Universidade de Aveiro
Ano 2019

Departamento de Biologia

Leandro Tomás Pereira

MICROBIOLOGICAL ANALYSES FOR SAFETY AND QUALITY ASSESSMENT OF FOODS AND LUNCH BOXES

ANÁLISE MICROBIOLÓGICA PARA AVALIAÇÃO DE SEGURANÇA E QUALIDADE DE ALIMENTOS E LANCHEIRAS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Maria de Fátima Filipe Tavares Poças, Investigadora Coordenadora do CINATE da Escola Superior de Biotecnologia da Universidade Católica Portuguesa e coorientação da Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro.

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agradecimentos

Queria agradecer a todos aqueles que contribuíram para que este estágio fosse possível, apoiando, dessa forma, o meu desenvolvimento e formação profissional.

À orientadora, Doutora Fátima Poças agradeço por me ter aceitado no CINATE e pela orientação e acompanhamento durante o estágio e na redação e revisão crítica do relatório.

À coorientadora, Professora Doutora Adelaide Almeida agradeço pela sua disponibilidade e apoio na redação e revisão crítica do relatório.

Um grande agradecimento à Doutora Cristina Mena pela orientação do trabalho experimental e constante partilha de conhecimentos para além do apoio importante na redação do relatório.

Queria agradecer também a toda a equipa do CINATE que me acolheu da melhor forma, incluindo as Técnicas Superiores Luísa Carneiro e Isabel Santos pelo apoio e ensinamentos valiosos durante a fase experimental no laboratório de microbiologia.

À Feliciana, pela amizade e apoio durante o período de estágio e aos meus amigos e colegas, principalmente os que conheci durante o mestrado cujo apoio e companheirismo foi indispensável durante esta etapa.

Por fim, queria agradecer aos meus pais e irmão pelo amor incondicional e apoio constante.

palavras-chave

segurança e qualidade alimentar, critérios microbiológicos, indicadores de higiene, patogénicos, lancheiras.

resumo

Este relatório apresenta as atividades realizadas e competências adquiridas durante os 10 meses de estágio curricular no Laboratório de Microbiologia do CINATE, um laboratório de análises e ensaios a alimentos e embalagens da Escola Superior de Biotecnologia (ESB) da Universidade Católica Portuguesa.

Este estágio permitiu a realização de várias funções, sendo as mais relevantes a realização de ensaios de pesquisa e contagem de microrganismos em alimentos, águas e zaragatoas de superfícies para verificar estados de higiene e contaminação, mas também as funções de manutenção de um laboratório de microbiologia. Este estágio provou ser enriquecedor e proveitoso, pois permitiu o desenvolvimento de competências laboratoriais e analíticas na área da microbiologia aplicada à segurança alimentar.

Adicionalmente, o relatório apresenta conceitos de segurança alimentar, aborda importantes patogénicos transmitidos por alimentos, bem como as entidades reguladoras de critérios microbiológicos de segurança e qualidade da indústria alimentar.

Paralelamente, foi realizado um estudo acerca dos aspetos de segurança alimentar relacionados com o uso de lancheiras no transporte de refeições.

A utilização de lancheiras no transporte de refeições tem aumentado pela maior preocupação da população com a alimentação, no entanto, pode tornar-se um potencial vetor de transmissão de microrganismos patogénicos caso não sejam adotadas práticas corretas de manutenção e higiene. Para a determinação do conhecimento da população sobre este assunto, foram aplicados inquéritos online e em pessoa respondidos em paralelo com a avaliação da qualidade microbiológica deste modo de transporte e armazenamento de refeições, na qual foram analisadas lancheiras (n=102) de alunos e funcionários da ESB, da região do Porto. As amostras recolhidas foram analisadas para contagens de unidades formadoras de colónias (UFC) de microrganismos totais a 30 °C, *Enterobacteriaceae*, *Escherichia coli* e deteção de microrganismos patogénicos (*Listeria monocytogenes* e *Salmonella* spp.) através de cultivo em meios apropriados e testes de confirmação bioquímicos segundo procedimentos baseados em normas nacionais e internacionais. Detetou-se *E. coli* em apenas uma amostra com uma concentração de 1,0 log UFC/100 cm^2 de área interna da lancheira. Não se detetou *Salmonella* spp. nem *L. monocytogenes*, no entanto, obteve-se crescimento de outras espécies de *Listeria* em 8% (n=8) das lancheiras. Os resultados dos indicadores de higiene, microrganismos totais a 30 °C e *Enterobacteriaceae* foram comparados com os valores-limite estabelecidos por normas encontradas para superfícies de contacto com alimentos e descobriu-se que a maioria das lancheiras (59,8%) apresentou boas condições de higiene segundo as contagens de microrganismos totais a 30ºC. Este estudo exploratório é indicador da perceção e atitude da população relativamente aos cuidados de higiene e segurança alimentar no transporte de refeições que, em alguns casos, de acordo com os resultados dos inquéritos, eram inadequados. Os resultados da análise microbiológica indicaram, contudo, que as condições de higiene são maioritariamente aceitáveis. Maiores esforços deveriam então ser dirigidos à informação da população acerca das boas práticas de utilização de lancheiras de modo a assegurar a segurança alimentar.

keywords

food safety and quality, microbiological criteria, hygiene indicators, pathogens, lunch boxes.

abstract

This report presents the activities and skills acquired during the 10-month curriculum internship at the Microbiology Laboratory of CINATE, Laboratory for food analyses and packaging studies at the Escola Superior de Biotecnologia da Universidade Católica Portuguesa (ESB). This internship allowed the accomplishment of several functions, the most relevant one being the performance of microbiological assays in food, water and surface swab samples, for the detection and quantification of microorganisms indicative of hygiene and contamination conditions. The internship also included maintenance functions of a microbiology laboratory. This internship proved to be enriching and fruitful, as it allowed the development of laboratory and analytical skills in the field of microbiology applied to food safety, sparking my interest for a potential career in this field.

In addition, the report also presents concepts of food safety, indicating important foodborne diseases, regulations and regulatory entities of microbiological safety and quality criteria in the food industry.

At the same time, a study focusing on the aspects of food safety related to the use of lunch boxes in the transportation of meals was carried out. The use of lunch boxes in the transport of meals has increased, due partly to an increased trend of healthy eating, however, it can become a potential vector of transmission of pathogens if proper maintenance and hygiene practices of lunch boxes are ignored. To evaluate population knowledge on this matter, an online survey was employed as well as an in-person version which was answered simultaneously with evaluation of the microbiological quality of this mode of transportation and storage of meals in which lunch boxes (n=102) of ESB students and staff from the Porto region were analysed. The collected samples were analysed for the count of colony-forming unit (CFU) of Total Viable Count (TVC), *Enterobacteriaceae* and *Escherichia coli* hygiene indicators. Pathogen detection of *Listeria monocytogenes* and *Salmonella* spp. was also carried out. These parameters were determined using appropriate culture media and biochemical confirmation tests according to procedures of CINATE based on Portuguese and International standards. *Salmonella* spp. and *L. monocytogenes* weren't detected, however, growth of other species of *Listeria* spp. was detected in 8% (n=8) of the lunch boxes. The results of Total Viable Count and *Enterobacteriaceae* were compared to limit values established by standards found for food contact surfaces and from this, it was concluded that most of the lunch boxes (59.8%) presented good hygiene conditions according to the obtained low TVC counts. This exploratory study is thusly indicative of the perception and attitude of the population regarding hygiene and food safety practices in the transportation of meals in lunch bags, which in some cases, according to survey results, were inadequate. However, the results of microbiological analysis indicated that hygiene conditions of the analysed bags are mostly acceptable. Greater efforts should therefore be directed towards population information about correct practices to assure food safety during lunch bag use.

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Abbreviations

AAB – Acetic Acid Bacteria

ALOA – Agar Listeria Ottavani & Agosti medium

API 20NE – Analytical Profile Index for identification of Gram negative non-*Enterobacteriaceae*

ASPW - Alkaline Saline Peptone Water

BCA - *Bacillus cereus* Agar

BEAA - Bile Esculin Azide Agar

BHI - Brain Heart Infusion

BPA – Baird-Parker Agar

BPA+RPF - Baird-Parker Agar + Rabbit Plasma Fibrinogen

BPW – Buffered Peptone Water

CCA - Chromogenic Coliform Agar

CCP – Critical Control Point

CFU - Colony Forming Unit

DBDM - Dekkera/Brettanomyces Differential Medium agar

EC – European Commission

EFSA – European Food Safety Agency

ELFA - Enzyme-Linked Fluorescence Assay

ESB - Escola Superior de Biotecnologia

EU – European Union

FAO - Food and Agriculture Organization

FDA – United States Food and Drug Administration

FSIS - Food Safety Inspection Service

HACCP - Hazard Analysis of Critical Control Points

Half-Fraser Broth - Fraser Broth with half concentration

IPAC - Instituto Português de Acreditação

ISO - International Organization for Standardization

LAB – Lactic Acid Bacteria

Lab. - laboratory

M-Broth – Mannose Broth

MKTTn - Muller-Kauffmann Tetrathionate-novobiocin broth

MMGB - Minerals (modified) Glutamate Broth

MPN – Most Probable Number

MRS - De Man, Rogosa and Sharpe agar

MSA - Mannitol Salt Agar

NIAS – Non-Intentionally Added Substances

NSW – New South Wales

NUTS - Nomenclature of Territorial Units for Statistics

OF – Oxidative Fermentative

ONPG - O-Nitrophenyl-β-D-Galactopyranoside

OR - Odds Ratio

PALCAM - Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesculin Mannitol agar

PCA – Plate Count Agar

pH – Acidity level

Pseudomonas CN – *Pseudomonas aeruginosa* Agar with Cetrimide and Nalidixic Acid

RBCA - Rose-Bengal Chloramphenicol Agar

RODAC - Replicate Organism Direct Agar Contact

RVS - Rappaport Vassiliadis Soya peptone broth

S&B - Slanetz and Bartley agar

TBX - Tryptone Bile X-glucuronide agar

TCBS - Thiosulfate Citrate Bile Salts Sucrose agar

TSC - Tryptose Sulfite Cycloserine agar

TSI – Triple Sugar Iron agar

TVC – Total Viable Count

UCP - Universidade Católica Portuguesa

USDA – United States Department of Agriculture

UV- Ultra-Violet

VRBD – Violet Red Bile Dextrose agar

VRBL – Violet Red Bile Lactose agar

WHO – World Health Organisation

XLD - Xylose Lysine Deoxycholate agar

YE -Yeast Extract agar

YM - Yeast and Mould agar

1. Introduction

This report was developed to present an overview of the curricular internship I chose to complete my Master of Science (M.Sc.) degree in Microbiology from the University of Aveiro. This internship occurred during the second year of the course in a food and packaging quality and safety laboratory of the Escola Superior de Biotecnologia (ESB) of the Universidade Católica Portuguesa (UCP) in Porto, Portugal. This department is denominated CINATE and consists of a group of accredited laboratories that specialise in food safety and quality control of foods and packaging. This internship lasted approximately 10 months, starting in October 2017 and ending at the beginning of August 2018.

This report was structured to approach five main topics, the first one being informative section in which the definition of food safety was presented, as well as the regulating entities which are responsible for the standards by which food safety regulations are established and how microbiological sciences are relevant for these situations, with the mention of relevant microorganisms.

This is followed by the description of the department in which I presented main activities and services it offers.

Succeeding this, I went in depth about the work developed in the lab, explaining the workflow and the different activities which were carried out, while also mentioning the activities for which I was responsible.

During this period, I was also given the opportunity to participate in a food safety study which focused on obtaining information about the Portuguese population's use of lunch boxes and associated food safety practices while also sampling volunteers' lunch boxes for microbiological analysis. In this case, the sampled bags belonged to students and employees of ESB. The results of this study were mentioned and discussed in this report.

Concluding this document, the final topic chosen was the discussion and conclusion in which I discussed the competencies and skills I acquired during the 10 months in CINATE and whether my goals for this internship were reached.

I chose to participate in this internship in the Microbiology Laboratory of CINATE as opposed to a common research laboratory because of my personal interest in other equally important applications of microbiology besides research, which in this case was food safety and quality studies targeting services to industries. I therefore established the following goals to be reached during my time in CINATE:

- Gain experience in a microbiology laboratory and consolidate previous knowledge of traditional microbiology methods which are essential for all microbiology labs;
- Comprehend the workings of a certified laboratory, including maintenance and the application of standardized methods for detection and quantification of different microorganisms;
- Develop some level of confidence and autonomy in a professional laboratorial setting;
- Possibly discover a career path in microbiological quality control.

1.1. Food Safety and Quality

Food safety can be defined as the evaluation of the food production process for the inspection and prevention of consumers' health risks when consuming the final product. Its assurance is of utmost importance in the food production industry, from the raw ingredients up until the final product distribution and consumption. Three main categories of hazards may appear along the food production chain. Such hazards may be of biological, chemical or physical origin (World Health Organisation - WHO, n.d.; Rooney & Wall, 2003).

Biological hazards include the presence of pathogenic microorganisms such as bacteria, virus, fungi, protozoa or viruses and metabolites these may produce. The product might be contaminated by the food source, equipment and food-handlers during processing of the product (Rooney & Wall, 2003). Chemical hazards are compounds resulting from pollution from various activities, including the agricultural activities which release pesticides, fertilizers as well as residues from food-producing animals containing veterinary drugs into the environment, thus contaminating food sources. Other compounds include naturally occurring compounds such as mycotoxins (fungal toxins) which are simultaneously biological and chemical hazards. Physical hazards are explained as foreign matter such as glass, stones, metal, etc which may end up in the food product due to environmental contamination (WHO, n.d.; Rooney & Wall, 2003).

Food quality, on the other hand, is also of high importance but encompasses evaluation of other parameters which may alter the characteristic properties of the products, not necessarily affecting the consumer's health, however, affecting its consumer value. These parameters include food spoilage and changes in product's sensory, nutritional and physical-chemical properties (Food and Agriculture Organization -FAO/WHO, 1997).

Food safety and quality are obtained by the combination of efforts on behalf of many factors including legislation which should lay down minimum hygiene requirements and official controls which should be in place to check food business operators' compliance, as well as food safety programmes and procedures based on the Hazard Analysis and Critical Control Point (HACCP) principles which are established and operated by food business operators as stated in the Commission Regulation (EC) Nº 852/2004 for the case of the European Union (E.U.).

HACCP is a preventive planning method which is employed by food industry operators to maintain food safety throughout the overall food production from food ingredients up to the final product, also encompassing retail and food services of the food chain. HACCP systems analyse the food production chain, determining different Critical Control Points (CCP), establishing acceptable measures for them and maintaining continued monitoring. If a standard for a CCP is not met, the product is considered unsafe and corrective measures, also defined by the HACCP, are taken. This system saves resources by directing them towards the more relevant aspects which would represent an increased food safety risk (FAO/WHO, 1997).

In the European Union, in order for food industries to ensure the quality and security of food products, they must follow legislation which includes the Commission Regulation (EC) Nº 178/2002, whose main objectives are protection and health of human life as well as free movement of food in the community. Food industries also follow other important legislation concerning hygiene

processes, namely Commission Regulations (EC) Nº 852/2004 on the hygiene of foodstuffs and (EC) Nº 853/2004, which lays down specific rules for the hygiene of different foodstuffs. Another important law, Commission Regulation (EC) Nº 854/2004 is conjointly followed and in which specific rules are laid down for the organisation of official controls on products of animal origin intended for human consumption.

1.2. Microbiological Criteria

Microbiological criteria for food safety and quality are guidelines which are based on internationally accepted principles, being established by legislation and used to assess the microbiological levels in food products and their manufacturing processes, including performance of hygiene processes. The criteria provide objectives and reference points for food businesses and competent authorities to manage and monitor foodstuffs. This is made by determining limit values for the quantity of specific microorganisms, their metabolites or associated markers present in certain food products or batches along different sectors of the food production chain. These criteria also describe the appropriate methods of detection of the microorganism and sampling plans as well as the recommended corrective measures to be taken (Viegas *et al.*, 2017). These are thus of utmost importance for the evaluation of good practices and the development of food safety preventive methods such as HACCP implementation. In Europe, the Commission Regulation (EC) Nº 2073/2005 establishes the microbiological criteria for food products (Viegas *et al.*, 2017).

1.3. Importance of Food Safety/Quality Microbiology Labs

Analysis of microbiological criteria in food products have important repercussions in public health and in the economy since they can determine if products are microbiologically safe and if they should be commercialized or recalled from the markets. Food safety and quality laboratories are thus a crucial part of food control systems since they are responsible for monitoring samples of food products as well as environmental samples along the food production chain for these established microbiological criteria.

These laboratories are integrated into quality assurance programs and accredited by accreditation agencies which allow an improvement of their performance and consequently improvement of result reliability, accuracy and repeatability. To guarantee result quality, accredited laboratories perform inter-laboratory testing, and internal quality control (FAO/WHO, 1997).

Pertaining to the microbiological analyses, samples are tested for the presence and quantification of various microbiological parameters which include indicator/index organisms as well as detection of food-borne pathogens. Indicator organisms are defined as microorganisms, groups of microorganisms or even a product of microbial metabolism (ex: toxins) which, if present in the food product/surface sample, indicates the increased probability that the sample has been exposed to conditions which increased the risk of a pathogen contamination or allow pathogen proliferation. (WHO,2017)

Indicator microorganisms are ideally and usually non-pathogenic and methods used for their detection should be rapid, inexpensive and widely available, with the achievement of clear results. Such methods include colony forming units (CFU) count of microorganisms grown on appropriate culture media. To be a good indicator, they must occur in the samples frequently enough so as their

levels may be monitored in a food safety system, allowing for establishment of baseline levels and maximum levels as well as detection of out-of-control conditions when such indicator levels increase.

Index microorganisms, on the other hand, allow direct correlation between their presence in a food sample and the presence of a certain pathogen. Listed below are some common indicators/index microorganisms and food-borne pathogens which are detected and quantified:

- Total Viable Count at 30 °C

Total Viable Count (TVC) at 30 °C is a parameter which indicates non-specific microbial growth (bacteria, yeasts and moulds) under aerobic conditions at a standard 30 °C. It is used as a general sanitation indicator, with standard values presented in microbiological criteria. This parameter has also been used for the evaluation of effectiveness of intervention steps, microbiological quality and spoilage of different foods such as ready-to-eat foods. Waters may also be evaluated for this parameter, however, at temperatures varying from 20 to 37°C (WHO, 2017).

- *Enterobacteriaceae*

The members of the family *Enterobacteriaceae* are found ubiquitously, in many ecological sources like soil, water and vegetation, with many species being part of the normal microbiota of animals including humans. This family also includes pathogens which are important public health concerns (i.e. *Salmonella* and *Eschericia coli*) (Jenkins 2017). Enumeration of this parameter by colony count on Violet-Red Bile Dextrose (VRBD) agar may be indicative of the effectiveness of sanitation processes and postprocessing contamination in foods, albeit not necessarily faecal contamination since it englobes a wide diversity of microorganisms including those of environmental origin (Tortorello, 2003).

- Total coliforms

Coliforms are bacteria belonging to the *Enterobacteriaceae* family and which encompass a wide range of aerobic and facultatively anaerobic, Gram-negative, non-spore-forming bacilli that are able to grow in relatively high concentrations of bile salts and ferment lactose with acid and gas production within 24 hours and at 30 °C - 37 °C with production of the enzyme β-galactosidase. Many coliforms are found in the environment, in water, soils and grains, while some are also of faecal origin, being found in the gastrointestinal tract of some animals. Therefore, in analysis of food products or waters, coliform enumeration does not necessarily indicate faecal contamination; however, it may indicate postprocessing contamination of foods that have been processed (heating, irradiation, or chlorination) for safety when high colony counts are present postprocessing. They are also useful indicators in guidelines at critical control points, particularly after heat processing, since they are sensitive to heat (Tortorello, 2003, WHO, 2017).

- Faecal/Thermotolerant coliforms and *E. coli*

These coliforms are thermotolerant, that is, they are able to grow and ferment lactose at higher temperatures, more specifically, 44 °C. *E. coli* is also considered a thermotolerant/faecal coliform distinguished from the other coliforms by production of indole from tryptophan or presence of the

enzyme β-glucuronidase. Their presence in food products and waters is associated with faecal contamination and presence of other possible enteric pathogens. This microbiological group in food products is indicative of ineffective heat processing of foods (since these bacteria are easily destroyed by heat) or cross-contamination with contaminated equipment/surfaces which weren't properly sanitized beside also indicating poor disinfection or posterior contamination of waters (WHO, 2017). Although *E. coli* is a normal bacterium of the gut microbiota, some strains are considered pathogenic and can cause acute diarrheal diseases, such as the O157:H7 serotype which commonly causes outbreaks where people develop enterohemorrhagic symptoms resulting from the production of toxins (Shiga toxins). Unlike other strains, this *E. coli* is known to not produce β-glucuronidase (Ratnam *et al.*, 1988).

- Yeasts and Moulds

Yeast and moulds are widespread and can contaminate foods through inadequate sanitation processes and airborne contaminants. Enumeration of yeasts and moulds are particularly important in food products with low pH levels such as fruit juices and or low water activity such as sugars, which stimulate growth and proliferation of fungi as opposed to bacterial growth. These fungi often cause food spoilage with yeasts producing off-flavours and causing excessive gas production while moulds may cause off-odours (Tortorello, 2003).

- Psychrotrophic microorganisms

Psychrotrophic microorganisms englobe all types of microorganisms which can grow under, albeit not ideally, refrigeration conditions. Their culture and quantification, on non-selective growth media and at refrigeration temperatures, are of increased importance for quality control since they affect the quality of refrigerated perishable foods. Psychrotrophs are known to cause off-tastes and off-odours when high levels of contamination are obtained, reducing significantly the shelf-life of products (Tortorello, 2003; Ribeiro Júnior *et al.,* 2017).

Other than the previous microbial groups, samples are also evaluated for the presence of many pathogens, with many common ones being presented below.

- *Staphylococcus aureus*

Staphylococcal species are Gram-positive, nonmotile, catalase-positive, small, spherical bacteria (cocci), which appear characteristically bunched in grape-like clusters in microscopic imaging. They are ubiquitous bacteria found mainly on the skin and mucous membranes of animals, including humans with many of the species and subspecies being potentially found in foods due to environmental, human, and animal contamination. All coagulase-positive staphylococcal species produce highly heat-stable enterotoxins that cause gastroenteritis in humans. *Staphylococcus aureus* is predominantly associated with staphylococcal food poisoning as well as other illnesses such as toxic shock syndrome, pneumonia, postoperative wound infection, and nosocomial bacteraemia (United States Food and Drug Administration-FDA, 2012).

- *Listeria* spp. and *Listeria monocytogenes*

Listeria genus members are Gram-positive, rod-shaped, flagellated, motile bacteria being wildly distributed in the environment, water, soil, sewage, vegetation and faeces of animals and humans. The presence of this genus in foodstuffs and surfaces may indicate poor hygiene procedures (Lakicevic *et al.*, 2010) with the *Listeria monocytogenes* species being one of the leading causes of death from foodborne illness. Despite not having an increased incidence, infections from *L. monocytogenes* are one of the leading causes of death from food-borne illnesses presenting and a non-invasive gastrointestinal illness form and another much more invasive form which may cause septicaemia and meningitis. *L. monocytogenes* is ubiquitous in the environment, being mostly found in moist environments, soil land and decaying vegetation. Additionally, it's salt-tolerant and survives and proliferates under 1 °C, putting at risk food products kept in refrigeration. This bacterium is associated with many products including dairy products (raw, unpasteurized milks, cheeses) as well as raw vegetables and meat (FDA, 2012).

- *Salmonella* spp.

Salmonella is a motile, Gram negative non-spore forming, rod-shaped bacterium from the *Enterobacteriaceae* family. The genus *Salmonella* is divided into two species that can cause illness in humans, *S. enterica and S. bongori* with the former being of most concern and divided into several subspecies which are then again divided into serotypes according to antigenic properties. Salmonella spp. causes two types of diseases, nontyphoidal salmonellosis and typhoid fever, with the latter presenting a higher mortality rate despite the former also being severe but mostly in vulnerable populations such as the immunocompromised or the elderly. *Salmonella* is found in the gastrointestinal tract of vertebrates including food-producing animals, being spread through the faecal - oral route when contaminated food products including meats, poultry, dairy products, seafood and spices as well as contaminated water are consumed (FDA, 2012).

- Enteropathogenic Vibrio species

Members of the *Vibrio* genus are Gram-negative, curve-shaped rods which occur naturally in preferably maritime and estuarine waters despite also growing in other aquatic environments. These bacteria are thus an important food-borne pathogen associated with consumption of contaminated seafood (fish and molluscs) and water. There are many species of human pathogenic *Vibrio* species including *Vibrio cholerae*, the causative agent of cholera, a severe diarrheal disease with high mortality rate if untreated and Non-cholera *Vibrio*. In this last group other species such as *Vibrio parahaemolyticus*, cause mostly mild gastrointestinal disease, which is cured relatively fast, although that would not be the case for vulnerable populations (young children, elderly, pregnant women, immunosuppressed) (FDA, 2012).

- *Bacillus cereus*

Bacillus cereus is a Gram-positive, facultatively anaerobic, endospore-forming, large bacilli (rod shaped) bacteria which is widespread in the environment, being found in soil and vegetation. *B. cereus* is tolerant to a wide range of temperatures (4 - 48°C), pH levels (4,9 - 9,3) and high salt concentrations (7.5%) making it prolific in various food products. It is known to cause diarrheal-type

food poisoning associated with consumption of meats, fish, vegetables and vomiting symptoms associated generally to rice consumption as well as other starchy foods. (FDA, 2012)

- *Campylobacter* spp.

Campylobacter jejuni is a non-spore-forming, Gram-negative rod with a curved shape morphology, which may present motility due to flagellums located at polar ends of the cells. Members of the *Campylobacter* genus are microaerophilic, that is, they grow preferably in lower than atmospheric oxygen concentrations (3-5%) making them quite fragile in ambient environment and more difficult to culture in laboratories. *C. jejuni* is also sensitive to freezing, drying as well as disinfectants and acidic environments. Infections with this bacterium has been associated with undercooked, poorly handled poultry since *Campylobacter* is part of the natural microbiota of food-producing animals such as chickens and their presence indicates poor hygiene practices and inadequate processing. Infections have also been linked to unchlorinated pond water and unpasteurized dairy products (milk and cheese). (FDA, 2012)

Many hygiene indicators of foods are also detected in various types of water samples (drinking-water, ponds, recreational waters, waters from food processing systems, etc) with many indicators also being used mostly in water samples and which can include the following.

- Intestinal Enterococci

Intestinal enterococci are Gram-positive, facultatively anaerobic cocci which may appear in short chains and are known for moderate toleration for elevated salt concentrations and elevated pH levels as well as being found in faeces of warm-blooded animals. Although the presence of intestinal enterococci is indicative of recent faecal contamination in waters since these bacteria can be found in sewage and sewage contaminated waters, their presence may indicate a more previous faecal contamination compared to other indicators (faecal coliforms) since they are more resistant to water conditions(WHO, 2017).

- *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative rod bacterium which is polarly flagellated and aerobic. It is a member of the *Pseudomonadaceae* family and a common environmental organism found in faeces, soil and water, being able to grow and multiply in water environments and surfaces of organic materials in contact with water. Nevertheless, ingestion of water is not an important source of infection by *Pseudomonas aeruginosa*, despite possibly causing spoilage with production of off-odours and turbidity in the water. This being said, the bacterium may also colonise damaged tissue like cuts, possibly eventually provoking severe infections such as septicaemia and meningitis (FDA, 2012).

- *Clostridium perfringens*

Members of *Clostridium* spp. are Gram-positive, anaerobic, sulphite-reducing rods which produce spores that are resistant to extremely unfavourable conditions, that is, extreme temperature, pH and Ultra-violet (UV) radiation levels, in water environments. *Clostridium perfringens*, a characteristic species of the genus, is a member of the normal gut microbiota of warm-blooded

animals, including humans, contrasting with other *Clostridium* species with aren't exclusively of faecal origin. *C. perfringens* is thus a strong index of not so recent faecal contamination in waters as its spores are resistant towards extreme conditions including disinfection processes such as chlorination (FDA, 2012).

Microbiological analyses are also performed on alcoholic beverages including wines and beers, although mostly for the presence of spoiler-organisms since these beverages present unfavourable conditions for growth of pathogens (increased pH levels).

- Enumeration of microorganisms - Colony Count at 30 °C

The quantification of total microbial count in these beverages are used in wineries mostly in the context of hygiene testing in addition to evaluation of wine instability if present in large numbers by contributing to sulphur dioxide degradation. These bacteria include water bacteria transmitted through rinsing, cleaning processes, hygiene indicators and airborne bacteria (Just & Regnery, 2008).

- Enumeration of Yeasts and Moulds at 25 °C

This parameter allows for the quantification of fungal cells, yeasts and moulds, which are indicative of incorrect processing since in principle, they are undesirable in the finished product (bottles of wine). These yeasts are spoilers which can decompose the alcohol and cause off-tastes and odours. Moulds are usually present in wines and don't normally cause issues except if the grapes are bruised beforehand, and moulds can cause off-odours (Just & Regnery, 2008).

- Lactic Acid Bacteria and Acetic Acid Bacteria

Lactic Acid Bacteria (LAB), Gram-positive cocci or bacilli and Acetic Acid Bacteria (AAB), Gram-negative bacilli, some of which originate from leaves and grapes and are indicative of spoilage in wines. Although LAB may be used in fermentation processes, they should be absent in the final bottled wine. Bottled wines offer optimum conditions for LAB growth since these are anaerobic and acid- tolerant. If present, they cause turbidity and off-tastes as well as acid degradation with subsequent slimy consistency of the wine. AAB are also acid-tolerant growing strictly in the presence of oxygen and despite their reduced survival with the continuation of fermentation processes with reduced oxygen concentrations, they may leave off-tastes in wines which cannot be removed (Just & Regnery, 2008).

- *Brettanomyces*

Brettanomyces spp., is a genus of yeasts, known for causing spoilage in fermented products from cheese, fermented milks and also alcoholic beverages (wines, beers) by producing many compounds which alter the organoleptic properties of the products. This yeast is known to produce volatile fatty acids and phenols, the latter of which are important in wine spoilage in which production of undesirable flavours and aromas is also observed. These beverages are contaminated when in contact with contaminated areas such as wooden barrel interiors in which wines are aged (Tubia *et al.,* 2018).

2. Internship Location - CINATE

CINATE is a laboratory of UCP with its facilities located in the Escola Superior de Biotecnologia (ESB), which was founded in 1990. It consists of a group of laboratories accredited by the Portuguese Institute of Accreditation (IPAC) and which develop work in food safety and quality control of foods and packaging. This department, therefore, serves an important role in the innovation and analytical support of the food industry, being the interface between the university and companies.

The laboratories are all certified according to the NP EN ISO/IEC 17025 Portuguese standard, hence the use of national and international standards to execute assays. Internal standards, which have been developed and validated, are also applied. To maintain certification, CINATE is regularly monitored through quality audits and by conducting tests with certified reference materials, reference cultures and regular participation in interlaboratory trials (ESB, n.d.).

CINATE executes an array of tests for many clients, including restaurant facilities and food manufacturing and processing industries. These tests are divided into physical, chemical and microbiological tests, sensory evaluations and package testing, as is specified below:

- **Physical-chemical tests**

Analysis of characteristic compounds and quality indicators; food composition and nutritional characterization; legal and specification compliance assessment; analysis of residues and contaminants; research and evaluation of food and oenological additives; allergen search.

- **Microbiological tests**

Microbiological characterization of food products; quantification of microorganisms that indicate contamination; search for pathogenic microorganisms; monitoring hygiene of personnel and facilities; validation of disinfection processes.

- **Sensory evaluation tests**

Characterization of the sensorial food profile; analysis of consumer preference, acceptance and expectations; origin, characterization and organoleptic defects and deviations.

- **Packaging studies**

Determination of barrier properties against oxygen and water vapor; overall and specific migration of residual monomers, pigments and additives; quantification of contaminants and Non-Intentionally Added Substances (NIAS); assessment of recycled materials.

In addition to performing these tests, CINATE also performs shelf-life studies, problem diagnosis and offers technical advice and tailor-made training programmes (ESB, n.d.).

Focusing on the Microbiology Lab of CINATE, since the internship was held there, work premises were divided into seven sectors according to the different functions performed.

A) Sample Reception Room

Room in which samples were received, some of which maintained in a refrigerator or in a cupboard, for posterior analysis.

B) Sample Preparation Room

Room containing equipment for sample preparation, including a digital scale and a sample blender to prepare food sample suspensions.

C) Main Laboratory

Area in which the assays for different microorganisms were performed. This sector also contained incubators set to varied temperatures: 25 °C; 30 °C; 37 °C; 41,5 °C and 44 °C and refrigerators for storage of sterile growth media as well as an optical microscope and two water baths also for sample/media incubation.

D) Culture Media Preparation Room

Sector where all growth media were prepared. This room has cabinets filled with dehydrated growth media and reagents, heating plates and an autoclave used mostly for sterilization of utensils, materials and growth media. This room also had a tap for deionized, treated water for media hydration.

E) Pathogen Analysis Room

Separate room from which the assays for pathogenic microorganisms such as *Listeria monocytogenes* and *Salmonella* spp. were performed. This room also included equipment for Enzyme-Linked Fluorescence Assays (ELFA) connected to a computer.

F) Decontamination/Cleaning Room

Room where lab material was decontaminated in an autoclave. Reusable material would then be cleaned in a dish washing machine unlike other decontaminated waste which would then be placed in a waste bin.

G) Microscopy Room

Room with an optical microscope connected to a digital camera, allowing clearer and more detailed images for microorganism identification through morphology.

The microbiology lab followed general practices according to the International Organization for Standardization (ISO) 7218 standard entitled "Microbiology of food and animal feeding stuffs-General requirements and guidance for microbiological examinations".

3. Internship Activities

My functions during the internship consisted primarily of maintenance of the laboratory and the performance of assays for the detection and quantification of microorganisms in food and surface swab samples. These functions were further detailed below.

3.1. Sample Reception and Preparation

At time of reception, each sample was labelled with the number it was attributed and with which it was registered in CINATE'S database.

Proceeding to sample preparation, this process was performed in the sample preparation room with clean surfaces, disinfected with a diluted hypochlorite solution or 70%(v/v) ethanol solution and under aseptic conditions, i.e., in the proximity of an active Bunsen burner. For sample analysis, an initial suspension was prepared, in order to obtain a sample with homogenous microorganism distribution. Using disinfected knives, scoops or spatulas, laboratory personnel would weigh representative portions of the sample, normally 25 g, into a sterile stomacher bag placed in a gravimetric diluter and then fill the bag with a certain quantity of diluent solution, 9 times superior than that of the sample in order to obtain a 1 in 10 (10^{-1}) suspension. In CINATE'S Laboratory, the diluent solutions were mainly:

- **¼ strength Ringer's Solution** - Isotonic buffer solution containing physiologic concentrations of sodium (Na+), potassium (K+), calcium (Ca2+), and chlorine (Cl–) (Oxoid, n.d.).
- **Buffered Peptone Water (BPW) -** Buffer solution containing protein extract and used as a pre-enrichment medium in the detection of *Salmonellae*. BPW could be the diluent also when the sample was to be analysed for the enumeration of other microorganisms as well as *Salmonellae* detection (Biorad, n.d).
- **Half-Fraser Broth -** Buffer solution with a mixture of peptones, which is a selective enrichment medium for primary enrichment of *Listeria* spp. (bioMérieux, 2007).

The sample would finally undergo a homogenization process in the room's Stomacher blender during approximately 30 seconds to 3 minutes, after which it could be used for microbiological analysis.

This sample preparation was suitable for most types of samples, more specifically solid samples (meats, cheeses, prepared dishes, biscuits, powders) excluding waters, wines and environmental samples including swabs and contact plates. In the case of water and wine samples, appropriate culture media would be directly inoculated with predetermined volumes measured directly from the samples. In the case of swab samples, the diluent in which the swab was immersed would be the base point for analysis and in the case of contact plates, the growth media was already directly inoculated with the sampled surface.

3.2. Culture Media and Reagent Reception and Preparation

Despite not being a function I took part in, this was an essential part of normal laboratory activities, namely, purchase and reception of growth media and reagents. This would be followed by verification of information such as name, brand, supplier and number of packages which would then be inserted in CINATE's database. After this, date of material reception and opening was also registered and written on the culture medium/reagent container with stock and validity verification being performed on a regular basis.

I did, however, participate in growth media and reagent preparation which were all prepared in the culture media preparation room. Before any measurements, digital scale and pH meter calibration were necessary, followed by the measuring of deionized, filtered water used in the media hydration.

Digital scale verification consisted of measuring the mass of a check weight of known mass. This measurement was repeated two more times and all three measurements were registered in a document. For pH meter calibration, pH levels of three different buffer solutions of known pH were measured. These were buffer solutions which had pH 4.01 and pH 7.00, with the third solution also being of pH 7.00 and used as a solution for calibration confirmation. Following this calibration, the pH levels of the deionized water, which was also filtered with an UV light treatment, was measured. pH levels were measured while the water was vortexed on a magnetic stirrer hot plate to thus obtain a representative value of the water, which, according to standards, should be between 5 and 7, preferably as neutral as possible. If any growth medium or reagent was prepared on the following day, the scale calibration, pH meter calibration with water pH measurement would be repeated and registered again.

Ensuing these processes, the desired dehydrated medium or reagent could be prepared in clean Schott glass bottles which were also kept in cabinets in preparation room. The bottle was placed on the digital scale and the medium weighed into it. The weighed medium was then hydrated with deionized water. If the culture media was liquid, dissolution was executed on the magnetic stirrer hot plate with subsequent distribution into clean glass tubes with the aid of a peristaltic dispensing pump which distributed equal volumes of media into each tube. This also occurred in the case of preparation of Ringer's solution.

Culture media pH levels were measured in certain situations: after a new package of dehydrated media was opened, after the preparation of certain media with different reagents and after the preparation of diluents (e.g. Ringer's solution).

3.3. Sterilization

An important activity in laboratory was also the sterilization of materials such as pipette tip boxes and utensils for sampling as well as prepared growth media. Sterilization was performed by autoclaving (humid sterilization) normally at 121 ℃ for 15 minutes, except for certain growth media which needed to be autoclaved at different temperatures. Each autoclaved item had a piece of autoclave tape stuck to it and containing information about the date and autoclave cycle number for the day with additional identification of the culture media in the cases when containers with these media were sterilized. This autoclave tape also had a heat indicator which would turn black

if the temperature inside the autoclave reached high levels, thus indicating that the sterilization process was successful. All sterilized material would be registered in a journal, indicating material/growth medium, quantity, date and autoclaving cycle of the day. This would be accompanied by a graph of sterilization indicating temperature variation during the sterilization cycle.

3.4. Microbiological Assays and Result presentation

As an IPAC certified laboratory, CINATE's microbiology laboratory performs a diverse number of validated assays for the detection and or enumeration of various microbiological parameters. Most procedures were based on traditional culture-based methods of microbiological identification, with the use of selective and differential culture media including enrichment stages. Some procedures would also be followed by bio-chemical confirmation methods.

In CINATE, all results, including CFU count, detection or absence and identification test results, are registered on the result sheet of the respective sample. Each sheet also contains information about the sample type with corresponding sample description as well as identification of the personnel who prepared the sample suspension and dilutions, who performed the assay and who analysed the results. The sheet additionally includes the dates of the end of the assay (date when results were obtained). Proceeding result register on the sheet, this information is uploaded to the CINATE informatic system.

Assays for detection of microorganisms can be considered either quantitative or qualitative.

3.4.1. Quantitative Methods

Quantification of the viable microorganisms in the sample could be executed from solidified agar-based culture media by means of the pour plate method or spread plate method (Figure 1). Microorganisms could also be quantified in liquid culture media by the Most Probable Number method (MPN) (Figure 2) For these methods, initial (1:10) suspensions would normally be prepared, as well as decimal serial dilutions with sterilized tubes containing isotonic diluent, i.e. Ringer's solution.

Pour plate method - This method consisted of pipetting, normally, 1 mL of the sample's main suspension and decimal dilutions into a sterile Petri dish after which molten agar culture medium would be poured into the plate and then mixed in with the sample. This procedure would be executed under aseptic conditions, that is, on disinfected surfaces, in a sterile area in the proximity of a Bunsen burner. The medium would then solidify, and the Petri dish inverted and incubated for a determined amount of time and temperature according to the target microorganism. Subsequently, on the surface of the medium, CFU, resulting from viable cells which were spaced out far enough to form independent colonies, would appear, allowing for enumeration (McClure, 2008).

Spread plate method - This method comprised of pipetting a reduced volume of 100 µl of the sample suspension or decimal dilution onto the surface of the solidified culture medium inside the Petri-dish, followed by spreading, all under aseptic conditions. After the inoculant was absorbed into the medium, the Petri-dish would be inverted and incubated accordingly and resulting CFU would have grown on the medium's surface. This method was more advantageous than the pour-

plate method, because colonies were more easily sub-cultured from the medium surface and the risk of underestimating CFU count was reduced since the risk of exposure to heat stress was reduced (McClure, 2008).

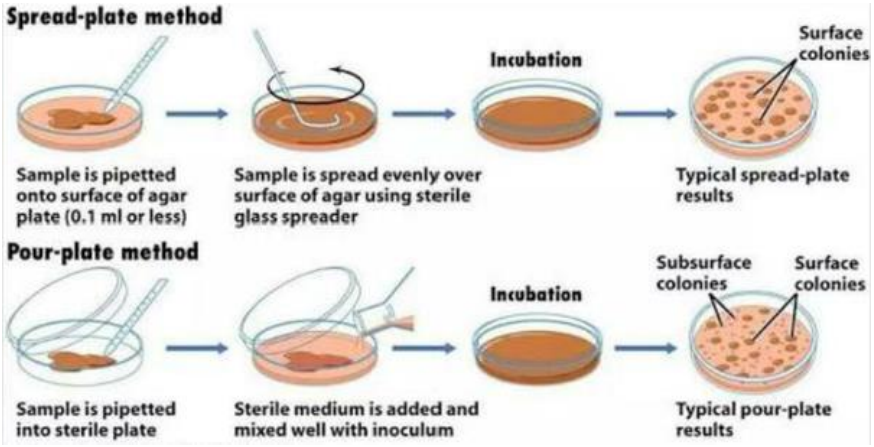

Figure 1 - Comparison of Pour plate and Spread plate Method. Retrieved from: https://www.quora.com/What-is-the-difference-between-the-pour-plate-method-and-the-spread-plate-method-in-isolation-of-bacterial-colonies

For enumeration methods (from either the pour plate or spread plate method), plate count limits would be between 10 and 300 CFU while these limits would be 10 to 150 CFU in assays for enumeration of presumptive colonies with further identification steps. Preferably, the enumeration of microorganisms in the sample would be the mean CFU count between plates of two successive dilutions with valid plate counts using the following equation:

$$\frac{\Sigma\, CFUcount}{(n1+0{,}1n2)\times d1} \qquad (1)$$

In which:

Σ CFU count - is the sum of CFU count of plate of two successive dilutions

n1 - is the number of Petri dishes of the lowest dilution

n2 – is the number of Petri dishes of the highest dilution

d1 – is the first countable dilution

If only a plate of a certain dilution obtained valid CFU count, the results would be obtained by multiplying the CFU count with the dilution factor of the counted plate.

In assays with confirmation steps after presumptive colony count, final CFU count would be obtained by the multiplication of the presumptive count with the proportion of positively confirmed colonies, that is, the proportion of positive colonies. This altered count would then be in place of "Σ CFU count" in the equation (1). All enumeration results would then be presented as a number between 1.0 and 9.9 to the appropriate power of 10, with two significant figures and expressed in

function of the sample quantity (mass (g), volume (mL), per swab or even by area (cm^2), as Table 1 shows.

In enumeration assays, when obtained CFU count was outside the quantification limits, that is, less than 10 CFU in the lowest dilution or more than the maximum limit (150 or 300) in the highest dilution, results were also presented as shown in Table 1:

Table 1- Expression of results of CFU results.

CFU count	Result Presentation (/g or /mL)
0	$<1.0 \times 10^1$
1-4	Present but $<4.0 \times 10^1$
4-10	$<1.0 \times 10^2$ EN= $4.0 – 9.0 \times 10^1$
10 -150 or 10 - 300	$1.0 – 9{,}9 \times 10^X$
>150 (for presumptive colonies)	$>1.5 \times 10^{d+2}$
>300 (for total count)	$>3.0 \times 10^{d+2}$

Note: EN =estimated number; x = appropriate exponent; d = highest considered dilution.

In the case of surface sampling with the use of Replicate Organism Direct Agar Contact (RODAC) plates and Dip slides, plate count limits were different than those of other assays, being between 1-100. These tools contained solidified agar media with specific surface contact areas, and which would be pressed onto the analysed surface and then incubated accordingly, followed by CFU count. Results would be presented as CFU count per analysed area which would be 25 cm^2 in RODAC plates and 9 cm^2 on each side of the dip slide. Thus, if plate count results surpassed the valid count limits, results would be presented as the Table 2 demonstrates.

Table 2- Expression of results for CFU counts in RODAC and Dip slide methods for surface sampling.

CFU count	Result Presentation	
	RODAC (CFU/25 cm^2)	Dip slide (CFU/9 cm^2)
0	<1	<1
0 -100	0 – 100	0 - 100
>100	>100	>100

Most Probable Number (MPN) method - This method consisted of preparing a number of serial decimal dilutions of the sample, according to estimated level of sample contamination, which was followed by transfer into culture broth tubes and appropriate incubation conditions. This would be replicated normally three times and tubes would be analysed for signs of growth (turbidity, gas production, pH alteration). Results would then be compared to probability tables in which the different combination of positive replicates for each dilution would correspond to an estimated contamination level. Despite being more labour intensive and imprecise, this method is adequate for situations in which low counts are expected or sample quantity is too high (McClure, 2008).

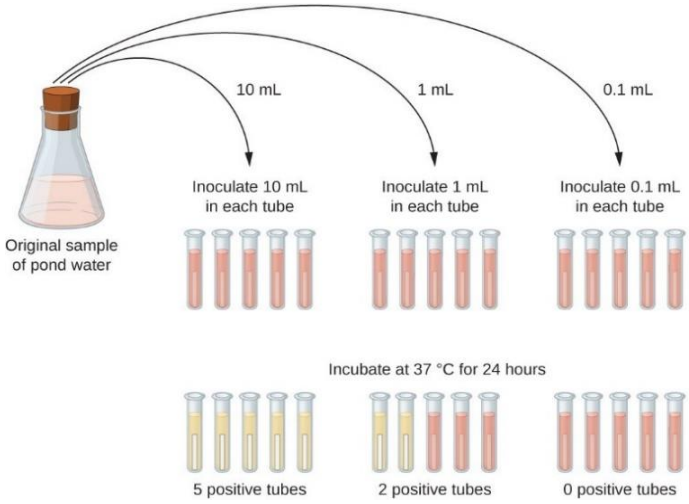

Figure 2 - Representation of Most Probable Number (MPN) technique. Retrieved from: https://microbeonline.com/probable-number-mpn-test-principle-procedure-results/

3.4.2. Qualitative Methods

Other performed assays included qualitative methods, when the main goal of the assay was to indicate the presence or absence of a microorganism in the sample. These methods were most commonly used for pathogen detection but also for some situations of hygiene indicator detection and would consist of four phases: primary or pre-enrichment; selective enrichment; detection/selective plating and finally confirmation. In these assays, a microbiological group would be considered either present or absent in the sample. Results were presented as present/absent, (positive/negative) in analysed sample quantity, that is mass (g); volume (mL); swab stick or even by area (cm^2) if the sampled surface area was known.

3.4.3. Microbiological assays applied to general foodstuffs and food products

CINATE executes assays on a variety of food samples including, fruits and vegetables, meats, food powders, milks, dairy products as well as ready-to-eat meals, waters and wines with procedures based on ISO standards. The main microbiological assays performed in CINATE and of which I took part are summarized further on:

- Enumeration of microorganisms – TVC at 30 °C

Primary sample suspension and decimal dilutions were inoculated by the pour plate method in non-specific, nutritive Plate Count Agar (PCA) and incubated at 30 °C for 72 hours followed by CFU count.

- Enumeration of *Enterobacteriaceae*

Primary sample suspension and decimal dilutions were inoculated in Violet Red Bile Dextrose Agar (VRBD) and incubated at 37 °C for 24h by the pour plate method followed by CFU count. Confirmation tests are performed on characteristic colonies and include Oxidase test and oxidative fermentative (OF) test to detect - glucose fermentation.

- Enumeration of Total coliforms/Thermotolerant coliforms

Primary sample suspension and decimal dilutions were inoculated in Violet Red Bile Lactose Agar (VRBL) by the pour plate method and incubated at 30 °C and 44 °C, respectively, for 24 hours followed by CFU count.

- Enumeration of *E. coli*

Primary sample suspension and decimal dilutions were inoculated in Tryptone Bile X-glucuronide (TBX) medium and incubated at 44 °C for 24 hours, followed by CFU count of β-glucuronidase positive colonies.

- Enumeration of *E. coli* in depurated shellfish

MPN technique was executed with Inoculation of five replicates of each of three dilutions into tubes containing Minerals (modified) Glutamate Broth (MMGB) at 37 °C for 24 hours. Aliquots of tubes positive for growth (yellow colour indicative of acid production) would then be streaked onto TBX agar for confirmation.

- Enumeration of Yeasts and Moulds

Primary sample suspension and decimal dilutions were inoculated onto Rose-Bengal Chloramphenicol Agar (RBCA) by the spread plate method at 25 °C for 5 days followed by separate count of yeast and mould colonies.

- Enumeration of Coagulase – positive *Staphylococcus aureus* with confirmation

Primary sample suspensions were inoculated at 37 °C for 48 hours on Baird-Parker Agar (BPA) medium prepared with tellurite egg-yolk emulsion. Confirmation of characteristic and non-characteristic colonies would occur after Brain Heart Infusion (BHI) inoculation and incubation (37 °C 24 hours) which is followed by Coagulase test.

- Enumeration of Coagulase – positive *Staphylococcus aureus* without confirmation

Primary sample suspensions were inoculated onto Baird Parker Agar + Rabbit Plasma Fibrinogen (BPA+RPF). 37 °C for 48 hours and characteristic colonies were quantified.

- Detection of Coagulase Positive/Negative *Staphylococcus aureus*

Pre enrichment in Chapman broth at 37 °C for 24 +24 hours. After, broth was inoculated onto BPA after 24 and 48 hours of incubation. Coagulase confirmation follows with confirmation of characteristic and non-characteristic colonies after inoculation on BHI at 37 °C for 24 hours.

- Enumeration of *Listeria* spp./*Listeria monocytogenes*

Primary enrichment (1:10) in ½ Fraser was inoculated on Agar Listeria Ottavani & Agosti medium (ALOA) and Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesculin Mannitol (PALCAM) agar by spread plate method followed by incubation at 37 °C for 48 hours. Confirmation of characteristic colonies from the previous media onto Blood Agar for confirmation of β-haemolysis as well as fermentation of Rhamnose, Xylose, Mannitol sugars in Purple Agar as well as Gram and Catalase Test.

- Detection of *Salmonella* spp

Pre- enrichment in (1:10) sample suspension were prepared in Buffered Peptone Water (BPW) and incubated at 37 °C for 24 hours following selective enrichment in Muller-Kauffman Tetrathionate-novobiocin broth (MKTTn) and Rappaport Vassiliadis Soya peptone broth (RVS), incubated at 37 °C and 41.5 °C respectively, for 24 hours. Then aliquots of these broths were streaked onto chromogenic selective media plates containing Xylose Lysine Deoxycholate (XLD) agar and RAPID *Salmonella* agar with incubation at 37 °C for 24 hours. Presumptive colonies would then be confirmed by biochemical tests including Triple Sugar Iron (TSI), urease presence, lysin decarboxylation, O-Nitrophenyl-β-D-galactopyranoside (ONPG) test for β-galactosidase presence, as well as serological tests, namely antigen agglutination tests.

- Detection of enteropathogenic *Vibro* spp.

Pre-enrichments were prepared in Alkaline Saline Peptone Water (ASPW) and incubated at 41.5 °C for 18 hours and second incubation at 37 °C also for 18 hours. The enrichment would then inoculate Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar plates. Presumptive colonies would be confirmed by: Gram coloration, fresh exam, TSI, Indole production and by Analytical Profile Index (API) 20NE test for identification of Gram negative non-*Enterobacteriaceae*.

- Enumeration of *Bacillus cereus*

Sample suspension and decimal dilutions were inoculated on *Bacillus cereus* Agar (BCA) by the spread plate method and incubation at 37 °C for 48 hours followed by CFU count. Confirmation was then performed by isolation onto 5% Sheep's Blood agar for haemolysis detection.

- Enumeration of Psychrotrophic microorganisms.

Main suspension and decimal dilutions were inoculated on plates with PCA medium by the spread plate method. Plates were then incubated in a refrigerator at 6.5 °C during 10 days after which CFU count was executed.

Membrane filter method for liquid samples - When analysing water or wine samples, the membrane filter method was preferred. For this method a filter unit consisting of a filter holder, filter funnel and suction flask as well as vacuum was used. A membrane filter would be placed on the filter holder with the use of flame sterilized forceps. The sample volumes or decimal dilutions were poured into the filter funnel and transferred into the suction flask by applying a vacuum.

Membrane filters with pore sizes of 0.45 µm were used since they allowed bacterial cell retention besides other microorganisms (yeasts and moulds). The microorganisms would be retained on the filter surface and concentrated from the filtered volume after which the filter was placed on a suitable culture medium and incubated, followed by subsequent CFU count. Before, between and after sample filtrations, the filter unit would be sterilized by burning off alcohol in the filter funnel and all membrane filters were held using the sterilized forceps (Just & Regnery, 2008).

In CINATE, a three-branch manifold filter unit was employed, allowing for simultaneous filtering of three sample/dilutions, similarly to the system in Figure 3.

Figure 3 - Three branched manifold membrane filter system used for waters and wines. Image adapted from Just & Regenery (2008).

In CINATE, all types of water samples were analysed, including drinking water, domestic use water, as well as waters used in food production processes. Analysed microbiological parameters were:

- Enumeration of culturable microorganisms at 22 °C and 37 °C

One millilitre of water was pipetted from the sample and inoculated into two petri dishes containing Yeast Extract (YE) agar according to the pour plate method, with each dish incubated in different conditions, 22 °C for 72 hours and 37 °C for 48 hours. Results would be the average CFU count of each plate.

- Detection of *Salmonella* spp.

Membrane filter method was used for the filtration of 1000 mL of sample and the membrane would be placed into a sterile bag containing BPW which was incubated at 37 °C for 24 hours for the normal procedure for *Salmonella* detection to be continued.

- Enumeration of total coliforms and *E. coli*

Membrane filter method was used for filtration of 100 mL and the membrane was placed onto Chromogenic Coliform Agar (CCA) and incubated at 37 °C for 24 hours with subsequent CFU count of coliforms and *E. coli* separately.

- Enumeration of *E. coli*

Membrane filter method was used for filtration of 100 mL and the membrane was placed onto CCA and incubated at 44 °C for 24 hours with subsequent CFU count.

- Enumeration of intestinal Enterococci

Membrane filter method was used for filtration of 100 mL and the membrane placed on to Slanetz and Bartley (S&B) agar and incubation at 37 °C for 44 hours. Presumptive CFU were quantified and the membrane transferred onto another medium, Bile Esculin Azide Agar (BEAA) for confirmation.

- Enumeration of Staphylococci

Membrane filter method was used for filtration of 100 mL and the membrane placed on to Mannitol Salt Agar (MSA) and incubated at 37 °C for 48 hours. Following confirmation would be necessary, that is, Gram staining, coagulase and catalase testing.

- Enumeration of *Pseudomonas aeruginosa*

Membrane filter method was used for filtration of 100 mL and the membrane placed on to Pseudomonas agar with cetrimide and nalidixic acid (Pseudomonas CN). Presumptive colonies were confirmed with oxidase test.

- Enumeration of *Clostridium perfringens*

Membrane filter method was used for filtration of 100 mL samples and the membrane placed on to Tryptose Sulfite Cycloserine (TSC) agar with incubation at 44 °C for 24 hours and under anaerobic conditions. Colonies were then confirmed by inoculation of Lactose Broth, of Nitrate media and by mobility test.

Wines

Wines and other fermented alcoholic beverages were analysed for:

- Enumeration of microorganisms- Colony Count at 30 °C

Membrane filter method was used for filtration of 100, 10, 1 and 0.1 mL. Obtained membranes were inoculated onto Yeast and Mould extract (YM) agar and without additives at 30 °C for 72 hours.

- Enumeration of Yeasts and Moulds at 25 °C

Membrane filter method was used for filtration of 100, 10, 1 and 0.1 mL. Obtained membranes were inoculated into YM agar with a chloramphenicol (antibiotic) additive with incubation at 25 °C for five days.

- Enumeration of Lactic Acid Bacteria and Acetic Acid Bacteria

Membrane filter method was used for filtration of 100, 10, 1 and 0.1 mL and membranes prepared and incubated on De Man, Rogosa and Sharpe (MRS) agar with cycloheximide (anti-fungal) additive.

- Enumeration of *Brettanomyces*

Membrane filter method was used for filtration of 100, 10, 1 and 0.1 mL and filters prepared and incubated on Dekkera/Brettanomyces Differential Medium (DBDM) agar.

Environmental sampling - CINATE executed environmental sampling for the monitoring of a diverse number of locations along the food production chain including food-contact surfaces and food-handlers' hands. Most samples would be obtained using cotton swabs with applicator sticks such as those represented in Figure 4. The sampler at location would swab the determined surface and then place the swab in a tube containing sterile buffer solution. The inoculated solution would then be analysed for the specified parameters below. Before sample aliquots were transferred from the tubes, they were vortexed for approximately 30 s to allow maximum transfer of microorganisms from the swab to the diluent in the tube. Many of the microbiological groups evaluated in foodstuffs were also evaluated in the surface swab samples, with the inoculated diluent being considered the main suspension from which decimal dilutions were prepared and plated. Following some resumed procedures for swab samples are presented:

- Total Viable Count at 30 °C

The initial sample and decimal dilutions were inoculated on petri dishes with PCA medium by the pour plate method, followed by incubation at 30 °C for 72 hours and posterior CFU count.

- Detection of total coliforms/faecal coliforms and *E. coli*

An aliquot of 1mL of the suspension would be transferred from the swab tube into a tube containing the liquid culture medium, Lactose Broth and a Durham tube which would be incubated in a water bath at 30 °C for 48 hours. After this, if growth was detected in the tube (gas production detected in the Durham tube and broth turbidity), a 0.1 mL aliquot of the Lactose Broth was transferred to tube containing another culture medium, Brilliant Green Broth and incubated in the water bath at 30 °C for 48 hours. If growth was also detected in the Brilliant Green Broth, coliforms were present in the sample.

If for the same sample, it was necessary the detection of thermotolerant coliforms, a 0.1 mL aliquot of the Lactose Broth with positive result would be transferred to tube containing another culture medium, Brilliant Green Broth and incubated in the water bath at 44 °C for 48 hours. If growth was also detected in the Brilliant Green Broth, this indicated faecal coliforms were present in the sample.

For the detection of *E. coli*, a similar procedure would be executed, except, 0.1mL aliquots would be transferred to a tube with Brilliant Green Broth as well as another tube containing Peptone Water. These would be incubated in a water bath at 44.5 °C for 48 hours and then the Brilliant Green broth was evaluated for cell growth while a few drops of Erlich-Kovacs reagent were added to the Peptone water tube to execute the Indole test. If Brilliant Green tube had cell growth and the Indole test was positive (red ring at the surface of peptone water broth, sign of tryptophan breakdown and subsequent Indole presence), *E. coli* would be present in the sample.

- Detection of coagulase positive *Staphylococcus*

An aliquot of 1mL of the swab sample would be transferred to a tube containing Chapman liquid medium for a selective pre-enrichment phase. The tube would be incubated at 37 °C for 48 hours. With a loop, BPA agar plates were streaked with an inoculant taken from incubated Chapman broth. This procedure was executed after 24 and 48 hours of Chapman incubation. BPA plates were

incubated at 37 °C for 48 hours. Characteristic and non-characteristic *S. aureus* colonies would then be transferred into tubes containing BHI, followed by another incubation for posterior Coagulase test. If a positive result was obtained, coagulase positive Staphylococcus aureus were present in the sample.

For the detection of *Listeria* spp./L. monocytogenes and *Salmonella* spp. the procedures are the same to the stated previously for food products, however, in this case, the sample is a swab.

As mentioned before, other methods of environmental sampling executed in CINATE included the use of RODAC plates (Figure 5) and Dip slides (Figure 6).

(RODAC) plates are pre-prepared plastic plates containing specific solidified media for a determined microbiological group and used for hygiene monitoring of surfaces. These plates have the agar medium projecting over the opened plate, allowing a rapid contact of the medium when pushed onto the surface to be analysed. This contact is maintained for 10 seconds and then the cap is placed again on top of the plate (Steris Laboratories, n.d.). The plate area is normally 25 cm^2.

Dip slides contain paddles with solidified agar medium on each side of said paddle, which is kept in a tube, as Figure 6 shows. To analyse a surface, the paddle is removed from the tube, followed by the pressing of the agar medium of either side onto the analysed surface. The inoculated dip slide is then placed inside the tube again and transported to the lab. If each paddle side contained different culture media, two parameters could be simultaneously evaluated. Each side of the dip slide would have an area of approximately 9 cm^2 (Merck, n.d.).

At arrival to the lab, the inoculated contact plates and slides would be immediately incubated at the prescribed time according to the evaluated microbiological groups, (normally hygiene indicators such as Total Viable Count (TVC) at 30 °C, *Enterobacteriaceae*, coliforms and *E. coli*) followed by CFU count.

Additionally, CINATE executed pathogen detection using a VIDAS (bioMérieux, n.d.) equipment based on an automated version of an immuno-assay technique, ELFA, in which the presence of the pathogen was detected by a fluorescent signal which was emitted when an enzyme linked to antibodies which in turn were bound to the pathogen and degraded a fluorescence-emitting substrate. If the sample didn't emit a signal, the pathogen wasn't present. However, to confirm the presence of the pathogen, traditional confirmation methods would then be used. The main pathogens analysed in VIDAS in CINATE were *Listeria* spp. and *L. monocytogenes* as well as *Salmonella* spp., the latter of which I was able to observe during my internship and for which the following procedure was executed.

Figure 4 - Swab stick with accompanying tube with sample diluent. Retrieved from: http://www.foodtest.co.uk/environmental-swab-microbiology-testing.asp

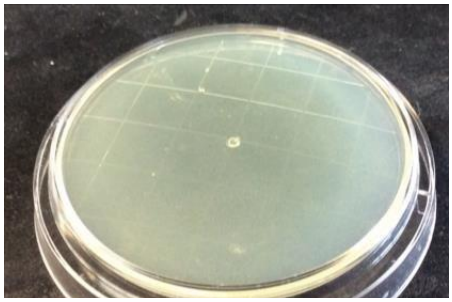

Figure 5 - RODAC- Replicate Organism Direct Agar Contact plate. Retrieved from: https://www.researchgate.net/figure/RODAC-Plate_fig4_325616125

Figure 6 - Dip slide test kits. Retrieved from http://www.merckmillipore.com

- VIDAS *Salmonella*

For the sample detection for *Salmonella* spp. by VIDAS, pre-enrichment and selective enrichment of the normal *Salmonella* spp. detection is followed (pre-enrichment in 1:10 suspension of BPW and subsequent selective enrichment in RVS and MKTTn. This is followed by an additional step of transferring 0.1mL of MKTTn culture into a tube with Mannose Broth (M-Broth) and transferring of 1 mL of RVS culture in another M-Broth tube, both of which then incubated at 41.5 °C for 24 hours.

After this process, 0.25 mL aliquots of each broth tube are heated in the VIDAS Heat and Go program, at 131 °C for 15 mins and left to cool. This final heated sample would then be loaded onto the VIDAS and analysed. If the sample obtained a low fluorescent signal, *Salmonella* would be absent in the sample. If a higher fluorescent signal were obtained, it wouldn't be necessarily indicative of *Salmonella* presence and further confirmation was necessary therefore, the previous MKKTn and RVS cultures would be streaked onto XLD and RAPID *Salmonella* agar, and confirmation tests executed if presumptive colonies appeared in the chromogenic selective media.

3.5. Cleaning Procedures/Hygiene Control

It was laboratory personnel's responsibility, including mine, to clean work surfaces, equipment surfaces and management of contaminated material.

3.5.1. Surface Hygiene

On the daily basis, before and after use, work surfaces were sanitized using a 20% diluted sodium hypochlorite solution or a solution of 70% (v/v) ethanol and a clean damp cloth. Weekly sanitation was performed and consisted of a more thorough procedure in which all the surfaces of the laboratory rooms would be cleaned excluding the floor. Unlike the daily sanitization, the weekly sanitization was kept record and included identification of the person cleaning, which rooms were cleaned and the date in which the procedure occurred.

3.5.2. Hygiene of containers used for sample transportation

After sample reception the used cooler-boxes in which they were transported from the collection point, were washed with dish soap and water, followed by a wipe down with a cloth and a diluted bleach solution. Sanitization was recorded with a sticker on the box with the person who cleaned and the date. Weekly, a container would be chosen arbitrarily, and the surface control was performed with a RODAC plate with PCA medium for TVC at 30 °C. Analysed carriers would be selected rotationally to allow testing of all of CINATE's containers. After incubation, surface hygiene results were considered satisfactory if CFU count was below 100 on the analysed surface.

3.5.3. Air Quality Control

On a weekly basis, air quality control was performed and in which four parameters were detected and quantified: TVC at 30 °C, *Enterobacteriaceae*, Yeasts and Moulds and coagulase-positive *S. aureus*. Plates with appropriate culture media for each of the four parameters were placed and left open in each of the rooms of the laboratory which were kept shut from each other for 15 minutes. After this, the plates would be closed and recovered, in the same order in which they were placed (from room A to G) followed by incubation according to the evaluated parameter. This activity would then be registered in the laboratory activity journal, as well as the results in a specific document for this analysis. Different rooms had different limit values as Table 3 demonstrates.

Table 3- Maximum limit of CFU count for environmental exam parameters according to room and installation tested.

Different installations of the Microbiology Laboratory of CINATE	Total Plate Count at 30°C Yeasts and Moulds (CFU/plate)	*Enterobacteriaceae* *S. aureus* (CFU/plate)
A – Sample reception room; B - sample preparation room; C - Main laboratory area; D - medium preparation room; E – pathogen assay room; G – microscopy room	15	0
F – cleaning/decontamination room	30	0
Incubators	10	0

3.5.4. Decontamination and Waste Management

Contaminated material, including contaminated Petri-dishes, used test tubes, used food samples and swabs were placed in an autoclave appropriate plastic bag and decontaminated at 121 °C for 30 minutes in the autoclave found in the cleaning room of the laboratory. All reusable material, including test tubes and Schott glass bottles were then washed in a dish washer in the same room.

Glass pipettes were cleaned in the sink with water and detergent after being kept in a 2-litre burette containing a diluted solution of bleach with detergent in the main laboratory area following its use in assay procedures. All laboratory rooms also had containers with the same solution, but in which used micropipette tips and used microscope slides were placed. This material would then be disposed of in the waste bins along with the non-reusable decontaminated material. The waste bags were then finally collected by university cleaning staff daily.

3.6. Internal Quality Control

In CINATE, all aspects of laboratory activity were regularly verified, with varying frequencies according to the maintenance plan and in order to maintain internal quality control, which therefore assured the reliability of the laboratory activity. Such controlled aspects included the assurance of correct:

- Sample transportation and reception - temperature and microbiological control in fridges and carriers.
- Laboratory equipment maintenance – temperature control in refrigerators, incubators and water baths with additional microbiological quality of incubator interiors. Verification and reproducibility of measurements made on scales, gravimetric diluters, pH meter, VIDAS equipment, autoclave, thermometers and also the pipettes.

- Culture media preparation – pH levels and microbiological quality of water used for media preparation, culture media pH levels, as well as positive and sterility controls. Culture media performance, stock and validity were also verified.
- Air and work surface quality – microbiological quality in the different lab sectors (A, B, C, D, E, F, G).
- Assay Results – determination of reproducibility of results in Interlaboratory assays and repeatability of results in Duplicate assays.

Some of the internal quality assurance procedures had been specified in the previous sections, however, other procedures, of which I also took part in during the internship are explained in more detail below.

3.6.1. Water Quality Control

The purified water used for culture media preparation was also subjected to regular testing. Once a month, total microorganism growth was evaluated in aliquots of 0.1mL and 1mL of the water on YE agar, by the pour plate technique, following incubation at 22 °C for 72 hours with subsequent CFU count. Results would be considered satisfactory according to levels determined by the lab's criteria.

3.6.2. Temperature Verification

Another function in the laboratory was the daily verification of the internal temperatures of the working incubators and refrigerators used in the laboratory. All the equipment contained sensors which constantly registered the temperature, which would be shown on a program in the computer of the main laboratory room in which most of the equipment was also located apart from one refrigerator maintained out of the laboratory due to its ample dimensions. The verification was then registered in an appropriate document. The temperature of each equipment was considered acceptable if included in the established ranges of temperature.

Water baths also had temperature verification, however, with the temperature measurement using a standard glass thermometer and only during assays when said baths were utilized.

This temperature analysis was important, since it could indicate equipment failure, when temperature measurement was outside the established range. This would then affect the assay results. In these situations, the laboratory would then implement corrective measures to reset the correct functioning of the equipment. All the assays would need to be analysed and, if necessary, repeated to assure result reliability.

3.6.3. Micropipette Verification

To evaluate the graduated micropipettes' accuracy, that is, to check if they delivered correct volumes, these would be verified on a trimestral basis. This would consist basically of measuring a water mass in an analytical scale, accompanied by measurement of water and ambient temperature with a glass thermometer and measurement of atmospheric pressure with a barometer. Micropipettes would be adjusted for the wanted volume, a beaker would be placed on the analytical scale which would then be tarred. The micropipette would then collect the wanted volume of water from a beaker outside the scale and pour the volume into the beaker on the scale. The resulting mass of the water would subsequently be documented, and this measurement

process would be repeated to obtain a total of 10 measurements. Water and ambient temperature as well as atmospheric pressure were measured before measuring water mass and after all the other 9 repetitions. These values were later used in an Excel spreadsheet in order to obtain the conversion value Z(µL/mg) which was used to convert the water mass values into volume. Along with volume values, the spreadsheet also calculated average water volume, accuracy and precision. Lab standards considered acceptable all micropipettes whose relative error and precision were equal to or below 5%. If the values deviated significantly from expected, verification was repeated and if then the results continued abnormal, calibration from manufacturer was required.

3.6.4. Interlaboratory Testing

In the case of the interlaboratory testing, the laboratory would receive samples from which technicians would analyse them for many different microbiological parameters. When enough sample amount was sent, it was advised that more than one technician analyse the sample. A report containing information about the technicians' name, performed assays and respective results would be submitted to the organisations, such as Public Health England which coordinate this testing. This testing would allow to evaluate the performance of the laboratory as well as determination any systematic error. If the obtained results were different than the expected, a non-conformity would be defined and registered for the development of corrective measures to be applied. During the internship, I participated in an interlaboratory testing exercise for samples contaminated with *Campylobacter.* spp.

3.6.5. Duplicate Assays

These assays were performed preferentially in naturally contaminated samples and consisted in preparing 2 plates for each of the sample dilutions and from which obtained results would be used for the definition of the laboratory's precision criteria, which took into consideration the average difference of results between duplicates and defined the maximum acceptable control limit.

Repeatability of testing would then be evaluated by introducing CFU counts of at least 15 samples into a spreadsheet which would indicate if the values were contained in the acceptable range of results, that is , if the duplicate assay's results weren't too varied If not, the lab technician who performed the assay had to evaluate the results of other samples analysed on the same day as the other and repeat duplicate sampling. If again the results were unacceptable, a non-conformity would be confirmed and registered for the application of corrective measures.

4. Lunch box Project

4.1. Introduction

A lunch box/lunch bag can be defined as a reusable container that is used to transport food products (Cambridge Dictionary, n.d.). Nowadays, an enormous variety of lunch bags are available on the market, being made from diverse materials and with all types of shapes and styles. These bags include rigid plastic boxes, metal boxes, thermos containers, neoprene (wet-suit material) bags and the more common insulated bag, among many others, as a quick web browsing can corroborate (Lunchbox.com., n.d.).

The habit of lunch bag use is normally associated to school-aged children but also with the working class. Furthermore, in recent years, an increased number of the latter group have been following the trend of meal-prepping (Shamsian & Dreyfuss, 2016) which is the planning and preparation of meals ahead of time, normally for consumption along a weekly period. This habit is said to be quite beneficial since it allows for timesaving and money-saving as well as being an important tool for portion-size control and the promotion of a nutritionally balanced diet as is confirmed by Mills *et al.* (2017). Since the consumers are eating more home cooked meals as opposed to take away/fast-food options (Harvard T.H. Chan, 2017) from the increase of this practice, it is obvious that more and more lunch bags would be used besides the frequent users who normally prepare their foods on a regular basis as opposed to just one day a week. Further confirmation of the rise of lunch bag use can be supported by what is stated by Cooke, K. (2018) who evidenced that in 2017, in the United Kingdom, there was a notable increase of consumption of packed lunches, namely 65 million, compared to the preceding year.

The importance of correct lunch bag use has been evidenced by public health/food safety agencies around the world including the United States (Food Safety Inspection Service- FSIS, United States Department of Agriculture -USDA, 2016) as well as the United Kingdom and Republic of Ireland (*Safe*food, 2018) and Australia (New South Wales (NSW) Food Authority, 2015a). These entities present indications for correct lunch bag use in order to maintain the food safety of the carried products and the health of their users. These are mostly directed towards children but are applicable to all lunch bag users. Such measures include correct hygiene and preparation of foods before transportation and then the use of insulated lunch boxes containing icepacks/frozen water bottles or thermos containers to prevent cold and hot foods, respectively, from reaching the "danger zone" established as a temperature interval (4,4 - 60,0) ℃ in which microorganisms proliferate and multiply exponentially (FSIS/USDA, 2016). Additionally, responsible hygiene practices including cleaning the lunch box daily with hot soapy water as well as sanitation with disinfectants such as diluted solution of chlorine bleach would also be recommended. Some of these measures can be observed in Figure 7.

If these aforementioned food safety measures aren't followed, there can be increased risk of illness since incorrect food preparation, storage and bag cleaning, as well as uncontrolled temperature variations can occur and provoke increased microbial contamination of foods with possible growth and proliferation of foodborne pathogens in the transported products. Such food related microorganisms could include bacteria such as *Listeria monocytogenes*, *Salmonella* spp., or *Campylobacter* spp. among others (FDA, 2012).

According to the European Food Safety Agency (EFSA, 2017) almost 40% of food-borne outbreaks in 2016 in the European Union (EU) were of domestic origin. This most likely resulted from improper food handling in the domestic environment in which many behaviours like poor hygiene and incorrect storage of food allow for microbial growth, including proliferation of food pathogens in foods and on surfaces due to cross-contamination (Fischer *et al.,* 2007; Azevedo *et al.*, 2014). Therefore, since microbial contamination and possible food pathogens may be found on surfaces of the domestic environment, including kitchens in which lunch bags are normally prepared, it was questioned if the bags' interior surfaces could also influence consumer's food safety by possibly retaining pathogenic bacteria. Scarce literature can be found in which food safety and microbiological analysis of lunch boxes are performed and the literature that exists is mainly aimed at the younger, more vulnerable populations as was evidenced in Hudson & Walley (2009) in which lunch bags of school-aged children in the UK were analysed.

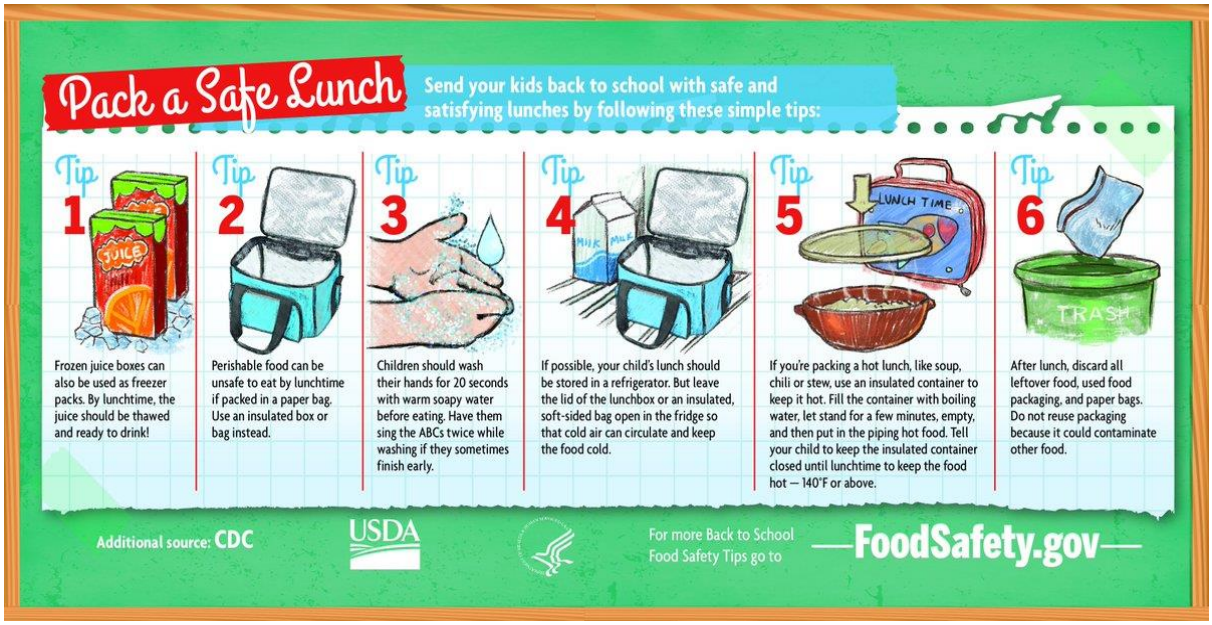

Figure 7. Poster with food safety advice for packing lunch boxes, recommended by the United States Department of Agriculture (USDA). Retrieved from FSIS/USDA (2013).

Thus, with the increased popularity and use of lunch bags on behalf of adults, this study was aimed to evaluate food safety habits regarding adults' use of lunch boxes in a sample of the Portuguese population and research if lunch boxes could be considered vectors for the transmission of foodborne pathogens. This study was integrated into the SafeConsume project which collaborates with many European countries and receives funding from the European Union's (EU) Horizon 2020 programme. This project was developed to reduce the health burden of food illness by investing in research and education initiatives to improve food safety in the E.U. (SafeConsume, n.d.).

To reach the study goals, the following objectives were established:

- Application of an online survey to determine the profile of food hygiene and safety habits of lunch box use in a sample of the Portuguese population.

- Sample lunch box interior surfaces for the detection and count of microbiological hygiene indicators (TVC at 30 °C, *Enterobacteriaceae*, *E. coli*) as well as the detection of important foodborne pathogens (*Listeria monocytogenes* and *Salmonella* spp.)
- Correlate microbiological results with lunch box hygiene and food safety information obtained from in-person surveys of the volunteers whose lunch boxes were sampled.

4.2. Materials and Methods

4.2.1. Online Survey

To understand the habits and food safety practices of lunch box use in the Portuguese population, an online survey was developed. Participants were recruited via social media publications and official university emails of the Aveiro University and of ESB, providing details about the study as well as a link to the online survey in their messages. This questionnaire (additional file) was created using the Google Forms application, being developed to take only a few minutes to answer (5-10 minutes) and include various questions organised in the following manner: 1) sociodemographic information questions; 2) twenty-four questions relating to lunch bag characteristics and habits of use; 3) four questions about associated cleaning habits; 4) two questions about recent gastrointestinal illness.

Following response to sociodemographic questions, participants completed the second part of the survey, in which they were questioned about lunch box use, frequency of use, the type of lunch bag, storage recommendations of foods in them, existence of different compartments and price of their bags. In this section they were also questioned about duration of use, which products were transported, who prepared the bags, time the food was normally stored, use of ice packs and finally if food was kept in direct contact with the bags' interior surfaces or if any waste was kept in them.

The third sector focused on the sanitation conditions of lunch boxes, questioning the respondents' preferred cleaning procedures as well as their frequency. In this sector, it was also inquired if the lunch box was ever in contact with the floor surface during its use.

The last portion of the survey was created to question about any recent gastrointestinal problems the volunteers may had suffered, and which could possibly have been result of incorrect storage of food and sanitation of their respective lunch boxes.

The survey's questions consisted mostly of yes/no and multiple-choice questions, many of which followed by another question to which the respondent would justify their answers, or indicate another option not contained in the multiple-choice. The online questionnaire can be viewed in Annex 1, having been written in Portuguese since it was developed for the inquiry of the Portuguese population.

4.2.2. Lunch box Sampling

In parallel with the online survey, determination of the microbiological quality of lunch boxes was conducted. In this phase of the study, participants were recruited from ESB community. Sampling was preferably made in the school's canteen, breakrooms and offices, during the lunch period, normally between 12:45-14:00 h due to the high affluence of people with lunch boxes under these conditions.

The volunteers willing to participate in the study were asked to remove all contents of their lunch boxes to proceed to the swabbing which was performed according to International Standard Organization (ISO) 18593:2004(E) methodology. Cellulose sponge-stick swabs (7.6 by 7.6 by 3.8 cm; 3M, USA) (Figure 8) pre-moistened with 4 mL of sterile Ringer's solution (Oxoid, UK) were used. The sampler swabbed a 100 cm^2 area of the interior surface of the lunch box, with the aid of a sterilized template with the bottom surface being the preferred for analysis since it was where possible food residue could accumulate more. The sampler made sure to use both sides of the sponge, breaking it off from the stick after sampling, to then be stored inside the sample bag which was then sealed. The bags were subsequently placed in a refrigerator a 4 °C, to be analysed within the hour.

While swabbing occurred, the volunteers were also asked to complete an in-person version of the online survey. This particular survey also included an item that wasn't mentioned in the online survey: "How is your cutlery kept inside the lunch box?". If interested in knowing their results, the volunteers were also asked to provide their email addresses. Following sampling, each sponge was aseptically immersed in 10 mL of Buffered Peptone Water (BPW; Bio-Rad, USA) poured into each sample bag, followed by homogenization in a sample blender (Smasher, AES; bioMérieux, France) for approximately 30 seconds. The sample volume was then divided for microbiological testing.

Figure 8. Photo of an example of the Sponge stick kit used in this study for lunchbox sampling. Retrieved from: https://www.3m.com/3M/en_US/company-us/all-3m-products/~/SPGESTK-3M-Sponge-Sticks/?N=5002385+3293785595&rt=rud

4.2.3. Microbiological Analysis

All samples were evaluated for the presence and quantification of three hygiene indicators: Total Viable Count (TVC) at 30 °C, *Enterobacteriaceae* and *E. coli* according to ISO 4833-1:2013, ISO 21528-2:2004 and ISO 16649-2:2001 methodology, respectively. The samples were also evaluated for the presence of two bacterial pathogens: *Listeria monocytogenes* according to ISO 11290-1:2017 and *Salmonella* spp. according to ISO 6579-1:2017. All ISO methodologies were the same used in the Microbiology Lab of CINATE.

Thus, decimal dilutions were then prepared from the obtained samples and used in the pour-plate method using Plate Count Agar (PCA; bioMérieux, France; Liofilchem, Italy), Violet Red Bile Dextrose Agar (VRBD; VWR, Germany) and Tryptone Bile X-Glucuronide Agar (TBX; Biokar, France) growth media for enumeration of TVC at 30 °C, *Enterobacteriaceae* and *E. coli*, respectively. For enumeration of *Enterobacteriaceae*, characteristic colonies in VRBD underwent further confirmation (Oxidase test (oxidase test stick (Liofilchem, Italy), and glucose fermentation (OF) (Liofilchem, Italy). In the procedure for detection of *Salmonella* spp., the sample was incubated at 37 °C for 24 hours, followed by enrichment in Muller Kauffmann Tetrathionate Broth (MKTTn, bioMérieux, France) (37 °C/24h) and Rappaport-Vassiliadis Soya Peptone Broth (RVS, bioMérieux, France) (41.5 °C/24h) and then streaking in two different selective media: Xylose Lysine Deoxycholate (XLD, VWR, Germany) agar and RAPID Salmonella (Bio-Rad; USA) from which characteristic colonies, when existent, would then be confirmed through biochemical confirmation methods according to the standards procedure. For *L. monocytogenes* detection, sample aliquots underwent enrichment in Half-Fraser Broth (bioMérieux, France) (30 °C/24 h) followed by streaking in selective media ALOA (bioMérieux, France) or PALCAM (VWR, Germany) (37 °C/24+24 h) from which presumptive colonies would be confirmed accordingly.

The results for enumeration parameters were presented as CFU per 100 cm^2 of the analysed lunch bag surface. Since there aren't any established microbiological criteria for lunch bag hygiene, the results from lunch bag surfaces were thus classified according to guidelines for food contact surfaces found in a compilation of microbiological food standards from the Basque Health Department - Spain (Moragas *et al.,* 2019). Lunch bags' hygiene were thusly categorized as excellent, good, unclean or very unclean if values for TVC at 30 °C were as follows: ≤ 100 CFU/100 cm^2; 100 - 1000 CFU/100 cm^2; 1000–10000 CFU/100 cm^2 or >10000 CFU/100 cm^2, respectively. From this document, it was also established that if *Enterobacteriaceae* count was ≤ 200 CFU/100 cm^2, the surface was considered satisfactory.

4.2.4. Statistical Analysis

Statistical Analysis was conducted using SPSS software (PASW Statistics 25.0; IBM SPSS, Armonk, NY) with the execution of Pearson's Chi-square tests to determine independence of all categorical variables. The Fisher Exact and Likelihood Tests were also executed in specific situations in the cross-tabulations. These were respectively, when an expected value was less than 10 and when more than 20% of expected values were under 5. Additional calculation of odds ratios (OR) for the determination of associations' strength were executed, when possible, for comparison of dichotomous variables. For all tests, *P*-value ≤ 0.05 was established to determine statistical significance. Demographic information (gender, education and age group) was compared with the online survey answers of some of the most relevant questions which inquired food safety behaviours. For microbiological data analysis, corresponding hygiene status was used with further dichotomisation of categories into "clean" or "unclean" relating to TVC at 30 °C. These results were then analysed for association with demographic information as well as survey items of the in-person survey considered most relevant to food safety behaviours.

4.3. Results

4.3.1. Online Survey – Population Tendencies

From March to October 2018 a total of 247 responses to the online survey were obtained, of which only 239 were considered since 8 respondents did not use lunch bags or prepare them for others, thus being excluded from analysis. Table 4 summarizes sociodemographic characteristics of the studied population (n=239). Participants had ages between 17 and 63 years, resulting in average age of 31.7 ± 10.6. Furthermore, the majority of participants resided in the Northern region of Portugal (Nomenclature of Territorial Units for Statistics - NUTS II region) (66.9%), were female (79.9%), with age lower than 26 years (40.2%), were single (58.6%) and without children (67.8%). Concerning education levels, 84.9% (203) of participants had either completed or were enrolled in post-secondary courses, including Higher Education courses (undergrad, masters, PhD and post-graduate courses). Most participants were employed (54.8%), working in varied job sectors including health related professions (nurses, nutritionists, pharmacists), academic professions (professors and grant researchers) as well as others such as personal trainers, shop workers and drivers. This information wasn't analysed due to the immense diversity of answers which were obtained. Another significant part of the survey sample were students (37.2%). Almost all volunteers answered to the question concerning their financial stability, from which it was ascertained that with their current income, most participants had acceptable (45.2%) or even comfortable living conditions (36.8%).

Table 4 - General socio-demographic information of respondents to the online survey

		Nr	Frequency (%)
Gender			
	Female	191	79.9%
	Male	48	20.1%
Age group			
	[16-25]	96	40.2%
	[26-35]	61	25.5%
	[36-45]	56	23.4%
	[46-55]	19	7.9%
	[56-55]	7	2.9%
	Average ±standard deviation 31.7 ± 10.5		
	Range: 17-63		
Marital Status			
	Married	85	35.6%
	Divorced	14	5.9%
	Single	140	58.6%

		Nr	Frequency (%)
(Table 4 continued.)			
Work Situation			
	Employed/Self-employed	131	54.8%
	Student	89	37.2%
	Student worker	13	5.4%
	Unemployed	6	2.5%
Region of Residence			
	Alentejo	1	0.4%
	Algarve	2	0.8%
	Centre region	63	26.4%
	Lisboa region	12	5.0%
	Northern region	160	66.9%
	out of the country (France)	1	0.4%
Education Level			
	University Education	203	84.9%
	Highschool or less	36	15.1%
Children			
	Yes	77	32.2%
	1	28	11.7%
	2	44	18.4%
	3	4	1.7%
	undetermined	1	0.4%
	No	162	67.8%
Financial Stability			
	Confortable living conditions	88	36.8%
	Acceptable living conditions	108	45.2%
	Some difficulty	33	13.8%
	Strong difficulties	7	2.9%
	unanswered	3	1.3%
Total of Respondents		239	100.0%

As shown in Table 5, all participants confirmed either using a lunch bag (97.1%) or preparing them for others despite not using one themselves (2.9%). This last group was included in the 23.8% (57) of respondents who indicated preparing foods for others, namely, their children, partners and other family members. According to survey results, lunch bags were used mostly daily (68.2%), twice a

week (12.6%) or during special occasions (14.2%) including field trips, picnics or other recreational outings. Respondents widely preferred common insulated thermal bags (62.3%), plastic boxes (23.8%) and neoprene bags (10.9%) with the majority of them (68.2%) confirming that their bag had insulating interior lining, with the remaining stating they were unsure (8.8%) or knew that theirs lacked insulating lining (22.6%). Seventy-three percent of respondents (72.8%) denied and 23.9% were uncertain of the existence of any type of temperature or time guidelines for the lunch bag. Only 3 (1.3%) respondents confirmed having guidelines, of which only 2 presented indications, specifying that their bags could maintain the internal temperature for 2 hours or had to be kept at -20 or 4 °C. Further specifying lunch box characteristics, 90.4% (216) of the volunteers' bags had only one compartment, contrasting with the minority (9.2%) who indicated having bags with extra compartments which were used mostly to separate the main meals from fruits, salads, soups or drinks or to separate breakfast from lunch.

According to the participants, prices varied extremely, from 0 up to 100 € with some participants being unaware of price (5.9%). Despite the significant price range, participants spent mostly between 5 and 10 € (33.1%).

Table 5 – Responses from the online survey for lunch box characteristics and use

	Nr	Frequency (%)
Lunch bag use		
Yes	232	97.1%
No (but prepares for others)	7	2.9%
Preparation of other bags besides own		
Yes	182	76.2%
No	57	23.8%
Frquency of Use		
Daily	163	68.2%
2x week	30	12.6%
1x week	10	4.2%
Special Occasion	34	14.2%
unanswered	2	0.8%
Material of Lunchbag		
Thermal insulated	149	62.3%
Neoprene bag	26	10.9%
Plastic box	57	23.8%
Metalic box	2	0.8%
Various	4	1.7%
unanswered	1	0.4%

	Nr	Frequency (%)
(Table 5 continued)		
Insulating lining		
Yes	163	68.2%
No	54	22.6%
Unknown	21	8.8%
unanswered	1	0.4%
Conservation Indication		
Yes	3	1.3%
conserves temperature for 2 hours	1	0.4%
preserve at -20°C or 4°C	1	0.4%
undetermined	1	0.4%
No	174	72.8%
Unknown	57	23.8%
Unanswered	5	2.1%
Different Compartments		
Yes	22	9.2%
breakfast/lunch	1	0.4%
fruit or salad//meal	6	2.5%
beverages/meal	1	0.4%
soup/snack/meal	1	0.4%
doesn't use	2	0.8%
undetermined	11	4.6%
No	216	90.4%
Unanswered	1	0.4%
Price range (euros)		
[0,5]	67	28.0%
]5, 10]	79	33.1%
]10, 15]	42	17.6%
]15,20]	16	6.7%
]20, 25]	5	2.1%
]25, 30]	8	3.3%
>30	4	1.7%
Unknown	14	5.9%
unanswered/excluded	4	1.7%
Average ± Standard Deviation	10.1± 10.1	
Range (euros)	0 -100	
Total of Respondents	239	100.0%

Focusing on the duration of lunch bag use, as is shown in Table 6, only 216 out of 239 answers obtained were analysed as the remaining were too vague and inconclusive (“months”; ”years”). Lunch bag usage averaged at approximately 2.3 ± 2.8 years with a greatest part of respondents having used theirs for only 1 year or under (41.4%), while a few people (7.1%) had used theirs for over 5 years. This last group included participants who had used their bags for 20 years.

The vast majority of respondents (92.1%) confirmed that they prepared their bags themselves with 16 (6.7%) people having theirs prepared by a family member and 2 (0.8%) alternating preparation with the family members. The most common food items transported were cooked meals (87.0%), fruits (74.9%) as well as yoghurt or gelatine cups (56.1%) and beverages (44.8%) as Figure 9 evidences. Curiously, one person would also carry their medication in the lunch bag.

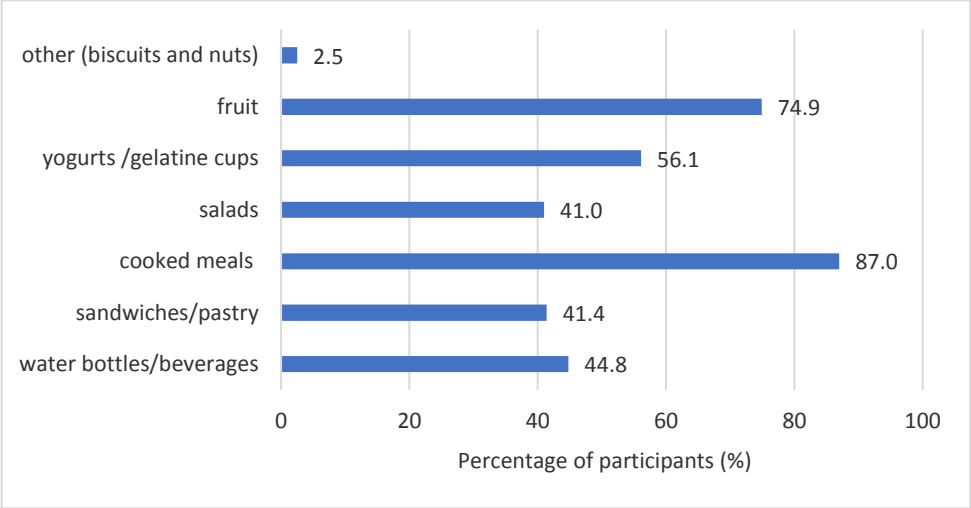

Figure 9 - Common food products transported in lunch boxes by respondents (online survey).

Relative to storage time, as Table 6 shows, it was found that food items were kept in their respective lunch bags during a vast range of hours, ranging from under an hour up to occasionally 24 hours. Of those whose food items were kept in the lunch bag for under an hour, two stated that their food was kept only for that short period of time since they would be placed in a refrigerator on arrival to work. Many people indicated time intervals when explaining the period of time their food items were stored as well as more than once, since people stored snacks longer than they did with main meals. Therefore, only mean values were used for the analysis. Two inconclusive answers were excluded (“Hours” and “Yes”). It was discovered that respondents stored their foods mostly more than 2 hours and up to 4 hours (36.4%) or between 4 and 6 hours (33.5%), averaging at 5.2 ± 3.6 h.

As mentioned before, many governmental public health agencies recommend the use of icepacks as an efficient way to preserve food products during their storage in lunch bags, however, results showed that the greater part of respondents, 190 (79.5%) did not use icepacks in their bags, with most of the remaining respondents using them only in specific occasions (12.6%) as opposed to regular use which was only the case for 18 people (7.5%) making a total of 48 (20.1%) users. The participants who used icepacks in specific occasions revealed using them in numerous different situations which were mainly, during the warmer seasons or when transporting a certain food product (mostly cold foods: salads, yoghurts, gelatine cups) and beverages as well as cooked meals and soups.

Icepack use was justified with numerous reasons including the objectives to maintain food products cold and preserved until reheating or immediate consumption and thus avoiding food spoilage. Additionally, three occasional users justified why they didn't use icepacks all the time, affirming that some foods didn't need refrigeration because they were eaten quickly or were canned and that during the colder seasons, the lunch bag would be adequately cold to preserve the food product until consumed (not shown in Table 6).

Table 6 - Responses from the online survey for further questions related to lunch box use

	Nr	**Frequency (%)**
Duration of use (years)		
[0; 1]	99	41.4%
]1; 2]	59	24.7%
]2; 3]	24	10.0%
]3; 4]	5	2.1%
]4; 5)	12	5.0%
>5	17	7.1%
unanswered/excluded	23	9.6%
Average ±Standard Deviation	2.3 ± 2.8	
Range	2 weeks - 20 years	
Who prepares the bag		
Self	220	92.1%
Family member	16	6.7%
Self+Family member	2	0.8%
unanswered	1	0.4%
Time food is kept in the bag (hours)		
[0; 2]	31	13.0%
]2; 4]	87	36.4%
]4; 6]	80	33.5%
]6; 8]	15	6.3%
]8;10]	3	1.3%
]10; 12]	12	5.0%
>12	8	3.3%
unanswered	3	1.3%
Average ±Standard Deviation	5.2 ± 3.6	
Range	<1 - 24	

		Nr	Frequency (%)
(Table 6 continued)			
Icepack use			
	Yes	18	7.5%
	In specific situations	30	12.6%
note: total sum of item more than Yes counts. Answers with >1 item	warmer weather	11	4.6%
	cooked meals and soup	8	3.3%
	cold foods and beverages (salad, yoghurt, gelatine, juices)	12	5.0%
	undetermined	5	2.1%
	No	190	79.5%
	Explanations for use (regular and occasional)		
note: total sum of item more than Yes counts. Answers with >1 item	prevent spoilage	9	3.8%
	maintain food fresh	16	6.7%
	maintain cold temperatures	7	2.9%
	excluded/unanswered	14	5.9%
Total of participants		239	100.0%

Still focusing on how food items were stored, when questioned if any food item was kept in direct contact with the bag's interior surface, 64.4% (154) of respondents confirmed this situation. Of those, all 136 people who specified what item, indicated storing fruits (apples, bananas, citrus fruits, pears) inside their lunch bags, with one person stating also storing bread and 11 (4.6%) (Table 7) specifying that their fruit was unpeeled, and therefore somewhat protected.

The following question of the survey pertained to the habit of withholding rubbish and waste from the meal inside the lunch bag and results confirmed that most participants (80.3%) didn't have this mentioned habit, in contrast with the remaining 47 (19.7%) respondents who did. Responses were quite diverse, indicating that people mostly kept dirty reusable food containers which were empty or had leftover foods or fruit (7.5%), used paper napkins (4.2%) as well as empty packages (gelatine or yoghurt cups) (2.9%) and used cutlery (0.8%). A few also stated keeping food scraps (2.1%), an answer which seemed inconclusive as they didn't indicate if these scraps were inside containers or in direct contact with the bag's surface.

Of the possible 239 answers for each of the following questions, only a maximum of 214 were obtained. Apart from correct storage of items and cutlery inside the lunch bag, their hygiene is also important for the food safety of the products. Most participants would actually clean their bags regularly, normally every time after they were used (38.5%), however, many also cleaned theirs

quite rarely (20.5%) or just once a week (21.3%). The prevalent cleaning method, used by 41.8 % (100) of all the participants was a simple wipe down with a damp cloth, with the succeeding most common methods being washing with water and dish detergent (18.0%) and using other detergents or disinfectants (16.3%). In lesser frequency, in the other options, respondents indicated using dishwashers (6.7%) and clothes washing machines (3.8%). The remaining 6 respondents stated just wiping down with kitchen paper, not cleaning, or just shaking out the bag after it was used.

To the following question, most participants answered keeping their lunch bags off the floor (72.4%) as opposed to other respondents (17.2%) who did. Situations in which this happened were diverse and included inside personal cars or public transportation, during classes, at work and in outings such as picnics and beach trips. 4 (1.7%) justified these moments as result of lack of storage space.

Finally, when questioned about any recent gastro-intestinal illness, the majority of respondents, 202 (84.5%) denied having one. Of the 12 (5.0%) who had suffered an illness, 6 (2.5%) confirmed having suffered from gastroenteritis ranging from 2 to 16 months prior to survey response. 3 (1.3%) others stated suffering from Irritable Bowel Syndrome with the remaining 3 having suffered from either a viral infection, gastritis and even anxiety-induced diarrhoea.

Table 7 - Responses of the online survey for lunch bag hygiene and related questions

		Nr	Frequency (%)
Food in direct contact with bag interior			
	Yes	154	64.4%
	Fruit (apples, banana, citrus, pears)	124	56.9%
	Unpeeled fruit	11	4.6%
	Fruit and Bread	1	0.4%
	undetermined	18	7.5%
	No	84	35.1%
	unanswered	1	0.4%
Waste retention			
	Yes	47	19.7%
note: total sum of item more than total counts because there are answers with >1 item	container with food/fruit scraps	18	7.5%
	empty packaging	7	2.9%
	napkins/paper	10	4.2%
	fruit peels	5	2.1%
	food scraps	5	2.1%
	cutlery	2	0.8%
	dirty containers	2	0.8%
	undetermined	5	2.1%
	No	192	80.3%

		Nr	Frequency (%)
(Table 7 continued)			
Frequency of cleaning			
	Rarely	49	20.5%
	1x week	51	21.3%
	>1x week	22	9.2%
	After every use (Everyday)	92	38.5%
	unanswered	25	10.5%
Cleaning method			
	Water+ dish detergent	43	18.0%
	detergent/disinfectant	39	16.3%
	dish washer	16	6.7%
	clothes washer	9	3.8%
	damp cloth	100	41.8%
	doesn't clean	6	2.5%
	unanswered	26	10.9%
Lunch bag in contact with the floor			
	Yes	41	17.2%
	public transportation	2	0.8%
	car	2	0.8%
	classes/workplace	6	2.5%
	outings/pic-nics	6	2.5%
	when no other option	4	1.7%
	during meal	2	0.8%
	undetermined	19	7.9%
	No	173	72.4%
	unanswered	25	10.5%
Recent cases of gastrointestinal illness			
	Yes	12	5.0%
	gastroenteritis (Dec.16, Nov.17, Dec.17; Jan.18)	6	2.5%
	Irritable Bowel Syndrome	3	1.3%
	viral infection (Jan.2018)	1	0.4%
	gastritis	1	0.4%
	anxiety induced diarrhoea	1	0.4%
	No	202	84.5%
	unanswered	25	10.5%
Total of Respondents		239	100.0%

4.3.2. In-person Survey in ESB

In total, 102 people participated in this part of the study, most of which were female (84.3%) being predominantly aged 25 years and under (50.0%), ranging from 18 to 54 years. Complete sociodemographic information is presented in Table 8. Participants of the in-person survey weren't questioned about their region of residence since it was presumed that they lived in region.

According to survey results, 79.4% (81) of respondents were single, 16.7% (17) married respondents and one (1.0%) respondent was divorced with only 15.7% of participants having at least one child As would be expected for a university setting, most of the respondents (59.8%) were students, with inclusion of PhD fellows, while 38.2% (39) were school staff (teachers, administrative technicians, lab technicians; IT specialists and economists) as well as investigators and research fellows. The remaining 2.0% (2) of respondents were student-workers who, besides studying, were also either a research fellow or working in customer service. All participants had at least a high school education with 58.8% (60) having pursued further education including undergrad, master's and doctorate degrees. Unlike the online survey, the question pertaining to financial stability of participants was excluded from this version of the survey, since less than half (43.1%) of the respondents answered it.

Table 8 - General socio-demographic information of respondents to in-person survey in ESB

		Nr	Frequency (%)
Gender			
	Female	86	84.3%
	Male	13	12.7%
	unanswered	3	2.9%
Age group			
	[16-25]	51	50.0%
	[26-35]	28	27.5%
	[36-45]	16	15.7%
	[46-55]	4	3.9%
	unanswered	3	2.9%
	Average ±standard deviation: 27.8 ± 8.3		
	Range: 18-54		
Marital Status			
	Married	17	16.7%
	Divorced	1	1.0%
	Single	81	79.4%
	unanswered	3	2.9%
Work Situation			
	Employed	39	38.2%
	Student	61	59.8%
	Student worker	2	2.0%
Education Level			
	University Education	60	58.8%
	Highschool	34	33.3%
	unanswered	8	7.8%

		Nr	Frequency (%)
(Table 8 continued)			
Children			
Yes		16	15.7%
	1	6	5.9%
	2	10	9.8%
No		81	79.4%
unanswered		5	4.9%
Total of Respondents		102	100.0%

Focusing on lunch bag use, all participants of this stage of the study used some type of bag or box to transport food, most of which prepared only their own bags (83.3%) as opposed to 13.7% of participants who indicated also preparing food bags for others (Table 9). The vast majority used lunch bags daily (77.5%) or twice a week (16.7%) and in lesser frequencies, 3 times a week (1.0%) or on special occasions (2.0%). Respondents preferred using a common insulated thermal bag (65.7%) or neoprene lunch bag (16.7%). A few volunteers also used plastic bags (3.0%). 2 other participants mentioned using either a thermal pouch or a bag made of cloth and plastic. Most participants, 74 (72.5%), stated that their lunch bags were lined with an insulating material congruent with the fact that almost all these participants had the insulated bag/pouch excluding one participant who stated carrying a cloth bag with this insulating lining.

Participants either denied (87.3%) or weren't certain of (2.9%) the existence of instructions on food transportation which would have come with the bags when they were purchased. The remaining volunteers (6.9%) who indeed confirmed their bags came with instructions, didn't specify them. Most of the participant's bags (86.3%) had only one compartment, while 9 (8.8%) had more, indicating that these compartments were used to separate the main meals from either snacks, fruit, cutlery or napkins as mentioned in Table 9

Pricewise, participants spent from 0 up to 30 € to purchase their bags however, most respondents spent only up to 10€ (69.6%).

Table 9 - Responses from the in-person survey for lunch box characteristics and use

	Nr	Frequency (%)
Lunch bag use		
Yes	101	99.0%
No	1	1.0%
Preparation of other bags besides own		
Yes	14	13.7%
No	85	83.3%

		Nr	Frequency (%)
(Table 9 continued)			
Frequency of Use			
	Daily	79	77.5%
	2x week	17	16.7%
	3x week	1	1.0%
	Special Occasion	2	2.0%
	unanswered	3	2.9%
Material of Lunchbag			
	Thermal insulated	67	65.7%
	Neoprene	17	16.7%
	Plastic bag	3	2.9%
	Cloth bag	1	1.0%
	Fabric	3	2.9%
	Fabric+plastic	1	1.0%
	Isothermal pouch	1	1.0%
	unanswered	9	8.8%
Insulating lining			
	Yes	74	72.5%
	No	25	24.5%
	unanswered	3	2.9%
Conservation Indication			
	Yes	7	6.9%
	No	89	87.3%
	Doesn't Know	3	2.9%
	unanswered	3	2.9%
Different Compartments			
	Yes	9	8.8%
	Lunch/Snack	1	1.0%
	Meal//Fruit	1	1.0%
	Doesn't use	1	1.0%
	Cutlery//Meal	1	1.0%
	Cultery +napkins/Meal	1	1.0%
	undetermined	3	2.9%
	No	88	86.3%
	Unanswered	5	4.9%

		Nr	Frequency (%)
(Table 9 continued)			
Price range (euros)			
	[0,5]	39	38.2%
	]5, 10]	32	31.4%
	]10, 15]	11	10.8%
	]15,20]	4	3.9%
	]20, 25]	2	2.0%
	]25, 30]	3	2.9%
	unanswered	11	10.8%
	Average ± Standard Deviation	8.0 ± 7.3	
	Range (euros)	0 - 30	
Total of Respondents		102	100.0%

In the following question relating to lunch bag use, most participants had used their lunch bags for only up to a year, being the case for 49.0% of participants (Table 10). Lunch bag use ranged immensely, from one case in which the bag was brand new up until the case in which the participant had been using their bag for 10 years.

From survey results it could also be stated that lunch bags were predominantly prepared by their own users (88.2%) while the minority had either their mothers prepare their food (3.9%) or prepared their food alternatively with a family member (husband or mother) (4.9%). The most commonly consumed products were cooked meals (94.1%), fruits (87.3%;) and yoghurts or gelatine cups (65.7%) besides other less frequently consumed products as is shown in Figure 10. Twenty-three respondents (22.5%) also transported other items in addition to the presented options, being mostly biscuits (10.8%).

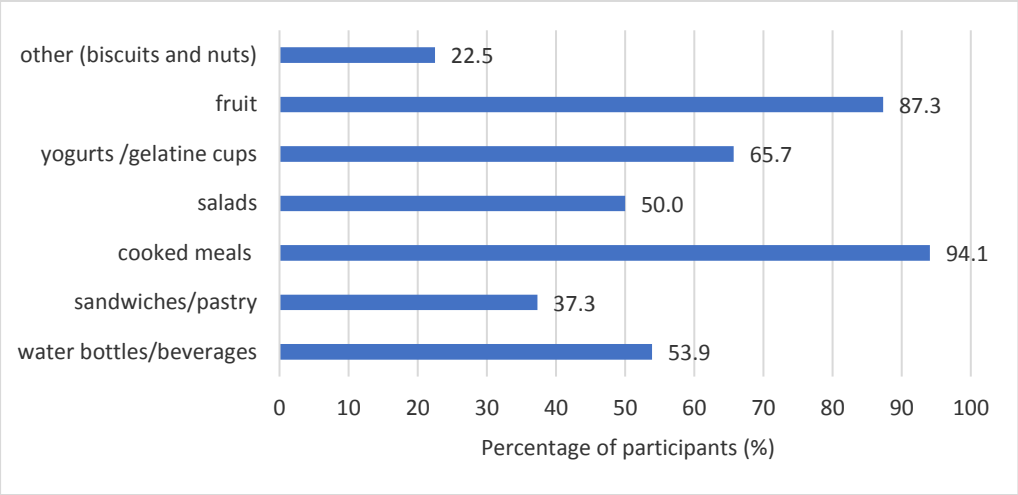

Figure 10 - Common food products transported in lunch boxes by respondents (in-person survey).

Relating to food storage time inside the lunch bag during a day, results ranged from 2 to 12 hours, with most respondents keeping their products stored between 4 and 6 hours or less (49.0%) (Table

10). Results also showed that one respondent indicated two different times, stating that the cooked meal and fruit were maintained in the bag for 5 hours while yoghurts consumed as snacks were kept inside for longer, up to 8 hours. For this situation, as well as those in which respondents indicated a time interval, mean times were used for analysis.

As the online survey also showed, the vast majority of participants (82.4%) did not use icepacks in their bags, with one person justifying their choice because they placed their food in a refrigerator at arrival to the workplace. Of the 18 (17.6%) participants who did use icepacks, only one person used them regularly, as opposed to the 10 other participants who explained when they were used. These other participants only used them during warmer weather, with one of them stating that they used icepacks because their bag had no insulating material.

Table 10 - Responses from the in-person survey for further questions related to lunch box use

		Nr	Frequency (%)
Duration of use (years)			
	[0; 1]	50	49.0%
	]1; 2]	23	22.5%
	]2; 3]	12	11.8%
	]3; 4]	3	2.9%
	>4	4	3.9%
	unanswered	10	9.8%
	Average ±Standard Deviation	1.7 ± 1.7	
	Range	1 month - 10 years	
Who prepares the bag?			
	Family member	4	3.9%
	Self	90	88.2%
	Self+Family member	5	4.9%
	unanswered	3	2.9%
Time food is kept in the bag (hours)			
	]2; 4]	39	38.2%
	]4; 6]	50	49.0%
	]6; 8]	9	8.8%
	]8;10]	2	2.0%
	]10; 12]	2	2.0%
	Average ±Standard Deviation	5.0 ± 1.7	
	Range(hours)	2 - 12	

		Nr	Frequency (%)
(Table 10 continued)			
Ice pack use			
	Yes	18	17.6%
	Always	1	1.0%
	Warm weather/Summer	10	9.8%
	undetermined	7	6.9%
	No	84	82.4%
	Explanation		
	Food in fridge at work	1	1.0%
	No thermal lining	1	1.0%
	unanswered	100	98.0%
Total of Respondents		102	100.0%

Pertaining to the storage methods inside the food-carriers, more than half of the respondents (52.9%) kept some food items in direct contact with their bag's interior surface (Table 11). In all cases, this food item was a piece of fruit, with only three respondents specifying which, having indicated apples, oranges, pears and bananas. In one case a respondent also transported bread.

Dirty cutlery could be a source of contamination of the lunch bags and, therefore, the survey included a question pertaining to the way the volunteers kept their cutlery inside their bags. The obtained results proved that the larger part of respondents (85.3%) kept their tableware covered, either wrapped in paper, plastic bag, cloth or even kept in a container, contrary to the other 3.9% (4) of participants who stated keeping their cutlery uncovered inside their respective bag.

Retaining waste and rubbish from meals could also be a food safety risk of the use of lunch bags. The survey results showed that 91.2% (93) of participants did not in fact withhold rubbish from their meals inside their bags besides empty reusable containers and cutlery. Contrasting with this, only 8.8% (9) maintained waste and of those, only 3 specifying that they withheld dirty paper napkins or crumbs.

From the achieved results as evidenced in Table 11, it was concluded that more than half (55.9%) of participants cleaned their bags quite infrequently, that is, less than once a week. This included 10 (9.8%) participants who had never cleaned their bags since they had begun using their lunch bag, one of which had used theirs for 7 years. the most used method of sanitation consisted of a wipe down with a damp cloth (50.4%). Other methods included cleaning with water and dish soap (11.8%) cleaning with other detergents/disinfectants (11.8%) and using the clothes washer (10.8%) or even using the dish washer (3.9%). It was expected that when detergents and disinfectants were used, cloths were also used to apply these products and wipe the bags as some of the participants specified (12.7%) indicated detergent/disinfectant use with cloths; 2.9% indicated cloth use after washing with water and dish detergent). Additionally, 4 (3.9%) participants indicated combining

different cleaning methods including, wiping down with a damp cloth or cleaning with detergent and water or use of other disinfectants/detergents in alternation with cleaning in the clothes washing machine. In one specific case, one respondent specified cleaning their food-carrier weekly with a disinfectant, and every three months in the washing machine. As opposed to all other respondents, 11 (10.8%) confirmed not cleaning their bag, including two who claimed they'd either change their bags regularly or only shake out the rubbish in substitution of cleaning.

The participants were additionally asked if their bag was ever left on the floor and in which situations this would occur. The vast majority (71.6%) stated keeping their bags constantly off the floor, while the remaining participants (28.4%) confirmed keeping their bags on the floor in certain situations including during classes (8.8%), on public transport (bus, metro, train)(4.9%), in their cars (5.9%) or even just at home (2.9%) (Table 11).

Lastly, when questioned about any recent gastro-intestinal illness, only two volunteers (2.0%) confirmed having one. One indicated having had a *Helicobacter pylori* infection while the other suffered from a viral infection in November 2017, 4 months prior to questioning.

Table 11 - Responses of the in-person survey for lunch box hygiene and related questions

		Nr	Frequency (%)
Food in direct contact with bag interior			
	Yes	54	52.9%
	Fruit (apples, oranges, pears and bananas)	53	52.0%
	Fruit and bread	1	1.0%
	No	48	47.1%
Cutlery storage			
	container/bag//paper napkin	87	85.3%
	directly	4	3.9%
	unanswered	11	10.8%
Waste retention			
	Yes	9	8.8%
	food crumbs	2	2.0%
	paper napkin	1	1.0%
	undetermined	6	5.9%
	No	93	91.2%
Frequency of cleaning			
	Rarely	57	55.9%
	1x week	34	33.3%
	>1x week	4	3.9%
	After every use (Everyday)	7	6.9%

		Nr	Frequency (%)
(Table 11 continued)			
Cleaning method			
note: no clean includes change bag/shake	Water+ dish detergent	12	11.8%
	detergent/disinfectant	12	11.8%
	dish washer	4	3.9%
	clothes washer	11	10.8%
	damp cloth	51	50.0%
	doesn't clean	11	10.8%
	method combinations	4	3.9%
Lunch bag in contact with the floor			
	Yes	29	28.4%
note: >1 option was mentioned in answers	public transportation	5	4.9%
	rooms/in class	9	8.8%
	car	6	5.9%
	home	3	2.9%
	undetermined	3	2.9%
	No	73	71.6%
Recent cases of gastrointestinal illness			
	Yes	2	2.0%
	Helicobater pylori	1	1.0%
	Virosis	1	1.0%
	No	100	98.0%
Total of Respondents		102	100.0%

4.3.3. Microbiological Results

Of the analysed microbiological parameters, significant CFU counts were obtained for the hygiene indicators, TVC at 30 °C and *Enterobacteriaceae* with the exception of *E. coli* which was only detected in one sample but at a reduced level (<4,0 CFU/100 cm^2). The analysed food pathogens, *Salmonella* spp. and *Listeria monocytogenes* were not detected in any lunch bag. Despite the absence of *L. monocytogenes, Listeria* spp. was detected in 8 samples.

Regarding the hygiene indicators, the CFU count for TVC at 30 °C ranged mostly (41.2%) between 2,0 and 3,0 log/100 cm^2 of lunch bag surface while *Enterobacteriaceae* count was inferior, with almost all lunch bags (95.1%) presenting levels below 2,0 log/100 cm^2, including many samples from which no CFU count was obtained (Figure 11).

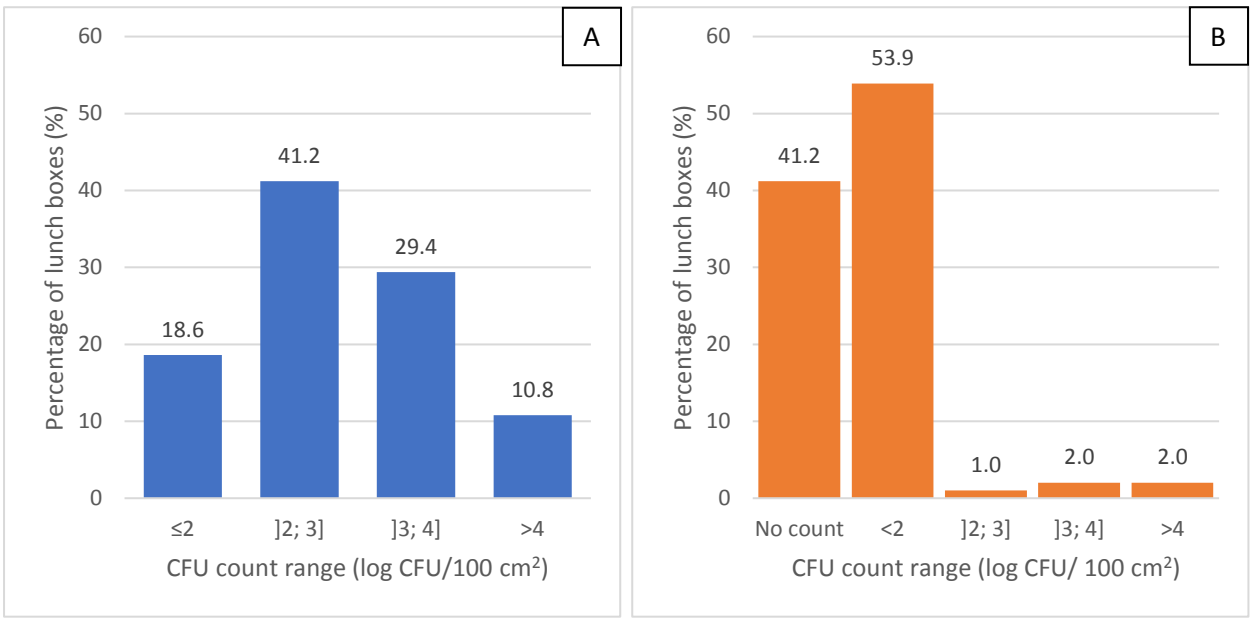

Figure 11 - Distribution of CFU count range in lunch boxes for: A) TVC at 30°C; B) *Enterobacteriaceae*

As Figure 12 shows and as aforementioned, CFU count values were categorized according to guidelines (Moragas *et al.,* 2019) which defined increasing levels of contamination for food contact surfaces. In the study's case, lunch bags, were considered excellent, clean, unclean and very unclean for levels of TVC at 30 °C. More than half of lunch bags (59.8%) were thus considered clean and even excellent while the remaining 40.2% presented bad hygiene conditions. The guidelines also allowed for the determination of hygiene as a response to *Enterobacteriaceae* count from which samples' levels were either at a satisfactory or unsatisfactory level. Figure 12 B evidences clearly that most of the samples (95.1%) had satisfactory levels of *Enterobacteriaceae*.

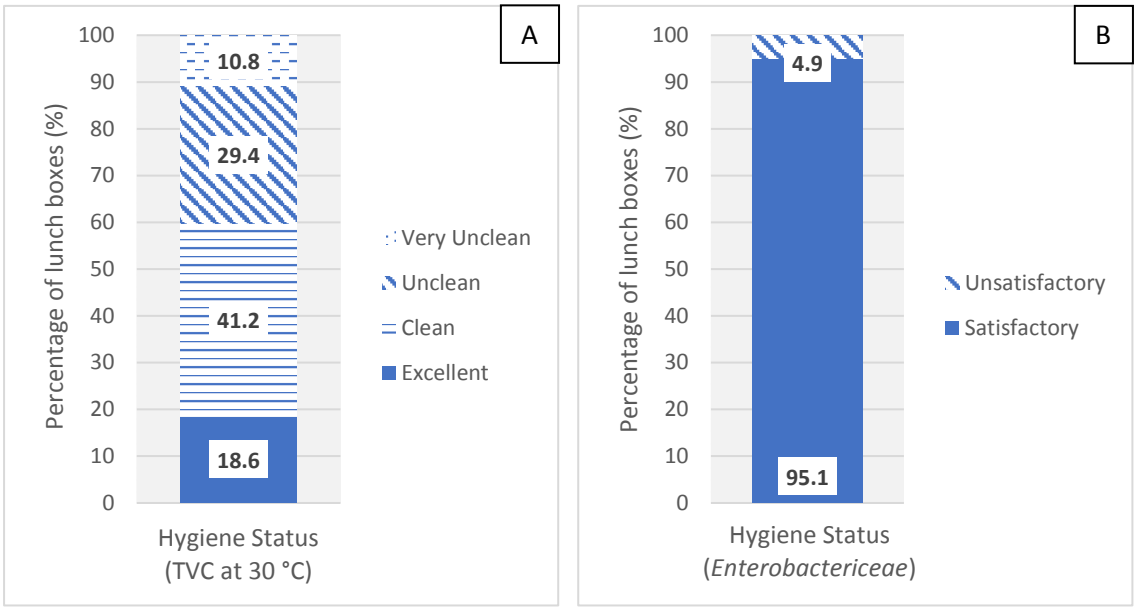

Figure 12 - Frequencies of hygiene status of lunch bags according to A) TVC at 30°C and B) *Enterobacteriaceae* count.

4.4. Discussion

According to self-reported results from the online survey, the sample population followed only a few of the correct practices and behaviours specified by the food safety entities mentioned before and which assure food safety during lunch bag use. Most participants (80.2%) correctly avoided retaining any waste in their respective bag after meal consumption and some (38.5%) frequently sanitised their bags, normally after every use. Additionally, participants primarily used the recommended insulated bag to transport their food (62.3%).

Nevertheless, in spite of these correct attitudes, most participants also followed improper behaviours. Although some cleaned their bags frequently, most didn't follow the recommended daily frequency, with many stating that they cleaned them rarely (20.5%) or only once a week (21.3%). Participants also did not follow correct cleaning procedures since they predominantly used a damp cloth to clean their lunch bags (41.8%) with only a lesser proportion of participants cleaning theirs in the recommended ways with water and soap (detergent) (18.0%) or only detergents/disinfectants directly onto the bag (16.3%).

Participants were also found to wrongly maintain their food products in their respective bags for an average of 5.2 hours, most of the times surpassing the recommended 2 hours (86.9%) for which perishable food products can be kept at room temperature, such as the cooked meals most participants carried (87.0%). As stated before, many participants used insulated lunch bags which supposedly should maintain food temperature low for some hours. After this time period, food products experience increased microbial growth with increased risk of possible food poisoning as can be shown in Borrusso & Quinlan (2017) in which higher incorrect refrigeration temperatures were associated with more microbial contamination (i.e. *Listeria* spp. growth) on refrigerator surfaces.

Gender was statistically associated with time control ($P = 0.006$) with a higher percentage of female participants (15.4%) maintaining food products less than 2 hours as opposed to the 4.2% of men who also did.

Evaluation of icepack use showed that only a few of the study participants (20.1%) seemed to follow the recommendations and used them when transporting their food products. Moreover, of these, most only used them on occasion (during warmer weather). The use of icepacks has proved to be important for temperature control inside lunch boxes, even in the case of insulated bags as studies have shown, including a study from NSW Food Authority in Australia (NSW Food Authority, 2015b) which measured the temperature of sandwiches stored in different conditions and which concluded that, with ambient temperature of 25 °C, temperature increase was considerably fast in sandwiches stored in paper bags as well as in insulated lunch bags without an icepack. On the other hand, sandwiches stored in insulated bags with icepacks or frozen beverage bottles showed a slower increase of temperature as well as a reduced microbial growth which was also analysed, thus indicating that, if foods were to be kept for longer than recommended inside the bags, as the current study evidenced (>2 hours), it is best to also carry an icepack to conserve low temperatures inside the lunch bag to prevent microbial proliferation in the transported food products. Relating to the current study, although not analysed for statistical significance, results showed that the majority of the icepacks users contained their foods for longer than 2 hours and also used insulated

bags. Most non-users, however, also used insulated bags which, despite being recommended, weren't complemented with icepack use as suggested by the previously mentioned study.

Most participants (64.4%) confirmed storing food items directly into the bags, being mostly whole fruits. Gender was statistically correlated (*P* = 0.041) with this practice, with female participants being less likely to not keep food (fruit) in direct contact with the lunch bag surface (O.R = 0.514). In other words, female participants were more likely to keep fruits in direct contact with the lunch bag surface sample. The food safety entities which establish the lunch bag food safety guidelines also promote fruit consumption, observed in most of the participants (74.9%), and also consider fruits to be low risk foods if consumed whole and washed under cool running water before being packed (Craig, 2018). Results from this survey, however, could not determine if the fruits transported by participants were previously cleaned.

Results from the online survey of this study clearly indicated that most participants showed some inadequate practices related to safe food storage and hygiene associated to lunch box use. This was consistent with findings made by Hudson & Walley (2009) who showed in their study that relating to their children's lunch boxes, parents did not always follow correct practices, despite being aware of many food safety risks. Most who had insulated lunch bags cleaned them only with a damp cloth, occasionally spraying with bleach or a surface cleaner and then wiping them. Similarly, to the current study, only 20% of the parents placed icepacks in their children's bags. In this mentioned study, the internal temperature of the lunch bags was also measured along the day during a whole school term and showed that the average temperature was low for only 1.5 hours in the morning and would rise to room temperature for the rest of the day, leaving bag contents exposed to dangerous temperatures. This was also aggravated by the fact that parents made their children keep leftover foods and packaging until arrival to home as a way for them to assure their children had eaten their meals.

In this current study, gender did reveal to be a predictor of correct food safety practices, at least concerning correct time of food storage, as referred above. This trend has been confirmed in many studies which have shown that women normally have better food safety knowledge and practices as opposed to men (Langiano *et al.*, 2012; Ruby *et al.,* 2019), with Kennedy *et al.* (2005) also having found that, in domestic environments, food handlers engaging in less hygienic practices, including incorrect cross-contamination prevention, were more likely to be male. A significant correlation was also established between age and frequency of cleaning, from the present study (*P* = 0.044), showing that a higher percentage of people aged 17 to 25 rarely clean their bags (30.2%) and a higher percentage of older participants aged 46 to 55 years cleaned theirs after every use (56.3%). Similar findings were obtained in Kennedy *et al.* (2005) which indicated that the food handlers who showed poor hygiene practices were also more likely to be aged under 45 years, considered young in the specific study. Further findings evidencing lack of knowledge and incorrect behaviours in younger populations could be found in many publications in which surveys to university students, normally aged between 18 and 24 years, showed that many had limited food safety knowledge, such as identifying high-risk foods, and didn't follow correct practices, including hand washing before food preparation. (Abbot *et al.*, 2009; Ferk, Calder & Camire, 2016). Relative to education level, this present study could not obtain a significant correlation with the food safety practices,

unlike in Ruby *et al.* (2019) which indicated that higher food safety knowledge was strongly associated with tertiary education, i.e. University or other post-secondary degrees.

Besides the aforementioned statistically significant correlations between survey answers and sociodemographic data from participants, only frequency of lunch bag use could also be correlated to gender ($P = 0.002$) and education level ($P = 0.040$) as is shown in Table 12. Female participants used their lunch bags on a daily basis or once a week much more frequently as opposed to the male counterparts. Participants with only a high school education or inferior would more frequently use their bags on special occasions as opposed to university-educated counterparts, despite also frequently using lunch bags.

Focusing on the microbiological results obtained from lunch bags analysed in the university setting of ESB, in all 102 lunch bag samples, the foodborne pathogens *L. monocytogenes* and *Salmonella* spp. were not detected. The absence of *Salmonella* spp. could be justified since lunch bags are expected to be prepared in kitchen environments, which have been shown to present low incidence of this bacteria in studies such as in Medrano-Félix *et al.* (2011) in which kitchen surfaces, sponges and dishcloths were shown to present a low incidence of this bacterium. Absence of *L. monocytogenes* could also be confirmed with other study findings in which this bacterium was undetected in domestic environments, including kitchen surfaces (Azevedo *et al.*, 2014) or infrequently detected in kitchens, more specifically, inside refrigerators as Azevedo *et al.* (2005) discovered. This indicates that, in the case of this study, lunch bags weren't vehicles or vectors for the transmission of these serious food-related pathogens, although hygiene indicator microorganisms were indeed detected and quantified.

In spite of the absence of *L. monocytogenes*, members of *Listeria* spp. were detected in 8 of the lunch bags. The presence of any Listeria *spp*. in food contact surfaces is a normal occurrence since this genus of bacteria is ubiquitous, being normally present in various food products including dairy products, various meats, fermented sausages, seafood products and fresh produce such as radishes and cabbage (Gandhi & Chikindas, 2007). Listeria *spp.* may also be found on surfaces in the domestic environment including kitchens (Azevedo *et al.*, 2014) and has been shown to be indicative of poor hygiene practices and sanitation. Therefore, its presence could also be associated with the hygiene conditions of the lunch bags in which it was found.

E. coli, a definitive indicator of faecal contamination, was absent in almost all the lunch bags, with the exception of only one lunch bag in which residual levels were detected. This may have been justified by the lower TVC most of the lunch bags (59,8%) presented, therefore indicating better hygiene conditions, as opposed to the remaining lunch bags which presented higher contamination levels and in which the one lunch bag positive for *E. coli* was included. Despite low incidence in the current study, *E. coli* has been commonly found in food preparation areas (kitchens) of household settings, more commonly in hand contact sites such as countertops, refrigerator door handles and cutting boards (Borrusso & Quinlan, 2017; Azevedo *et al.,* 2014) indicating potential for its transfer onto lunch bags. Azevedo *et al.* (2014) also discovered high counts of *E. coli* on kitchen taps. Thusly poor hygiene practices could cause probable cross-contamination situations during meal preparation, increasing potential contamination of lunch bag surfaces with this faecal contamination indicator.

Most of the analysed lunch bags were considered clean according to the guidelines established for food contact surfaces by the Basque Health Department – Spain. According to these guidelines, 59.8% and 95.1% of the lunch bags were considered as acceptable for TVC (≤3.0 log CFU/100 cm^2) and *Enterobacteriaceae* levels (<2.3 log CFU/100cm^2), respectively. In spite of this, a considerable proportion of bags (40.2%) were still considered unclean according to TVC levels, presenting increased microbial loads on their interior surfaces. Comparing the CFU count of the analysed microbiological groups, it was shown that all bags which presented high levels of *Enterobacteriaceae* and/or were positive for the presence of Listeria *spp.* were also included in the 40.2% of the lunch bags which were considered highly contaminated ("unclean"). In consequence, the determination of lunch bag hygienic conditions could be encompassed by evaluating the TVC levels which were then used for association with the in-person survey responses.

From this present study, as Table 13 shows, no statistically significant correlations could be determined between microbiological data and the analysed responses from the in-person survey (sociodemographic information and food safety questions related to lunch bag type, conditions in which food and cutlery were stored (covered/uncovered), waste retention and method and frequency of cleaning) although some conclusions could be made, as is shown ahead.

Findings for association of lunch bag material and microbiological contamination were statistically insignificant ($P = 0.863$). However, comparing the results for microbial contamination of the popular thermal insulated and neoprene bags, it was shown that the former were considered mostly clean (59.7%) while the latter, neoprene bags, were considered mostly unclean (58,8%). These results could be associated with their cleaning frequency (not shown), since a higher proportion (70.5%) of neoprene bag users rarely sanitised them, as opposed to 53.7% of insulated bag users. The study results contradict Hudson & Walley (2009) which were able to correlate microbiological data, although relatively limited, with the material of the lunch bag and cleaning practices. Their publication showed that the highest microbial counts were associated to insulated bags which were cleaned by wiping with a damp cloth and which were also positive for *S. aureus* contamination.

Results showed that almost half of the participants (47.1%) didn't maintain food products in direct contact with the lunch bags' surfaces and the larger part (64.6%) of these had bags which were considered clean. However, bags in which products, mainly whole fruits, (52.9%) were placed directly inside the bags, also presented clean conditions (55.6%). Consequently, carrying whole fruits in direct contact with the lunch bag surface could not be associated with their poor hygiene conditions. Even though results didn't indicate if these fruits were washed or not, the previously mentioned food safety organizations such as the FSIS, also recommended cleaning fruits and vegetables with running water before being consumed since this reduced the dirt and bacteria present on them (FSIS/USDA, 2013). Fruit surfaces aren't sterile as Seow *et al.* (2012) indicated with the observation that commercially sold fruits contained variable levels of aerobic bacteria count, i.e. TVC, coliform bacteria and yeasts and moulds. These surfaces were also shown to present a low incidence of pathogens such as *L. monocytogenes* and *Salmonella* spp. Therefore, if unclean whole fruits are stored directly into the bags, there may be potential for cross-contamination onto the bags' surfaces, reducing their hygiene conditions.

Another factor that could be connected to increased surface contamination of the lunch bag could be the practice of waste and food debris retention (i.e. leftovers, packaging) and incorrect storage of used cutlery as Hudson & Walley (2009) mentioned. Leftover food and dirty cutlery (with food debris) inside the lunch bag, when exposed to temperature-time abuse, allows the increase of the microbial load (Borrusso & Quinlan, 2017) and may promote the contamination of the bag surfaces. In the present study, participants predominantly kept their cutlery stored in either appropriate containers or wrapped in napkins or plastic bags (85.3%) and did not keep any leftover food or packaging in their bags (91.2%). Despite this, microbial contamination could be associated with these practices as most of the bags in which waste was kept, were considered unclean (66.7%) while most of the bags in which this wasn't observed were considered clean (62.4%). Even so, these results were not statistically significant (P = 0.152). Cutlery storage, on the other hand, could not be associated with microbial contamination, as most bags with packaged utensils showed mostly clean conditions (56.3%) and those in which utensils were kept uncovered, presented an even higher proportion of bags with clean conditions (75.0%).

Although not statistically relevant (P = 0.417), according to observed TVC levels, results showed that participants who frequently cleaned their lunch bags, that is, cleaned them after every use, also showed the highest percentage of clean bags (71.4%) compared to the other cleaning frequencies.

The online survey responses indicated that participants cleaned their bags after every use, contradicting most of the participants from the in-person survey in ESB who rarely cleaned their bags. Of these infrequent cleaners, more than half (57.9%) presented bags with good hygiene conditions, indicating that, in this study, cleaning frequency did not seem to influence the hygiene conditions of the lunch bags. These findings were consistent with Chen, Godwin & Kilonzo-Nthenge (2011) which reported that the most frequent cleaning of kitchen surfaces did not in fact show the least microbial contamination. They concluded that, besides frequency, correct methods were also important to ensure effective cleaning. Williams *et al.* (2010), on the other hand, evidenced the association between frequency of cleaning and microbial contamination by evidencing that samples of used reusable grocery bags which had very rarely been cleaned were contaminated with increased levels of microorganisms including many faecal coliforms. This study also evidenced the efficient reduction of the microbial load after hand or machine washing of the bags with additional detergent use.

The combination of different methods (washing with dish soap and water or use of detergents/disinfectants or wipe down with damp cloths in alternation with cleaning in the clothes washing machine), indicated the highest percentage of clean bags (75.0%). The lower microbial levels in this situation could have been result of the use of different detergents/disinfectants as well as thorough rinsing resulting from the washing machine use. This could possibly be corroborated by Cogan *et al.* (2002) who indicated that, after cleaning of kitchen surfaces (boards), hands and cloths with detergents and water, posterior rinsing helped reduce their microbial load. In Cogan *et al.* (2002), however, rinsing was described as leaving the item under cold running tap water for a few seconds. In this study it was also recommended that after cleaning and rinsing, disinfectants should be applied for an efficient sanitation, which was similarly recommended by food safety entities which enforce that lunch bags should be cleaned with soapy water (detergent

and rinsing) followed by the use of a diluted solution of bleach (disinfectant) (FSIS/USDA, 2016). Despite the obtained associations mentioned above, study results showed that the employed cleaning method was not statistically associated to the microbial levels present in the lunch bags (*P* = 0.863) since the different cleaning methods obtained similar proportions of clean bags (approximately 60.0%) as is shown in Table 13.

Wiping lunch bags with a damp cloth, the most used cleaning method and most mentioned in the online survey, resulted in good hygiene conditions of the respective bags according to TVC levels (59.4%), as did the bags which were cleaned with detergents and disinfectants with implied use of a cloth (60.0%). Yet publications have demonstrated that kitchen cloths are highly contaminated and are microbial reservoirs and vectors for spreading microorganisms onto various kitchen surfaces (Borrusso & Quinlan, 2017; Cogan *et al.*, 2002; Chen *et al.*, 2011). Borrusso & Quinlan (2017) discovered that kitchen cloths as well as sponges used for cleaning, presented high levels of hygiene indicator microorganisms including indicators of faecal contamination. Cogan *et al.* (2002) discovered the persistence of *Salmonella* on cloths, evidencing bacterial growth after overnight storage and with increased persistence on cloths even after their detergent-based cleaning. These methods could, therefore, potentially introduce microorganisms onto the bags instead of removing them. This would result possibly in a higher percentage of unclean bags, despite not being the case in this study. These results could show that although the incorrect cleaning methods were used, other correct practices such as not retaining waste and keeping cutlery appropriately stored may have helped compensate these bad practices and justify why most of the analysed lunch bags were considered clean.

The frequency of lunch bag use, as well as the time period participants had them, did not show any statistically relevant correlation to microbial contamination since cleaner interior surfaces of lunch bags were equally associated to rarely used or “new” bags as well as frequently used or older bags. Other questions included in the survey, such as icepack use and storage time of food inside the bag could not be associated with the microbiological results despite being possible important predictors for the hygiene and food safety conditions of lunch bags. The sole presence of an icepack inside the bag was insufficient to evaluate its influence on the surface microbial count since the measurement of the interior temperature variation of the bags would be required because the icepacks influence microbial levels by maintaining low temperatures for longer periods of time, which is not favourable for microbial growth. In turn, the influence of storage time of food could only be evaluated if microbiological levels of the bag surfaces were measured at different time points, which was not the case in this study.

Table 12 – Cross-tabulations between socio-demographic factors and online survey answers relevant to food safety. .

		Gender				Age range (Years)							Education			
		F	M	*P*-VALUE	ODDs RATIO	[16-25]	[26-35]	[36-45]	[46-55]	[56-65]	*P*-VALUE	ODDs RATIO	High school or less	University	*P*-VALUE	ODDs RATIO
Frequency of use	1x week	9 (4.7%)	1 (2.1%)	**0.002**		2 (2.1%)	3 (4.9%)	4 (7.3%)	1 (5.6%)	0 (0.0%)	0.061		0 (0.0%)	10 (5.2%)	**0.040**	
	2 x week	19 (10.0%)	11 (23.4%)			18 (18.8%)	5 (8.2%)	4 (7.3%)	3 (16.7%)	0 (0.0%)			5 (10.9%)	25 (13.1%)		
	Daily	140 (73.7%)	23 (48.9%)			57 (59.4%)	48 (78.7%)	42 (76.4%)	12 (66.7%)	4 (57.1%)			29 (63.0%)	134 (70.2%)		
	Special occasions	22 (11.6%)	12 (25.5%)			19 (19.8%)	5 (8.2%)	5 (9.1%)	2 (11.1%)	3 (42.9%)			12 (26.1%)	22 (11.5%)		
	Total	190 (100.0%)	47 (100.0%)			96 (100.0%)	61 (100.0%)	55 (100.0%)	18 (100.0%)	7 (100.0%)			46 (100.0%)	191 (100.0%)		
Duration of use (Years)	[0;1]	80 (46.0%)	19 (45.2%)	0.718		48 (53.9%)	21 (37.5%)	16 (32.0%)	10 (62.5%)	4 (80.0%)	0.214		19 (48.7%)	80 (45.2%)	0.072	
	]1;2]	50 (28.7%)	9 (21.4%)			24 (27.0%)	16 (28.6%)	15 (30.0%)	4 (25.0%)	0 (0.0%)			6 (15.4%)	53 (29.9%)		
	]2;3]	19 (10.9%)	5 (11.9%)			9 (10.1%)	9 (16.1%)	5 (10.0%)	1 (6.3%)	0 (0.0%)			4 (10.3%)	20 (11.3%)		
	]3;4]	3 (1.7%)	2 (4.8%)			2 (2.2%)	1 (1.8%)	2 (4.0%)	0 (0.0%)	0 (0.0%)			2 (5.1%)	3 (1.7%)		
	]4;5]	10 (5.8%)	2 (4.8%)			3 (3.4%)	3 (5.4%)	6 (12.0%)	0 (0.0%)	0 (0.0%)			1 (2.6%)	11 (6.2%)		
	>5	12 (6.9%)	5 (11.9%)			3 (3.4%)	6 (10.7%)	6 (12.0%)	1 (6.3%)	1 (20.0%)			7 (18.0%)	10 (5.7%)		
	Total	174 (100.0%)	42 (100.0%)			89 (100.0%)	56 (100.0%)	50 (100.0%)	16 (100.0%)	5 (100.0%)			39 (100.0%)	177 (100.0%)		

		Gender				Age range (Years)							Education			
(Table 12 continued)		F	M	*P*-VALUE	ODDs RATIO	[16-25]	[26-35]	[36-45]	[46-55]	[56-65]	*P*-VALUE	ODDs RATIO	High school or less	University	*P*-VALUE	ODDs RATIO
Time kept inside bag (hours)	[0;2]	29 (15.4%)	2 (4.2%)	**0.006**		7 (7.4%)	11 (18.0%)	9 (16.4%)	2 (10.5%)	2 (28.6%)	0.109		5 (10.6%)	26 (13.8%)	0.592	
	]2;4]	71 (37.8%)	16 (33.3%)			33 (35.1%)	20 (32.8%)	21 (38.2%)	10 (52.6%)	3 (42.9%)			22 (46.8%)	65 (34.4%)		
	]4;6]	64 (34.0%)	16 (33.3%)			33 (35.1%)	22 (36.1%)	21 (38.2%)	3 (15.8%)	1 (14.3%)			15 (31.9%)	65 (34.4%)		
	]6;8]	12 (6.4%)	3 (6.3%)			5 (5.35)	4 (6.6%)	4 (7.3%)	2 (10.5%)	0 (0.0%)			1 (2.1%)	14 (7.4%)		
	]8;10]	3 (1.6%)	0 (0.0%)			2 (2.1%)	1 (1.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)			1 (2.1%)	2 (1.1%)		
	]10;12]	6 (3.2%)	6 (12.5%)			7 (7.4%)	2 (3.2%)	0 (0.0%)	2 (10.5%)	1 (14.3%)			2 (4.2%)	10 (5.3%)		
	>12	3 (1.6%)	5 (10.4%)			7 (7.4%)	1 (1.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)			1 (2.1%)	7 (3.7%)		
	Total	188 (100.0%)	48 (100.0%)			94 (100.0%)	61 (100.0%)	55 (100.0%)	19 (100.0%)	7 (100.0%)			47 (100.0%)	189 (100.0%)		
Ice pack use	Depends on the food	27 (14.2%)	3 (6.3%)	0.331		13 (13.5%)	5 (8.2%)	9 (16.4%)	2 (10.5%)	1 (14.3%)	0.712		2 (4.3%)	28 (14.7%)	0.067	
	No	149 (78.4%)	41 (85.4%)			79 (82.3%)	51 (83.6%)	40 (72.7%)	15 (78.9%)	5 (71.4%)			39 (83.0%)	151 (79.1%)		
	Yes	14 (7.4%)	4 (8.3%)			4 (4.2%)	5 (8.2%)	6 (10.9%)	2 (10.5%)	1 (14.3%)			6 (12.8%)	12 (6.3%)		
	Total	190 (100.0%)	48 (100.0%)			96 (100.0%)	61 (100.0%)	55 (100.0%)	19 (100.0%)	7 (100.0%)			47 (100.0%)	191 (100.0%)		

(Table 12 continued)		Gender				Age range (Years)							Education			
		F	M	*P*-VALUE	ODDs RATIO	[16-25]	[26-35]	[36-45]	[46-55]	[56-65]	*P*-VALUE	ODDs RATIO	High school or less	University	*P*-VALUE	ODDs RATIO
Direct Contact	No	61 (32.1%)	23 (47.9%)	**0.041**	0.514	37 (38.5%)	23 (37.7%)	15 (27.3%)	4 (21.1%)	5 (71.4%)	0.098		19 (40.4%)	65 (34.0%)	0.411	1.315
	Yes	129 (67.9%)	25 (52.1%)			59 (61.5%)	38 (62.3%)	40 (72.7%)	15 (78.9%)	2 (28.6%)			28 (59.6%)	126 (66.0%)		
	Total	190 (100.0%)	48 (100.0%)			96 (100.0%)	61 (100.0%)	55 (100.0%)	19 (100.0%)	7 (100.0%)			47 (100.0%)	191 (100.0%)		
Waste Retention	No	153 (80.1%)	39 (81.2%)	1.000	0.929	79 (82.3%)	43 (70.5%)	48 (85.7%)	15 (78.9%)	7 (100.0%)	0.151		40 (85.1%)	152 (79.2%)	0.419	1.504
	Yes	38 (19.9%)	9 (18.8%)			17 (17.71%)	18 (29.5%)	8 (14.3%)	4 (21.1%)	0 (0.0%)			7 (14.9%)	40 (20.8%)		
	Total	191 (100.0%)	48 (100.0%)			96 (100.0%)	61 (100.0%)	56 (100.0%)	19 (100.0%)	7 (100.0%)			47 (100.0%)	192 (100.0%)		
Cleaning frequency	>1 x week	19 (10.8%)	3 (7.9%)	0.231		4 (4.7%)	8 (13.6%)	9 (18.0%)	1 (6.3%)	0 (0.0%)	**0.044**		2 (5.6%)	20 (11.2%)	0.445	
	1x week	45 (25.6%)	6 (15.8%)			18 (20.9%)	13 (22.0%)	15 (30.0%)	3 (18.8%)	2 (66.7%)			11 (30.6%)	40 (22.5%)		
	After every use (everyday)	70 (39.8%)	22 (57.9%)			38 (44.2%)	22 (37.3%)	22 (44.0%)	9 (56.3%)	1 (33.3%)			17 (47.2%)	75 (42.1%)		
	Rarely	42 (23.9%)	7 (18.4%)			26 (30.2%)	16 (27.1%)	4 (8.0%)	3 (18.8%)	0 (0.0%)			6 (16.7%)	43 (24.2%)		
	Total	176 (100.0%)	38 (100.0%)			86 (100.0%)	59 (100.0%)	50 (100.0%)	16 (100.0%)	3 (100.0%)			36 (100.0%)	178 (100.0%)		

		Gender				Age range (Years)							Education			
(Table 12 continued)		F	M	*P*-VALUE	ODDs RATIO	[16-25]	[26-35]	[36-45]	[46-55]	[56-65]	*P*-VALUE	ODDs RATIO	High school or less	University	*P*-VALUE	ODDs RATIO
Cleaning methods	Water + dish detergent	30 (17.1%)	13 (34.2%)	0.072		19 (22.4%)	15 (25.4%)	6 (12.0%)	1 (6.3%)	2 (66.7%)	0.122		4 (11.1%)	39 (22.0%)	0.272	
	Disinfectant/ detergent	36 (20.6%)	3 (7.9%)			8 (9.4%)	14 (23.7%)	14 (28.0%)	3 (18.8%)	0 (0.0%)			6 (16.7%)	33 (18.6%)		
	Dish washer	11 (6.3%)	5 (13.2%)			7 (8.2%)	3 (5.1%)	5 (10.0%)	1 (6.3%)	0 (0.0%)			4 (11.1%)	12 (6.8%)		
	Clothes washing machine	7 (4.0%)	2 (5.3%)			1 (1.2%)	2 (3.4%)	4 (8.0%)	2 (12.5%)	0 (0.0%)			1 (2.8%)	8 (4.5%)		
	Damp cloth	86 (49.1%)	14 (36.8%)			47 (55.3%)	23 (39.0%)	20 (40.0%)	9 (56.3%)	1 (33.3%)			21 (58.3%)	79 (44.6%)		
	No cleaning	5 (2.9%)	1 (2.6%)			3 (3.5%)	2 (3.4%)	1 (2.0%)	0 (0.0%)	0 (0.0%)			0 (0.0%)	6 (3.4%)		
	Total	175 (100.0%)	38 (100.0%)			85 (100.0%)	59 (100.0%)	50 (100.0%)	16 (100.0%)	3 (100.0%)			36 (100.0%)	177 (100.0%)		

Notes: Values presented as number of participants and respective percentage of the independent variables (sociodemographic factors) with accompanied significance(P-values) and Odds Ratio (O.R.) 2x2 cross-tabulation. F-female M-male

Table 13 - Cross-tabulations between hygiene status and socio-demographic factors and food safety answers of the in-person survey.

		Gender (n=99)		Age range (years) (n=102)				Education (n=94)		Frequency of Use (n=99)			
		F	M	[16-25]	[26-35]	[36-45]	[46-55]	University	High School	2x week	3x week	Daily	Special occasion
Hygiene status	CLEAN	51 (59.3%)	7 (53.8%)	34 (63.0%)	17 (60.7%)	8 (50.0%)	2 (50.0%)	33 (55.0%)	24 (70.6%)	10 (58.8%)	0 (0.0%)	46 (58.2%)	2 (100.0%)
	UNCLEAN	35 (40.7%)	6 (46.2%)	20 (37.0%)	11 (39.3%)	8 (50.0%)	2 (50.0%)	27 (45.0%)	10 (29.4%)	7 (41.2%)	1 (100.0%)	33 (41.8%)	0 (0.0%)
	Total	86 (100.0%)	13 (100.0%)	54 (100.0%)	28 (100.0%)	16 (100.0%)	4 (100.0%)	60 (100.0%)	34 (100.0%)	17 (100.0%)	1 (100.0%)	79 (100.0%)	2 (100.0%)
	p – value	0.768		0.796				0.137		0.272			
	Odds Ratio (OR)	1.249						0.509					

		Lunch bag type (n=93)				Duration of use (years) (n=92)					Time food was kept inside bag (n=102)				
		Neoprene	Plastic Bag	Fabric/ Cloth	Insulated Bag	[0;1]	]1; 2]	]2;3]	]3; 4]	>4	[2;4]	]4;6]	]6;8]	]8; 10]	]10; 12]
Hygiene status	CLEAN	7 (41.2%)	2 (66.7%)	4 (80.0%)	41 (60.3%)	28 (56.0%)	13 (56.5%)	9 (75.0%)	1 (33.3%)	2 (50.0%)	27 (69.2%)	27 (54.0%)	5 (55.6%)	2 (100.0%)	0 (0.0%)
	UNCLEAN	10 (58.8%)	1 (33.3%)	1 (20.0%)	27 (39.7%)	22 (44.0%)	10 (43.5%)	3 (25.0%)	2 (66.7%)	2 (50.0%)	12 (30.8%)	23 (56.0%)	4 (44.4%)	0 (0.0%)	2 (100.0%)
	Total	17 (100.0%)	3 (100.0%)	5 (100.0%)	68 (100.0%)	50 (100.0%)	23 (100.0%)	12 (100.0%)	3 (100.0%)	4 (100.0%)	39 (100.0%)	50 (100.0%)	9 (100.0%)	2 (100.0%)	2 (100.0%)
	p - value	0.351				0.653					0.093				

(Table 13 continued)		Ice pack use (n=102)		Food in direct contact with bag surface (n=102)		Cutlery storage (n=91)		Waste retention (n=102)	
		No	Yes	No	Yes	In container/bag/wrapped in paper	Directly into bag	No	Yes
Hygiene Status	CLEAN	51 (60.7%)	10 (55.6%)	31 (64.6%)	30 (55.6%)	49 (56.3%)	3 (75.0%)	58 (62.4%)	3 (33.3%)
	UNCLEAN	33 (39.3%)	8 (44.4%)	17 (35.4%)	24 (44.4%)	38 (43.7%)	1 (25.0%)	35 (37.6%)	6 (66.7%)
	Total	84 (100.0%)	18 (100.0%)	48 (100.0%)	54 (100.0%)	87 (100.0%)	4 (100.0%)	93 (100.0%)	9 (100.0%)
	p - value	0.793		0.353		0.632		0.152	
	Odds Ratio (OR)	1.236		1.459		0.430		3.314	

		Cleaning methods (n=102)							Cleaning frequency (n=102)			
		Damp cloth	Water and dish detergent	Detergent/ Disinfectant	Dish washing machine	Clothes washing machine	Method combination	No cleaning	>1x week	1x week	After every use	Rarely
Hygiene Status	CLEAN	19 (59.4%)	9 (60.0%)	15 (60.0%)	1 (25.0%)	7 (63.6%)	3 (75.0%)	7 (63.6%)	1 (25.0%)	22 (64.7%)	5 (71.4%)	33 (57.9%)
	UNCLEAN	13 (40.6%)	6 (40.0%)	10 (40.0%)	3 (75.0%)	4 (36.4%)	1 (25.0%)	4 (36.4%)	3 (75.0%)	12 (35.3%)	2 (28.6%)	24 (42.1%)
	Total	32 (100.0%)	15 (100.0%)	25 (100.0%)	4 (100.0%)	11 (100.0%)	4 (100.0%)	11 (100.0%)	4 (100.0%)	34 (100.0%)	7 (100.0%)	57 (100.0%)
	p - value	0.863							0.417			

Notes: Values presented as number of bags and respective percentage of the independent variables (sociodemographic factors and relevant food safety related answers to the in-person survey) with accompanied significance(*P*-values) and Odds Ratio (O.R.) when 2x2 cross-tabulation. F - female, M-male.

This study presented a few limitations including the diverse responses rates for different questions in both the online and in-person survey as can be exemplified by the online survey question of bag cleaning frequency which only obtained 214 of the possible 239 answers and by the in-person survey question about cutlery storage which obtained 91 of the expected 102. These situations could have possibly excluded responses which may have altered the statistical significance of this study's findings. Besides this, some questions were possibly misinterpreted and answered incorrectly, with inconclusive or incongruent answers obtained mostly for questions from the online survey. Such examples included the question about icepack use which a few participants mistook for the use of thermos to maintain their foods hot. Another example was the question about the retention of waste in the bag, to which some participants answered maintaining food scraps but not indicating if in the food container or directly inside the bag. The in-person survey presented less of these confounding responses since the sampling technician would aid in the interpretation of some questions participants may have had difficulty in understanding. To resolve these issues, a pilot survey should have been implemented as Ferk *et al.* (2016) did for a food safety survey aimed at students from the University of Maine. The pilot study could have evidenced the more difficult questions, and which could then be corrected in order to increase their clarity. Additionally, self-reported behaviours of the respondents could have been inaccurate, with respondents possibly having overestimated their correct practices as has been evidenced by Fischer *et al.* (2007) and Abbot *et al.* (2009) who compared self-reported food behaviour and knowledge of young adults with their actual observed food safety behaviour.

It is also suggested that for further studies focusing on this subject, a larger sample population should be obtained so that its analysis can further define stronger predictor variables and better descriptions of food safety behaviours related to lunch bag use. This could avoid what occurred in this study's results which could only associate some sociodemographic factors to a limited number of behaviours and neither of these factors or behaviours could be statistically associated with the microbial levels of the bags' surfaces. Besides this, the larger sample population could also increase the potential to generalise study findings to the Portuguese population, which may not have occurred in this study since participants were mainly recruited online, reaching mostly members (students, collaborators, teachers) of Aveiro University and ESB. Some responses, however, were obtained from a few more participants of diverse backgrounds through the Facebook publications. As mentioned before, temperature variation inside the bags wasn't analysed but is an important variable to be evaluated in further studies as has been shown in Hudson & Walley (2009), and which should be complemented with the corresponding analysis of microbial contamination for different time/temperature points in order to determine the influence of storage time and temperature on the contamination of the bags. Another aspect which should have been evaluated was the personal hygiene of participants, i.e. if they washed their hands before and after eating their meals from their bags, because hands have been shown to be vectors for the dissemination of pathogens. This makes hand washing important to avoid cross-contamination situations, as could be evidenced by Bloomfield *et al.* (2007). If correct handwashing was revealed to be frequent in the current study, it could also have justified why most of the bags were considered clean.

Despite these limitations, this study was important in evidencing some incorrect practices including incorrect food storage, with the majority of participants storing some foods without packaging

(fruit) and not using icepacks, as well as infrequent cleaning procedures and with inappropriate methods (wipe down with a damp cloth). These practices should be corrected to avoid food safety issues, such as cross-contamination situations when using lunch bags, as some of this study's findings evidenced and which have been grounded on other literature. The microbiological results, however, did not statistically confirm the risks of these bad habits since most of the bags had reduced microbial loads and subsequent acceptable hygiene conditions. Efforts should thusly be directed towards the population education to inform about correct lunch bag use. In addition to the pre-existing programs from food safety authorities which already help spread this information. Further methods should be applied in order to reach a larger demographic including young adults and people of working age, not only children and their caregivers. Other options could be the distribution of pamphlets accompanied with the lunch bags during their purchase, publications on social media and additional placement of posters in the schools/universities or workplace food areas. Population education would therefore result in better practices which in turn would result in cleaner, hygienic bags and subsequent reduced risk of microbial contamination and possible transmission of food borne pathogens.

5. Conclusion

Concluding the internship in CINATE, I believe that the objectives that were set out were indeed accomplished and truly fundamental for the completion of my degree since it allowed me to apply the knowledge that I acquired during the Master's in Microbiology into a professional workplace setting, more specifically in an accredited microbiological food quality and safety laboratory.

During this internship I developed skills encompassing the common maintenance practices in a microbiology laboratory including equipment verification, temperature and air quality verification as well as sanitation procedures and subsequent verification while also being enlightened on quality control practices such as interlaboratory testing and duplicate assays. Besides this, I also solidified important basic microbiology skills including culture media preparation and sterilization as well as the different inoculation techniques (spread-plate, pour-plate, filtration technique), which were used in the standardized methods for the detection and enumeration of different microbiological groups including hygiene indicators and important pathogens. During this experience I had the opportunity to participate in most of the steps of analysis of foodstuffs and surface samples, from the sample reception, preparation, execution of microbiological assays with subsequent interpretation and register of results, followed by decontamination and disposal.

Another fundamental stage of this internship was the lunch bag food safety project which gave me the opportunity to perform different activities such as the development of a survey and subsequent analysis and interpretation. It also allowed me to develop some level of confidence and autonomy in the approaching volunteers and collecting samples, as well as the preparation and execution of the microbiological assays from these samples. From this work, I obtained a significant amount knowledge about the habits of lunch bag use in the analysed population, from which I concluded that many of the online and in-person survey participants did not follow the appropriate practices. Despite this, lunch bag sampling results showed that these bags had mostly lower microbial loads, indicating adequate hygiene conditions. This study did indeed evidence the need for population education in order to reduce the frequency of incorrect practices and assure safer use of lunch bags which has become an increasing trend.

In summation, this internship in CINATE was a fulfilling experience, both professionally and personally, allowing me to participate in a wide variety of tasks and activities and motivating my curiosity and interest in this field. This has been crucial in helping me establish the following steps in my professional career which I believe shall pass through working in the field of microbiological food safety and quality.

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Annexes

Annex 1 - Online survey pertaining to lunch bag use and related food safety practices created with GoogleForms. (Document in Portuguese language since it was directed to the Portuguese population).

Annex 1

Aspetos de segurança alimentar na utilização de lancheiras

Os portugueses recorrem cada vez mais ao uso de lancheiras e marmitas no transporte de refeições para consumir nos seus locais de estudo/ trabalho.
Este questionário, dirigido a toda a comunidade, pretende contribuir para o melhor conhecimento sobre aspetos de segurança alimentar relacionados com a utilização de lancheiras e marmitas.
Realiza-se no âmbito do projeto SafeConsumE, cuja consulta dos propósitos pode ser feita pela respetiva Página Web: http://safeconsume.eu/.

Obrigado pela colaboração!
Leandro Pereira
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*Obrigatório

Identificação sociodemográfica

1. **Concelho de residência** *

2. **Género** *
Marcar apenas uma oval.
- ◯ Masculino
- ◯ Feminino

3. **Idade (anos)** *

4. **Estado Civil** *
Marcar apenas uma oval.
- ◯ Solteiro/a
- ◯ Casado/a ou em União de facto
- ◯ Divorciado/a
- ◯ Viúvo/a

5. **Tem filhos?** *
Marcar apenas uma oval.
- ◯ Sim
- ◯ Não

6. **Se sim, quantos?**

7. **Que idades têm?**

8. **Qual o grau de escolaridade mais elevado que frequentou?** *
Marcar apenas uma oval.

- ◯ Sem escolaridade
- ◯ 1ºciclo (4º ano)
- ◯ 2º ciclo (6º ano)
- ◯ 3º ciclo (9º ano)
- ◯ Ensino secundário
- ◯ Curso técnico-profissional
- ◯ Bacharelato
- ◯ Licenciatura
- ◯ Mestrado
- ◯ Doutoramento
- ◯ Outra:

9. **Concluiu esse grau?** *
Marcar apenas uma oval.

- ◯ Sim
- ◯ Não

10. **Situação ocupacional/profissional** *
Marcar apenas uma oval.

- ◯ Estudante
- ◯ Trabalhador-estudante
- ◯ Desempregado/a
- ◯ Reformado/a
- ◯ Trabalhador por conta de outrem
- ◯ Trabalhador por conta própria
- ◯ Outra:

11. **Qual a profissão atual ou última desempenhada?** *

12. **Qual das seguintes descrições se aproxima mais do que sente relativamente ao rendimento atual do seu agregado familiar?**
Marcar apenas uma oval.

- ◯ O rendimento atual permite viver confortavelmente
- ◯ O rendimento atual dá para viver
- ◯ É difícil viver com o rendimento atual
- ◯ É muito difícil viver com o rendimento atual

Dados sobre o uso de lancheira

13. **Utiliza lancheira?** *
Marcar apenas uma oval.

- ◯ Sim
- ◯ Não *Após a última pergunta desta secção, pare de preencher este formulário.*

14. **Prepara mais alguma lancheira para além da sua?** *
Marcar apenas uma oval.

- ◯ Sim
- ◯ Não

15. **Se respondeu "sim" indique para quem**

_____

16. **Qual a frequência com que usa a lancheira?** *
Marcar apenas uma oval.

- ◯ Diariamente
- ◯ 1 vez por semana
- ◯ 2 vezes por semana
- ◯ Em ocasiões especiais
- ◯ Outra: _____

17. **Se assinalou "ocasiões especiais", indique quais:**

_____

_____

_____

_____

_____

18. **Que tipo de lancheira possui?** *
Marcar apenas uma oval.

- ◯ Contentor plástico
- ◯ Contentor metálico
- ◯ Saco Térmico
- ◯ Saco Neopreno
- ◯ Outra: _____

19. **Se assinalou "outro", descreva**

_____

20. **Tem revestimento térmico?** *
Marcar apenas uma oval.
- ◯ Sim
- ◯ Não
- ◯ Não sabe

21. **Tem alguma indicação sobre o tempo ou temperatura de conservação?**
Marcar apenas uma oval.
- ◯ Sim
- ◯ Não
- ◯ Não sabe

22. **Se respondeu "sim", qual a indicação?**

_____

23. **Tem compartimentos para os diferentes alimentos?** *
Marcar apenas uma oval.
- ◯ Sim
- ◯ Não

24. **Se respondeu sim, como os usa?**

_____

_____

_____

_____

_____

25. **Quanto custou a lancheira (aproximadamente)?** *

_____

26. **Há quanto tempo utiliza a lancheira?** *
Indique, sendo o caso, meses e anos.

_____

27. **Quem a prepara ?** *
Marcar apenas uma oval.
- ◯ próprio
- ◯ familiar
- ◯ Outra: _____

28. **Que produtos costuma transportar na lancheira?** *
Marcar tudo o que for aplicável.

- [ ] Garrafas de água ou outras bebidas
- [ ] Sandes/Pastelaria
- [ ] Refeições cozinhadas
- [ ] Saladas
- [ ] Iogurtes/ Gelatinas
- [ ] Fruta
- [ ] Outra: _____

29. **Durante quanto tempo os alimentos são mantidos na lancheira (desde que são colocados até ao consumo)?** *
resposta em horas

_____

30. **Utiliza acumuladores térmicos?** *
Marcar apenas uma oval.

- ◯ Sim
- ◯ Não
- ◯ Depende do tipo de alimentos

31. **Se respondeu " depende do tipo de alimentos" indique as situações em que usa**

_____

_____

_____

_____

_____

32. **Se respondeu "sim" ou "depende do tipo de alimentos" explique porquê**

_____

_____

_____

_____

_____

33. **Coloca alimentos diretamente dentro da lancheira? (ex:fruta)** *
Marcar apenas uma oval.

- ◯ Sim
- ◯ Não

34. **Se respondeu sim , indique quais:**

35. **Costuma reter lixo das refeições dentro dela?** *
Marcar apenas uma oval.

- ◯ Sim
- ◯ Não

36. **Se respondeu sim indique qual:**

Limpeza

37. **Com que frequência limpa a lancheira?** *
Marcar apenas uma oval.

- ◯ Depois de cada uso (todos os dias)
- ◯ >1 vez semana
- ◯ 1x semana
- ◯ Raramente
- ◯ Nunca

38. **Se limpa a lancheira após o uso, como procede?** *
Marcar apenas uma oval.

- ◯ Limpar com pano húmido
- ◯ Lavar com detergentes/desinfetantes e enxaguado com um pano
- ◯ Lavar à mão com água e detergente de louça
- ◯ Lavar na máquina de louça
- ◯ Outra:

39. **Durante o uso, alguma vez a lancheira entra em contacto com o chão?** *
Marcar apenas uma oval.

- ◯ Sim
- ◯ Não

40. **Se sim, explique**

Casos de doença gastrointestinal

41. **Teve recentemente alguma doença gastrointestinal?** *

Marcar apenas uma oval.

- Sim
- Não

42. **Se sim, indique que doença e quando**

Obrigado pela sua colaboração!