates. In the microdilution test at 4 °C the bacteria was still viable at 2% of OLE concentration. To verify if the bacteria remain viable at higher concentrations, tests up to 6% OLE were performed. It was observed that the bacteria remain viable.

### *In situ* test:

The number for *Campylobacter* spp. in the Ringer suspension was 1.0x$10^8$-1.0x$10^9$ CFU $mL^{-1}$. In the meat pieces after the contamination step the number of *Campylobacter* spp. was 1.1x$10^8$ CFU $g^{-1}$.

When in contact for 24 h with the OLE (Figure 24) containing agar and stored at 41.5 ºC, the meat *Campylobacter* spp. count was <1.0x10 CFU $g^{-1}$, while in control agar without OLE the number was 8.5x$10^7$ CFU $g^{-1}$. Therefore, a 6-log reduction was achieved in meat stored at 41.5 ºC. However, when the meat was removed from the contact with OLE containing agar, some growth was observed upon incubation for 24 hours and 41.5 ºC and microaerophilic conditions in non-OLE containing agar. Therefore, these results also indicate that *Campylobacter* spp. growth is reduced but some

microorganisms remain viable. This may mean that the OLE affects either the number of viable bacteria (by killing some) or affects the cells metabolism (cells viable but not culturable).

In the meat stored at 4 ºC the results show that that the concentration of bacteria is high (> 1500 CFU $g^{-1}$) thus indicting that the potential reduction is not at the same level of that at 41.5 ºC. However, as only one dilution was performed, no further conclusions can be drawn. The additional dilutions needed were not planed because a higher effect of OLE in the reductions was expected.

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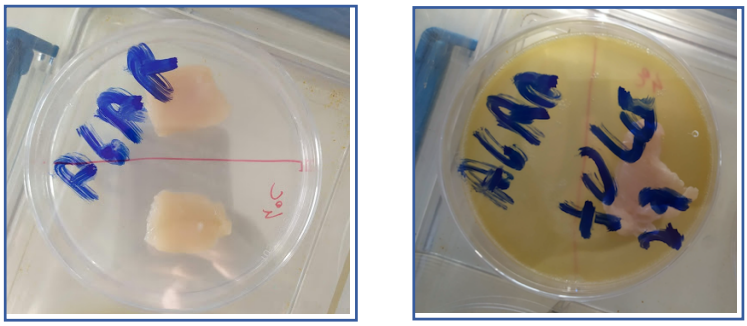

**Figure 24.** Chicken in an agar plate without (left) and with (right) OLE.

The results of the *in situ test* are therefore equivalent to those of the *in vitro* test, confirming once again that OLE has an effect. However, this effect is masked when the test is conducted in cold conditions, since cold also inhibits the growth of *Campylobacter* spp. The test at 4 ºC did not demonstrate whether OLE has a synergistic effect with low temperature. Reported studies in the literature indicate that aerobiosis and low temperature can assist on controlling the growth of *Campylobacter* spp. (Chynoweth et al., 1998; Garenaux et al., 2008). The biochemical changes of the bacteria at low temperatures must be investigated in order to develop better strategies to combat it.

## 6.4. Conclusions of the experimental part

- This study demonstrates that OLE is effective as a growth inhibitor, or bactericidal substance under optimal *Campylobacter* spp. growth conditions. At 4 ºC no significative antibacterial effect has been observed.
- In the study of Sudjana et al. (2009) OLE was found to have a MIC of 0.31% (v/v) against *C. jejuni* , therefore, in good agreement with that found in the present study (0.5%) if we take into consideration the differences in the type of extract and different oleuropein concentration in the extract, in both studies.
- *In vitro* and *in situ* assays at refrigeration temperature (4 ºC) have shown that the bacteria remain viable after being subjected to high concentrations of OLE. This may be due to possible defense mechanisms when the bacteria are at low temperatures.
- Agar dilution and microdilution methods allow for getting complementary information. The microdilution has the advantage of allowing for simultaneous determinations and using low amount of reagents, but does not allow for measuring the bacterial reduction. Validating the results with the two methods avoids method-related biases.
- The potential inhibitory effect of OLE against *Campylobacter* spp. at low temperatures was not possible to be validated in the present study. However, this can be done with some additional experimental work.
- The effect of OLE at high temperatures was observed. Its application can be useful in food products at risk of containing *Campylobacter* spp. and kept at temperatures higher than room temperature, more adequate for *Campylobacter* spp. growth, or in the case of abuse temperature. Likewise, it was found that the OLE can be a possible treatment for campylobacteriosis in humans or as a supplement in animal feed to reduce the bacterial proliferation of *Campylobacter* spp. in poultry.

# 7. Conclusions

-The preservation of food is still a challenge of the XXI century and food waste is a matter of concern. Although food packaging is criticized due to the use of plastic, it allows to preserve food for longer and therefore avoid waste. The search for new preservation methods is also a commitment to the environment.

-People increasingly values the fact that food products are preserved by natural methods instead of the use of synthetic chemical substances, many of which have been shown to have a negative effect on people's health.

-*Campylobacter* spp. it is still a bacteria that causes a lot of infections in the EU being one of the most important causes of foodborne diseases.

-The European legislation is increasingly severe, new solutions will have to be developed in order to comply with the legislation and avoid greater food waste which translates into greater economic losses for companies.

-There is a disparity between EU countries in relation to food safety, namely the availability and collection of information.

-The levels of *Campylobacter* spp. infection are seasonal, rise in summer and are proportional to the consumption of poultry between EU countries.

-Foods based on poultry meat are still the main cause of campylobacteriosis even other foods are also related to this bacterium like dairy products

-The combination of natural extracts can give synergistic effects that improve the results of the desired objective, much more than the use of extracts alone.

-The main research trends on antimicrobial packaging for poultry using natural products are the use of EO's, minerals, polysaccharides, modified atmosphere and the use of bacteriocins and bacterias. The most effective substances found in the literature against *Campylobacter* spp. are chitosan, carvacrol and metals such as zinc.

-Currently in the food industry the use of MAP predominates for the preservation of meat. Therefore, the combination of temperature and MAP remains the preferred method due to its ease of use, it does not affect the sensory properties and it does not release potentially harmful substances for health.

-Minerals such as zinc among others are a new branch in research for the development of antibacterial solutions for food. Zinc is beneficial for health because it strengthens the immune system, unlike aluminum, which has been shown to be an endocrine disruptor, therefore many minerals have the potential to be applied in food preservation.

-The interest of OLE is also due to the fact that it is a by-product of the olive industry that can be used. Today it is used a lot in herbal medicine because it has proven effects on health and therefore some companies use it to enrich their food. The main trends in research in relation to OLE are its use as an antioxidant, antibacterial and as a food fortifier.

-The strategies of OLE to preserve food that have been reported in the literature are mixed in the packaging, mixed with food or impregnation in the packaging, with the former having the greater effect.

OLE is effective in various types of food and against other microorganisms. It also has a clear antioxidant effect; it is one of the most powerful antioxidants that exist.

-The little homogeneity that exists between the extracts is a challenge, more studies must be done to optimize the extract standardization methods.

- The OLE is not authorized as food additive by EFSA but synthetic hydroxytirosol is authorized as food ingredient, It use can be only to fortify food.

-OLE has a clear inhibitory effect on *Campylobacter* spp. but not at all temperatures, these bacteria probably develop resistance mechanisms at low temperatures, which prevents multiplication but also makes killing more difficult.

# 8. Future work proposal

- More studies need to be done on the relationship of *Campylobacter* spp. with the temperature and the defense mechanisms it has when it is in adverse situations, only by knowing the bacteria better it will be possible to develop strategies to combat it.

- Toxicological studies of OLE have to be carried out in order to validate it as a food additive.

- OLE has proven to be effective as an antioxidant, antibacterial and antifungal. In spite of the results regarding *Campylobacter* spp. at low temperatures, it could be interesting to study the effect in other microorganisms such as *Salmonella* spp., *L. monocytogenes* and *E. coli.*

- Aerobiosis is effective against the growth of *Campylobacter* spp. but *Campylobacter* spp. it is found in areas of the chicken little exposed to oxygen such as under the skin. Due to this, studies have to be done in matrices that make different studies in chicken samples completely in aerobiosis and others that contain skin and other parts of the chicken that create a microaerophilic environment.

-The use of OLE as a treatment or as a preventive drug for campylobacteriosis in humans should be investigated. Since in this study its antimicrobial activity against this bacteria at 41.5 ºC is validated *in vitro.*

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