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**Novel insights on microbial communities relevant for
sustainability: metagenomic and culture-dependent
approaches**

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Abstract

Microbial communities are complex associations of microorganisms that coexist and interact with each other, interfering with the composition of the matrix they colonise. The study of microbiota can be conducted with conventional microbiological methods and/or with high-throughput sequencing techniques. In this thesis, the microbiota of different matrices (cooked ham packaged in a protective atmosphere (MAP), larvae of *Hermetia illucens*, and inflorescences of *Cannabis sativa*) was characterised by metagenomic analysis and/or classical microbiology techniques. In addition, a physiological and functional characterisation of *Leuconostoc carnosum*, a frequent colonizer of meat products, species still poorly studied, was performed. Thanks to the disclosure of *L. carnosum* genome, it was possible to resolve the phylogenetic relationships within the genus *Leuconostoc* through a phylogenomic approach.

A combined metagenomic and culture-dependent approach was used to study the evolution of the microbiota of cooked ham in MAP, in order to determine the secondary shelf-life. The quality of the cooked ham after opening, during 12 days of storage at 4 °C, was assessed through microbiological, molecular, sensory, and chemical analyses. The study highlighted a great variability in the composition of the microbiota, mostly associated to the producer, confirming that during the shelf-life the product is already abundantly colonised by a rather complex community.

Biochemical, physiological, and functional features of *L. carnosum*, frequently isolated from cooked ham and other meat matrices, were determined. This species can perform a dual function in meat products, either preserving or deteriorating them. Twelve strains that developed at temperatures between 4 and 37 °C and in the presence of NaCl up to 60 g/L, were analysed. Six strains produced exopolysaccharides responsible for the appearance of mucous exudates. A potential protective role of *L. carnosum* WC0321 and *L. carnosum* WC0323 emerged against *Listeria monocytogenes*. Three selected strains were tested for the ability to colonize a murine model, and their immunomodulatory properties were investigated. Despite the loss of viability during the passage through the gastrointestinal transit, the strains showed different immunomodulatory effects on dendritic cell maturation *in vivo*, suggesting a positive impact on the host's health.

Phylogenomic analysis of the genus *Leuconostoc* was carried out. 216 deposited genome sequences were subjected to phylogenomic analysis based on the Average Nucleotide Identity (ANI) and the core genome alignment, resulting in a proposal of phylogenomic reorganization of the genus.

Microbiological analysis of two diverse potential ingredients for novel foods/feeds and nutraceutical were carried out on larvae and prepupae of *Hermetia illucens*, and inflorescences of *Cannabis sativa*. The microbiota of larvae *H. illucens* fed with waste from agri-food industries has been determined, in order to develop innovative feed for laying hens. Three rearing temperatures of larvae were assayed to evaluate the influence on the insect microbiota. The higher temperatures favoured faster larval development and resulted in higher final biomass and mortality. The culture-dependent approach showed a high load of total mesophiles (10^8 cfu/g) and opportunistic pathogens, that can compromise the stability of the biomass and the safety of the product.

Inflorescences of 7 varieties of *C. sativa* for industrial purposes were analysed. They were collected and dried in two Italian locations, with the aim to produce biochemicals for manufacturing and cosmeceutical interest. Overall, the varieties collected in Rovigo showed higher contamination in total aerobes. Furthermore, the presence of *Salmonella* was highlighted in the samples obtained from Rovigo and Acireale.

Keywords: Microbiota; secondary shelf-life; *Leuconostoc carnosum*; metagenome; *Cannabis sativa*; *Hermetia illucens*

Sinossi

Le comunità microbiche sono complesse associazioni di microrganismi che coesistono ed interagiscono fra loro, interferendo sulla composizione della matrice che colonizzano. Lo studio del microbiota può essere condotto con tecniche microbiologiche tradizionali e/o con tecniche di high-throughput sequencing. Questo progetto si è incentrato sullo studio del microbiota di matrici diverse (prosciutto cotto confezionato in atmosfera protettiva (MAP), larve di *Hermetia illucens* e infiorescenze di *Cannabis sativa*) con metodi metagenomici e di microbiologia classica. Inoltre, è stata eseguita una caratterizzazione fisiologica e funzionale della specie *Leuconostoc carnosum*, frequente colonizzatore di prodotti carnei, specie a tutt'oggi poco studiata. A partire dallo studio del genoma di *L. carnosum*, è stato possibile risolvere le relazioni filogenetiche all'interno del genere *Leuconostoc* attraverso un approccio filogenomico.

Un approccio combinato metagenomico e coltura dipendente è stato utilizzato per studiare l'evoluzione del microbiota del prosciutto cotto in MAP, durante la shelf-life secondaria. È stata valutata la qualità del prosciutto cotto confezionato in MAP dopo l'apertura del pacchetto, durante 12 giorni di conservazione a 4 °C attraverso analisi microbiologiche, molecolari, sensoriali e chimiche. Lo studio ha evidenziato una grande variabilità nella composizione del microbiota legata soprattutto al produttore, confermando che durante la shelf-life il prodotto è già colonizzato da una comunità complessa.

Sono state determinate le caratteristiche biochimiche, fisiologiche e funzionali della specie *L. carnosum*, a cui appartengono numerosi ceppi isolati da prosciutto cotto e da altre matrici carnee. Nei prodotti a base di carne, questa specie può svolgere una duplice funzione, preservante o deteriorante. Sono stati analizzati 12 ceppi, che sviluppano a temperature comprese fra 4 e 37 °C, in presenza di NaCl fino a 60 g/L. Sei ceppi producono esopolisaccaridi, responsabili della comparsa di essudati. È emerso un potenziale ruolo protettivo dei ceppi *L. carnosum* WC0321 e *L. carnosum* WC0323 nei confronti di *Listeria monocytogenes*. Tre ceppi selezionati sono stati saggiati per la capacità di colonizzare un modello murino e sono state studiate le loro proprietà immunomodulatorie. I ceppi, nonostante la perdita di vitalità durante il transito gastrointestinale, hanno mostrato diversi effetti immunomodulatori sulla maturazione delle cellule dendritiche *in vivo*, suggerendo un impatto positivo sulla salute dell'ospite.

È stata effettuata l'analisi filogenomica del genere *Leuconostoc*. Le relazioni evolutive di 216 sequenze genomiche depositate sono state confrontate sulla base dell'identità nucleotidica media (ANI) e sull'allineamento del genoma centrale. Lo studio ha portato ad una proposta di riorganizzazione del genere.

È stato analizzato il microbiota di larve *H. illucens* alimentate con scarti agroalimentari, nella prospettiva di produrre mangimi innovativi per galline ovaiole. Sono state testate 3 diverse temperature di allevamento delle larve per valutare l'influenza sul microbiota dell'insetto, utilizzando tecniche coltura-dipendenti per la valutazione del rischio microbiologico. Le temperature più alte hanno accelerato lo sviluppo larvale e determinato una maggiore biomassa finale e una maggiore mortalità. Le tecniche coltura dipendenti hanno evidenziato un'elevata carica di mesofili totali (10^8 ufc/g) e di patogeni opportunisti che possono compromettere la stabilità della biomassa e la *safety* del prodotto.

Sono state analizzate le inflorescenze di 7 varietà di *C. sativa* ad uso industriale, raccolte ed essiccate in due località Italiane, destinate alla produzione di *biochemicals* di interesse manifatturiero e cosmeceutico. Nel complesso le varietà raccolte a Rovigo presentavano una maggiore contaminazione in aerobi totali. La presenza di *Salmonella* è stata evidenziata nei campioni ottenuti sia da Rovigo sia da Acireale.

Parole chiave: Microbiota; shelf-life secondaria; *Leuconostoc carnosum*; metagenoma; *Cannabis sativa*, *Hermetia illucens*

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1. OVERALL INTRODUCTION

Microbial communities are complex associations of microorganisms that coexist and interact with each other, interfering with the matrix composition they colonize. When the environments of growth are food products, bacteria can grow even at high concentrations thanks to the abundant nutrients and to the favourable pH. Consequently, they can compromise the safety of the food, cause harmful infections, or contribute to the deterioration of the food. The microbiota study of food matrices can be conducted with conventional and/or high-throughput sequencing techniques.

1.1. ROLE OF MICROBIAL COMMUNITIES

The term "food safety" refers to a set of precautions that should be adopted during food production, handling, and distribution. The product intended for humans is expected to be satisfactory, harmless, and healthy, to preserve its intrinsic characteristics, and to ensure the safety (Daskin, 2019). This last characteristic might be satisfied by adopting measures to prevent food from causing damage to health, being a carrier of pathogenic germs or toxic substances. However, food security is a permanent emergency and a real challenge. Many risk factors linked to the contamination exist. The raw materials, both of vegetable and animal origin, can be carriers of pathogenic microorganisms, toxic substances, agrochemical residues deriving from plants, drugs, additives, environmental contaminants such as heavy metals, mycotoxins, and processing-induced chemicals (Bleotu et al., 2018; Kamala and Kumar, 2018).

Microorganisms reach food as a result of contamination and find in it, rich by its nature in nutrients, an environment suitable to develop and multiply and consequently, over time in favourable environmental conditions, determine a series of alterations (Maukonen et al., 2003).

For these reasons, the European Union has matched the activities related to the official control of food products, prevention of public health risks, protection of the consumers, and avoiding of food fraud. In Italy, monitoring of food safety provides the official control of food destined to national and international markets. These control activities are carried out along the entire production chain, from primary production to transformation, storage, transport, trading,

administration, and consumption (Lasztity, 2009). The complete controls of the product include inspections, sampling, laboratory analyses on the production environment and the staff, and checks on the application of the HACCP practices that companies supply to identify critical points of the production chain (Mortimore and Wallace, 2013). Catering, farms, meat and flour processing are the most controlled and supervised areas.

Microbiological analyses represent an essential tool for assessing the level of safety and hygiene of food (Nauta, 2002). These analyses aim to establish the number and/or the presence of specific microbial populations (particularly bacteria called "indicators") and/or of a particular microorganism in food. The bacteria that make up the food can exert a role as pro-technologic, probiotic, pathogenic, anti-technological, or spoilage microorganisms. A fundamental phase of microbiological analysis is the preparation of the sample, which must be representative of the whole product to be analyzed, and attention must be paid to possible contamination by exogenous microflora (Mucchetti and Neviani, 2006).

1.1.1. Microbial communities of food matrices

Microorganisms for their growth require favourable conditions such as temperature, acidity, presence or absence of oxygen, water, and abundant nutrients for survival (Kilcast and Subramaniam, 2000). The higher the index A_w (water activity), the higher bacterial proliferation will be in any food matrix. Likewise, if high concentrations of nutrients are available in a food, the growth of a wide range of microorganisms is favoured (Cocolin et al., 2004).

Several microorganisms grow in abundance in food matrices without both being harmful to the consumer or altering the organoleptic properties. On the other side, specific spoilage microorganisms can cause food spoilage (called SSOs) (Cocolin et al., 2004; Benson et al., 2014; Fougy et al., 2016). Food matrices can be seriously compromised by the growth of foodborne pathogens such as *Listeria monocytogenes*, *Escherichia coli* O157: H7 (and other pathogenic enterotypes), *Yersinia enterocolitica*, and *Salmonella* spp., which cause severe damage to the consumer's health (Nyhan et al., 2018; Huang et al., 2018; Giovannini et al., 2004; De Cesare et al., 2007; Joutsen et al., 2012; Mir et al., 2018).

The growth of food pathogens occurs when there is poor hygiene created along each step of production (Mir et al., 2018; Kamala and Kumar, 2018). As a result, many of these bacterial species manage to end up in the final product before packaging and work to proliferate (Mills et al., 2014).

However, most food colonizers are not a danger for the consumer because they are neutralized by the acidity of pH when they reach the gastrointestinal tract (Zhu et al., 2006; Rossi et al., 2016).

The product is not suitable for sale also when, without risks in terms of safety, the organoleptic properties are altered by the microbial growth caused by changes in temperatures or other environmental factors (Arvanitoyannis, 2012; Höll et al., 2016). The expiration date of the products is related to both the number and type of bacteria that should be present at the beginning of shelf-life and their subsequent evolution depending on storage and environmental conditions (Iulietto et al., 2015). As shelf-life progresses, products begin to develop negative sensory characteristics related mainly to smell and colour, unwanted changes in appearance, texture, and taste, as well as exudates (Benson et al., 2014; Fougy et al., 2016, Kamala and Kumar, 2018). Defects in food products depend on the metabolic activities of the species that prevail foods, in conjunction with chemical and biological reactions related to raw materials, such as lipid oxidation (Croguennec, 2016; Luong et al., 2020). Some naturally occurring bacteria, such as LAB (Lactic Acid Bacteria), may produce bacteriocins preventing the proliferation of spoilage strains or pathogens (Chen et al., 2020; Rattanachaikunsopon and Phumkhachorn, 2010).

1.2. MICROBIOLOGICAL CONTROL

1.2.1. Cultures dependent methods

Conventional methods that detect and identify microorganisms and microbial pathogens are mainly based on specific biochemical, phenotypic, and genetic features, which description allows correct identification and typification (Jing et al., 2017). Traditionally, the presence and concentration of microorganisms in a particular sample were analyzed with culture-dependent approaches. These approaches can be sensitive, inexpensive, and provide qualitative and quantitative information on the microorganisms tested; however, they are severely limited by the requested time (Gokulakrishnan and Vergis, 2013). At the beginning of the 19th century, both liquid and solid cultural techniques were used for growth, enumeration, isolation, and identification of microorganisms or of a specific species according to the detection of certain metabolites (Gill, 2017). Viable plate count still represents one of the most common methods to enumerate bacteria. Serial dilutions of cells are plated onto agar Petri dishes, which are then incubated so that colonies can grow. The initial concentration of bacteria is often easily calculated and is typically expressed

in terms of cfu (colony-forming unit)/mL or cfu/g. However, these methods often fail to characterize populations of microorganisms for which selective enrichment is required.

Stressed or weakened cells often need specific culture conditions to be recovered and become cultivable (Gokulakrishnan and Vergis, 2013). Culture-dependent methods are still used because they are inexpensive and performing, provide a correct estimation of the microbial diversity of a sample under reproducible conditions, allow detection of foodborne pathogens and estimation of the prevalence of some species. The main drawback of conventional methods lies in the inability to detect non-culturable cells (Justé et al., 2008). Even in sample food matrices, such as fermented foods in which cultivable microorganisms generally dominate, at least 25-50% of the active microbial community cannot be grown *in vitro* (Ampe *et al.*, 1999). Moreover, most of the culture media are rich sources of carbon compared to the substrates that are usually found *in situ*, and this could influence the composition of the microbial community cultivated in favour of eutrophic microorganisms that grow in the presence of high levels of nutritional factors (Nocker et al., 2007).

1.2.2. Culture independent methods

In recent years, food microbiology has benefited from advances in molecular biology by adopting new strategies to detect, identify, and monitor microbes in food (Ercolini, 2013). Molecular culture-independent techniques are now available to study microbial ecosystems, allowing interesting perspectives to define microbial communities' composition and demographic dynamics in various environmental niches soil, water and food (Giraffa et al., 2001). The advances in biotechnology have allowed the development of robust tests to quickly identify specific bacterial strains directly inside the sample through the analysis of their DNA and RNA. This approach compensates the lack of knowledge about the natural habitat of microorganisms and the difficulty of developing specific cultivation media and tools that reproduce the natural conditions in which bacteria grow (Ercolini, 2004). Now, it is possible to identify uncultivable microorganisms, such as many microorganisms of the gastrointestinal tract, simply by studying bacteria starting from target DNA or RNA (Temmerman et al., 2004; Su et al., 2012). Culture-independent techniques are used to determine food products' microbiota, fermentations, and spoilage processes, and for research of foodborne pathogens (Cocolin et al., 2013; Wang and Salazar, 2015). Analysis of DNA makes possible to define how many and which microbial species are present in a certain sample. At the same time, analyzing the RNA makes possible to understand which are the metabolically active species (Coppola et al., 2003). These methods are based on cloning and direct sequencing of DNA

fragments or amplifying target sequences (16S or ITS) using PCR. Polymerase chain reaction (PCR) is undoubtedly the most widely used technique among independent cultural methods, capable of amplifying specific segments of DNA using a pair of primers (Mubarak et al., 2020).

PCR can be used to amplify specific genes for taxonomic groups of microorganisms and to detect genes involved in the virulence of foodborne bacteria (Finlay and Falkow, 1988; Bej et al., 1994). Several culture-independent methods have been applied in studies microbial ecology or fingerprinting of strains. Among them, denaturing gradient gel electrophoresis (DGGE) or restriction fragment length polymorphism (RFLP) have been largely exploited (Spiegelman et al., 2005). In recent years, sequencing of 16S rRNA gene amplicons using Next Generation Sequencing have been widely applied to study microbial communities, in terms of taxonomical characterization of the strains and metagenome analysis by amplicon sequencing.

1.2.2.1. RAPD-PCR

Randomly Amplified Polymorphic DNA-PCR (RAPD-PCR) is a type of PCR widely used since the 1990s to assess intra-specific genetic variation (Welsh and McClellan, 1990; Meyer et al., 2001).

A sole primer 8-12 bases long is used for DNA amplification. It anneals randomly to DNA whenever it finds a complementary sequence, simultaneously amplifying the fragments from 1-10 genomic sites. The amplification creates short random amplicons with a size range around 0.5-5 kb, which can be detected by gel electrophoresis. The resulting amplification profile comprises several bands and represents a sort of fingerprint that characterizes the strain, allowing to distinguish strains belonging to the same species and identification of diverse biotypes. Commonly utilized single primers are the minisatellite derived from the core-sequence of the wildtype phage M13 (5'-GAGGGTGGCGGTTCT-3'), or the microsatellite sequence 5'-GACAGACAGACAGACA-3' (Meyer et al., 2001; Hadrys et al., 1992).

Polymorphisms occur for variations within the primer annealing sites and might be generated by length differences within the amplified sequence between primer annealing sites.

The most significant advantage of RAPDs is that it is quick and straightforward to assay and requires only low quantities of template DNA. The most relevant drawback of RAPDs is the low reproducibility, and highly standardized experimental procedures are needed because of the sensitivity to the reaction conditions.

1.2.2.2. 16S rRNA gene

The most used approach in the study of microbial diversity is the analysis of 16S rRNA genes. These genes are ubiquitous in prokaryotes, present in a variable number of cell copies, essential for cellular functions. They contain highly conserved sites alternating with variable sequences. The conserved sites can be used to construct universal primers for amplification. The variable sequences, allow to quantify the phylogenetic distance between bacteria, measure the microbial diversity in a particular environment, and perform a taxonomic classification of microorganisms. Three types of rRNA are found in prokaryotic ribosomes: 16S, 23S, and 5S. Initially, the study of microbial diversity was based on the analysis of 5S rRNA molecules. However, these short sequences of 120 nucleotides contained few informative sites useful for taxonomic classification (Armougom and Raoult, 2009). The 16S rRNA gene is about 1540 bp long and consists of highly conserved sequences alternated with nine variable sequences (V1-V9) that include hypervariable regions (Figure 1). The length of hypervariable regions varies approximately from 50 to 100 bp, while the most conserved tracts from 1 to a maximum of 11 bp (Petrosino et al., 2009).

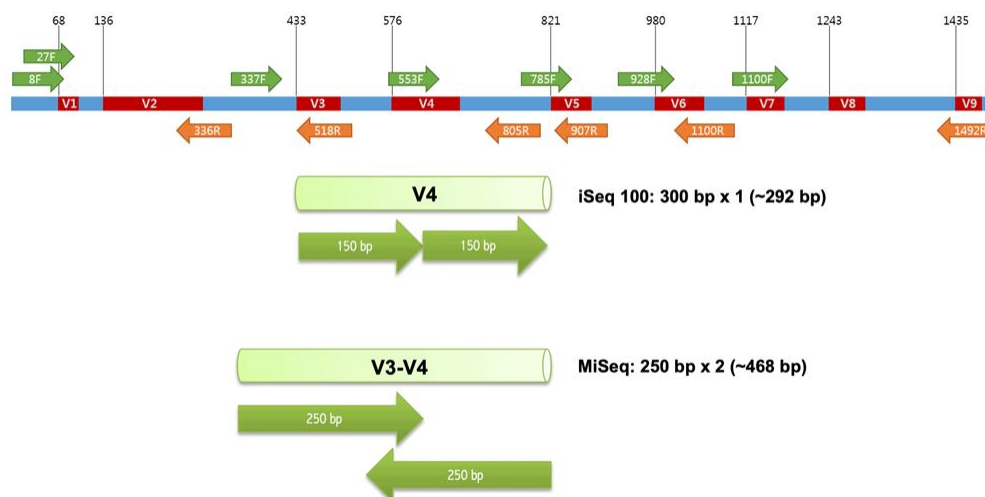


Figure 1 Structure of the 16S rRNA gene, in red the hypervariable and blue preserved regions are shown (<https://help.ezbiocloud.net>).

1.2.2.3. ITS gene

Ribosomes of eukaryotic organisms consist of two structural subunits, the 60S, and 40S made up of rRNA fragments and proteins. The 60S subunit (also called large subunit, LSU) consists of 28S, 5.8S, and 5S rRNA fragments and approximately 50 proteins. The 40S subunit features a single fragment of 18S RNA, also called the SSU (small subunit), and about 33 proteins. Upon translation of the mRNA, these subunits form 80S ribosomes (Areekit et al., 2008).

The genes that encode for the different rRNA fragments are repeated genes grouped into structural units within the chromosome, separated by non-coding spacer sequences, NTS (non-transcribed spacer) or IGS (intergenic spacer). Each structural unit consists of an external transcribed spacer (ETS) located at the 3' end of the 18S gene, and two internal transcribed spacers (ITS, internal transcribed spacer). ITS1 is located between the 18S gene and the 5.8S gene, and the ITS2 between the 5.8S gene and the 3' end of the 28S gene; the 5S gene is separated from the 28S gene by a non-coding space sequence. The 18S, 5.8S, and 28S genes are transcribed by DNA polymerase I into a 45S pre-rRNA, forming the rRNA fragments following maturation by eliminating the ETS and ITS spacer sequences. The 5S gene will be transcribed by DNA polymerase III into its corresponding rRNA (Guarro et al., 1999; Lourenço et al., 2003). Usually, the ITS1 and ITS2 regions are amplified to distinguish fungal species (Martin and Rygiewicz, 2005). The most common primers ITS1 and ITS4 amplify both ITS1 and ITS2 (Gardes and Bruns, 1993) (Figure 1.2).

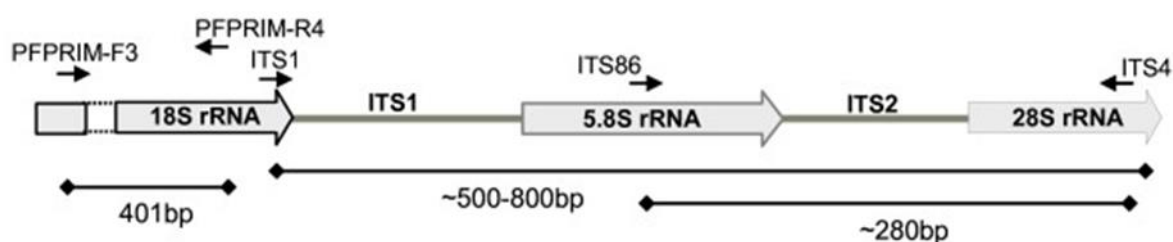


Figure 1.2 Structure of the ribosomal genes (Zunaina et al., 2008).

1.2.2.4. NGS techniques

Next-generation sequencing (NGS) techniques allow massive analyses of nucleic acids, facilitating obtaining more detailed information of microbial ecosystems. The breakthrough offered by NGS technologies is the ability to produce a large amount of data at an economical price (Metzker. 2010, Sarangi et al., 2019). The platforms currently used in sequencing projects are characterized by high throughput, sequence data accuracy (over 99% for some platforms), and substantially reduced costs compared to Sanger biochemistry-based capillary electrophoresis sequencers. The advantage of these technologies is the possibility to obtain the DNA sequence by amplifying the fragment without cloning it. These technologies can be used sequencing all the microbial nucleic acid (shotgun sequencing) or sequencing a specific gene (targeted sequencing/amplicon sequencing). For the latter approach, highly conserved DNA or cDNA segments are first amplified by PCR using universal or particular primers. Shotgun sequencing provides information on the phylogenetic composition, the number and potential function of specific genes (Campanaro et al., 2016).

The Sanger method (1977) was the first DNA sequencing technique capable of performing an analysis using pure DNA as starting material. However, this technique showed limitations, mainly related to the impossibility of being able to sequence samples containing mixtures of nucleic acids enclosed in a microbiota. In recent years, new sequencing technologies have been developed, among which the most used are 454 Roche pyrosequencing, Illumina and AB Solid.

454 Roche Pyrosequencing

The Genome Sequencer, commercialized in 2005 by 454 Science, was the first NGS sequencing platform available on the market. Sample preparation occurs by random DNA fragmentation into 300-800 bp (Margulies et al., 2006; Myllykangas et al., 2012). The first step consists of PCR amplification of the interest DNA region. During this phase, special primers are used, called fusion primers, which in addition to the target sequence for the gene of interest, have two adapters for the 454 Roche "A" and "B" sequencing, a short key sequence and a sequence Sample identification barcode (MID). Once the amplicons have been obtained, it is necessary to pool the samples under examination, creating a single library. Considering the MID used in amplification, the number of reads per sample to be obtained from the sequencing run, and the final concentration, the samples are mixed in an equimolar way until a single sample is obtained.

The amplicon library is subjected to a second amplification in an oil mixture (Figure 1.3). When emulsified, it creates micro-drops that constitute microreactors that contain only one Capture Bead and all the reagents for the amplification, including amplification enzymes. First, the ball is linked through adapter "B" to a single DNA template molecule. Then, the spheres bound to the strand are found in the aqueous phase of the water-in-oil emulsion for clonal amplification by PCR (Emulsion PCR, em-PCR) (Figure 1.3). It is a cyclic process in which DNA polymerase synthesizes the complementary strand, the double helix is denatured, and two new complementary strands are synthesized until tens of millions of copies of the fragment are obtained for each sphere (Shendure and Ji, 2008).

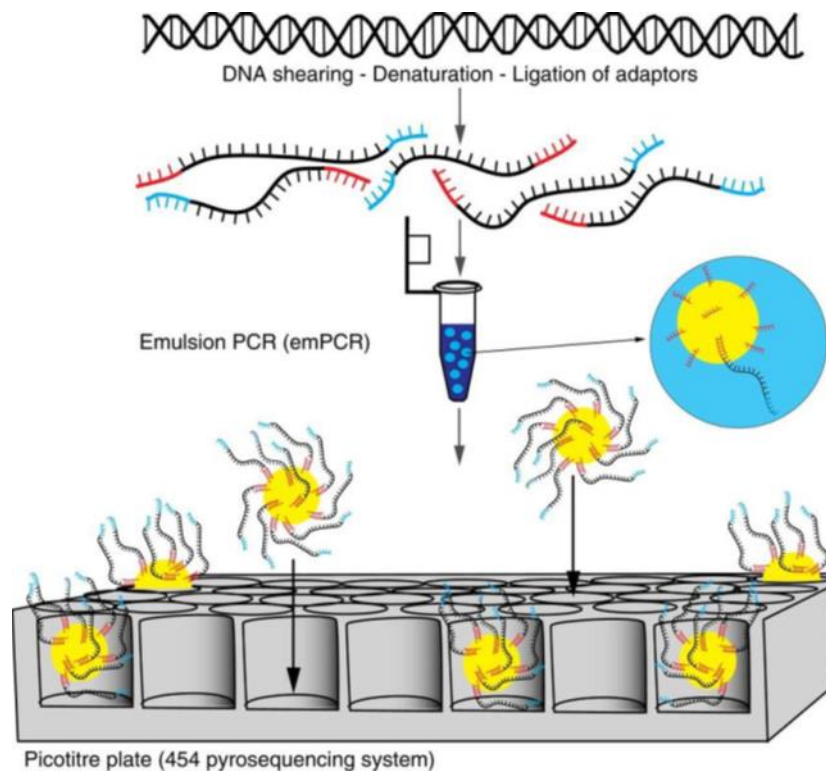


Figure 1.3 454 pyrosequencing, library preparation steps, emulsion PCR, PTP loading and sequencing (Wood et al., 2013)

At the end of the process, 10 to 50 million copies of dsDNA per bead will be present in each drop. Next, the DNA-free beads are removed by separating them from those bound to the magnetic beads (which have created a streptavidin-biotin bond with the enrichment primer and then with the capture beads) with a magnet. The denaturation of the bond between the double-strand takes place with the detachment of the enrichment primer bound to the magnetic beads and the recovery of the Capture Bead with the DNA, which is single-stranded.

The pyrosequencing involves the loading on the surface of a glass plate (Pico Titer Plate) of the beads bound to DNA, together with the enzymes sulfurylase and luciferase (Enzyme Beads), the PPases (PPase Beads), which eliminate the inorganic pyrophosphate (PP) and Packing Beads, which stabilize and keep all system components immobilized within the wells of the PTP plate during sequencing.

The spheres covered with the new filaments are placed on the sequencing plate. In the Roche 454 sequencer, the plate (called the PicoTiterPlate) has approximately 1.5 million wells, the diameter of which (44 μm) allows it to accommodate a single sphere. Genome Sequencer uses the pyrosequencing method (Marguiles M. et al., 2005), which involves completing the filament and the simultaneous reading of the signal emitted by the incorporated nucleotides. It is a cyclic process in which a single dNTP is added from time to time to the reaction. If dNTP is complementary to the template filament, its incorporation causes the release of a pyrophosphate, which is captured by the sulfurylase and converted into ATP, that triggers the luciferase reaction to light, and the emitted light signal is captured and measured by a CCD camera. Based on the number of incorporated nucleotides, more pyrophosphates and, therefore, more ATP molecules remain, generating more intense bioluminescence. Once the light intensity has been measured, it is possible to determine the number of nucleotides of the same type that follow one another in the sequence. For example, incorporating the AAA sequence will produce a light emission three times more intense than incorporating a single nucleotide A. If the dNTP is not complementary, it will not be included; no light emission will be generated; therefore, the absence of peaks indicates that dNTP is not present in the considered position. After recording the light emission, the remaining free nucleotides are degraded by apyrase, and the next dNTP is provided, continuing the polymerization reaction (Figure 1.4) (Breu, 2010).

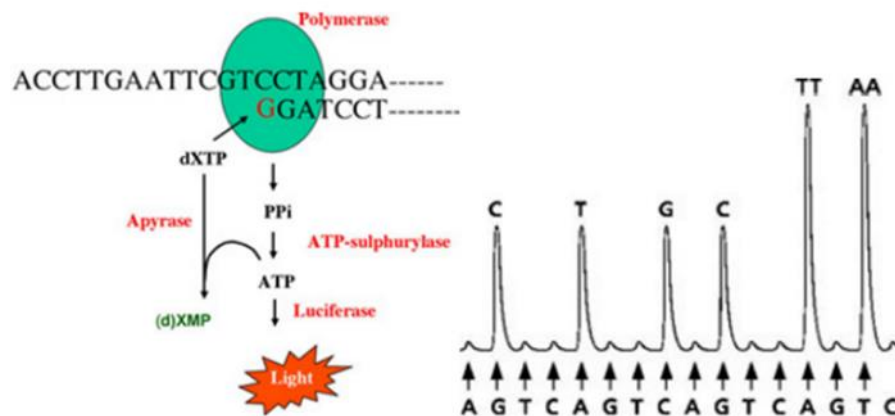


Figure 1.4 Representation of the pyrosequencing process. The incorporation of dGTP causes the release of a pyrophosphate which is converted into ATP by sulfurylase. ATP is involved in the bioluminescence reaction by luciferase which converts chemical energy into light energy. The light emission is captured by a CCD sensor and processed to obtain the nucleotide sequence. The unincorporated dNTPs and the ATP molecule are degraded by apyrase (Carlsson and Beldiceanu, 2004)

A single nucleotide base (A, C, G or T) is added to each cycle; at the following process, one of the remaining bases will then be introduced, repeating the introduction sequence for hundreds of cycles. The average error rate is between 10^{-3} and 10^{-4} , which is higher than the error rate of the Sanger method. Genome Sequencer allows to sequence 1.5 million spheres per run, determining sequences of variable length based on the number of sequencing cycles (limited to maintain the efficiency of polymerase and luciferase).

The advantage of the Roche 454 method is that reads up to 1000 bp long are easier to map having a reference genome: this is a positive aspect both for the assembly of a new genome and for metagenomic applications. In addition, the run time is relatively fast, around 23 h. On the other hand, there is a relatively low throughput of about 1 million reads and 100 Mb of data, combined with a high cost of reagents (Van Dijk et al., 2014).

Illumina sequencing

Illumina represents one of the most widely used NGS platforms (www.illumina.com). With this technology, numerous genomes of microorganisms, plants, humans, and animals have been sequenced over the years. This technology is based on reversible dye terminators added to the sequence by repeated cycles of polymerase-directed single base extension (Bentley et al., 2008). Contrary to the classic Sanger method, where the chain-terminator stops the sequence extension, the dye terminators used with Illumina are reversible, so they are removed after each imaging cycle, and a new nucleotide is added.

Technology

The sample preparation requires adding specific sequences as adapters to the ends of the DNA fragments. The production of the libraries begins with the fragmentation of the DNA, then enzymatic pull repairs the ends of the fragments, and adenines are added on the 3' end. The adapters are added, whose structure is unique for each end, and allow the DNA fragments to be immobilized on the rigid support. Immobilization occurs thanks to the hybridization between the adapters and the primers on the slide surface, that are complementary to the libraries. In this way, the DNA fragments form bridge structures that can start amplification through the PCR bridge, generating 100-200 million of physically separate clusters having a free end on which the specific primer for the sequencing reaction is hybridized (Metzker, 2010; Myllykangas et al., 2012) (Figure 1.5).

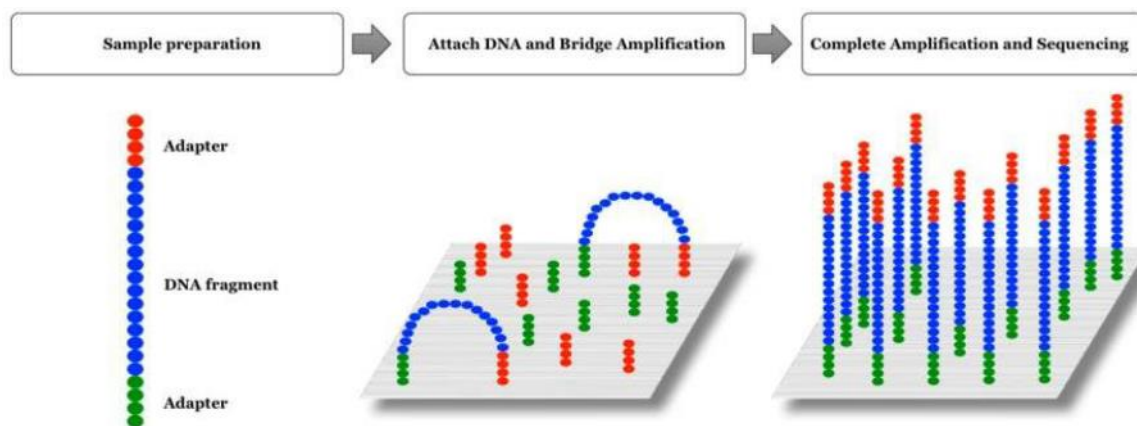


Figure 1.5 Fragments are attached to primers immobilized on a solid surface, by bridging amplification and generating DNA clusters with identical molecules (Garrido-Cardenas, et al., 2017).

In this platform, DNA sequencing is carried out by fluorescently labelled nucleotides which act as reversible terminators of the amplification reaction (Morozova et al., 2009). The four reversible termination nucleotides are simultaneously added and incorporated by the polymerase in each cycle. These nucleotides are chemically blocked by replacing the 3'-OH group with a 3'-azidomethyl group to prevent the polymerase from incorporating more nucleotides in each cycle. In the incorporation of a nucleotide, a fluorescence signal is emitted and measured with total internal reflection fluorescence (TIRF) using various laser channels. As for the next cycle, nucleotides that have not been incorporated are washed, and the chemical terminator of the 3' end is removed by the use of tris- (2-carboxyethyl) -phosphine to continue the synthesis of the chain (Bentley et al., 2008). With the high number of clonal fragments formed during amplification, the light signal is more intense and, therefore, more easily perceptible. When the labelled terminal nucleotides are inserted, the emitted signal will be visible by the CCD camera. Once the fluorescence signal is collected, a new cycle begins, repeating this dynamic until the sequencing of each fragment is finished. As previously indicated, the detection system used in the Illumina platform is Total Internal Reflection Fluorescence (TIRF), also known as evanescent wave microscopy. Its main advantage over other methods is that it can detect the fluorescence of molecules very close to a solid surface (glass or plastic) and is highly selective (Axelrod, 2001). With TIRF, it is possible to illuminate a very thin

layer of depth less than 100 nm, avoiding the excitation of other fluorophores that may be close but whose emission is not required for the measurement (Gould et al., 2011).

This same detection system is used by the Qiagen GeneReader platform, launched on the market in 2015. At present, the high sequence accuracy and low cost per Gb obtained, also for the large variety of equipment on the market, make Illumina sequencers the most widely used (Garrido-Cardenas et al., 2017). The length of the reads is about 300 bp, which is therefore compatible with most applications. On the other hand, sample preparation is particularly demanding due to the dispersion of the clusters within the flow cells, and the concentration of the library must be highly controlled.

HiScanSQ

HiScanSQ (Illumina, San Diego, CA) uses SBS chemistry to generate reads of 200 bp. Given the ability to sequence tens of millions of sequences in parallel, HiScanSQ technology is particularly suitable for sequencing whole genomes or transcriptomes. This approach uses reversible terminators to detect individual nucleotide bases as they are incorporated into the DNA strands to be sequenced. During each sequencing cycle, only one labelled deoxynucleotide triphosphate (dNTP) is added to the nucleic acid chain. The labelled nucleotide acts as a reversible terminator for the synthesis of the nascent DNA strand. After each incorporation, the fluorescence is detected by an optical system that allows the identification of the embedded base. Finally, the nucleotide is subjected to enzymatic cleavage to enable the incorporation of the next nucleotide. The HiScanSQ platform produces a maximum of 1.5 billion total reads per run, producing up to 150 Gb of output with an average of 60-70 million reads per sample when loaded into 3-sample pools per lane.

The SOLiD sequencer from Applied Biosystems

The SOLiD platform was introduced to the market in 2007 by Applied Biosystems. After denaturation of the double helix and the addition of universal adapters P1 and P2, the fragments are hybridized to complementary oligonucleotide primers, present on spheres, as in the Roche 454. However, in the SOLiD technology, the spheres are smaller (0.75 μm diameter instead of 28 μm), allowing a higher density of sequenced spheres per run.

The spheres are emulsified together with the PCR reagents and primers to obtain clonal amplification of the filaments, similar to the Roche 454 platform.

Unlike the latter technology, the SOLiD sequencing plate does not have wells; the spheres are instead bonded to the suitably treated surface of the sequencing plate by forming a covalent bond with the chemically modified ends of the amplified filaments.

The sequencing reaction is performed by DNA ligase, that covalently binds DNA. The acronym SOLiD means "Sequencing by Oligonucleotide Ligation and Detection". Each sequencing cycle involves the sphere, a sequencing primer, the ligase, and four dNTP probes. The probes are composed of 8 bases plus a fluorescent marker on the first two bases. The first bases of the probe are complementary to the template to be sequenced. (Garrido-Cardenas et al., 2017). Once the probe has paired with the template sequence, the marker linked to the probe is excited and the fluorescence emission is released. The instrumentation detects the light emission to identify the colour associated with the first two bases (Li and Homer, 2010). Each marker produces a specific light emission that identifies 4 of 16 possible pairs of nucleotides (Figure 1.6).

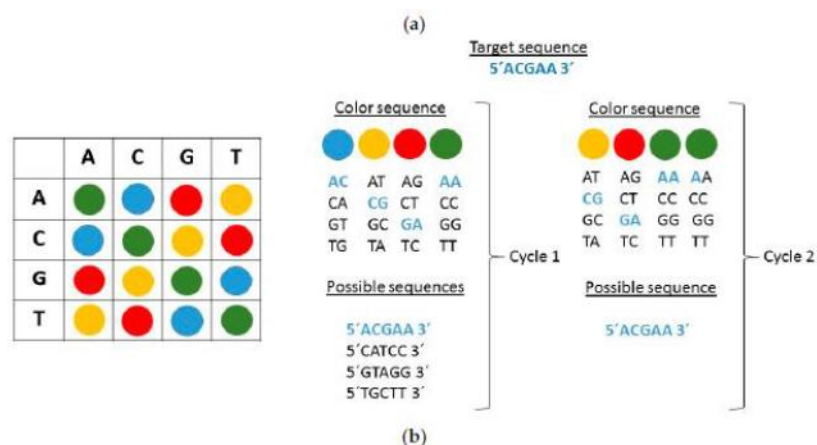


Figure 1.6 The possible combinations of nucleotides that the four colours of the fluorescent markers represent. Each colour does not uniquely represent a single pair of bases, but 4 out of the 16 possible combinations (Garrido-Cardenas et al., 2017).

After light detection, the fluorescent marker is removed and the cycle is repeated several times. The identification of nucleotide will be possible in a subsequent sequencing cycle by moving the primer to a defined position in the template sequence. After the pairing of the first probe, the process is repeated with the binding of new probes (Garrido-Cardenas et al., 2017). Overall, five sequencing runs are performed, each with the primer moved to a backward position each time. The five cycles allow for interrogating each sequence base twice (in two independent reactions with different primer positions) (Fig. 1.7).

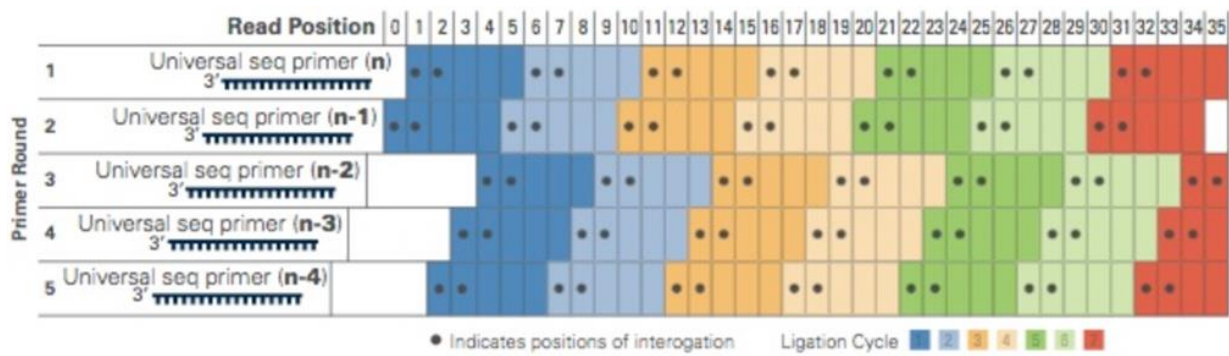


Figure 1.7 SOLiD sequencing scheme. The five sequencing cycles (one per row) are shown, each of which consists of six steps for adding new probes by the ligase. The black dots indicate the base pairs interrogated in each step (<http://seqanswers.com/forums/showthread.php?t=10>).

The colour of the fluorophore's emission allows the identification of the first bases pair, but it is not sufficient to distinguish one nucleotide from the other. In addition to the specific emission for each pair, one of the bases of the sequence must be known. The known base is incorporated in the last (fifth) sequencing cycle and corresponds to the last nucleotide of the primer. Therefore, since each colour represents four possible pairs of dNTPs and for each of them, the second nucleotide coincides with the first base of the next pair, knowing a base of the sequence allows us to interpret the whole sequence in its entirety.

1.3. METAGENOME BY AMPLICON SEQUENCING

In recent years, new techniques have been identified that allow the identification of microbial communities without proceeding with bacterial isolation or cultivation, that exploit the High-Throughput Sequencing (HTS) (Blow, 2008; De Filippis et al., 2013). The study of a microbial population without cultivation steps, when exploits sequencing of the genomes, is referred as metagenome analysis. It can use a single marker gene that is amplified and sequenced (Amplicon sequencing), or it is based on sequencing of all the genomes present in a sample (WGS Whole Genome Sequencing) (Połka et al., 2015; McLaren et al., 2019). Both the strategies need the direct extraction of the whole DNA from samples. On the other side, in amplicon sequencing, the DNA is amplified by PCR using the phylogenetic marker genes such as 16S rRNA for bacteria and ITS for fungi (McLaren et al., 2019). To gain information on microbial composition of the samples and to assess microbial diversity also among different samples, the sequences of the amplicons are

processed using specific bioinformatics tools such as Mothur (Schloss et al., 2009) or QIIME2 (Bolyen et al., 2019).

Amplicon sequencing presents a bias associated to the amplification step, that weakens the quantitative data. However, it is advantageous because of the lower costs and of the easier analysis, then it is the first choice to assess microbial composition, without targeting deeper investigation about functions. The raw sequences are cleaned and quality filtered before being clustered into ASV (Amplicon Sequence Variants) at 100% identity. ASVs are taxonomically assigned and those ascribed to the same taxon are aggregated into Operational Taxonomic Units (OTUs). SILVA (www.arb-silva.de) is the most common database used for this purpose (Quast et al., 2013).

Amplicon sequencing is important for detecting unique lineages, estimating diversity, and assessing the organization of microbial communities. However, it presents some drawbacks, such as a single sequence identity cut-off can be ineffective for distinguishing true taxonomic lineages at the species or genus level, it underestimates the number of substitutions when compared to multiple alignments, and it ignores the variability of the 16S rRNA gene or other conserved targets across the tree (Nguyen et al., 2016).

1.4. MICROBIAL DIVERSITY

Biodiversity is a tool to characterize and compare extremely diverse groups (Morris et al., 2014). Richness and evenness are two features of biodiversity. The number of distinct species encountered is characterized as species richness, which does not take into account the abundances of individual species. Species evenness, on the other hand, assesses how evenly dispersed the community is by measuring relative abundances of distinct species.

Whittaker (1972) developed three degrees of diversity: alpha, beta, and gamma diversity. At a local scale, alpha diversity (α) may simply be defined as the diversity of each sample. The changes in species composition across various samples are represented by beta diversity (β). The entire diversity of the sample is described by gamma diversity (γ), that may be connected to a mix of alpha and beta diversity.

1.4.1. Alpha diversity

The study of alpha diversity is based on investigation of two sample properties: the number of species (community richness) and the relative relevance of each species in the community. The creation of rarefaction curves (Gotelli and Colwell, 2011) in metagenomics determines if the sampling depth is adequate to define the microbial population properly. The formation of an increasing number of observed species may be predicted when more sequences linked to a specific sample are produced until no new species are obtained. This allows you to determine whether or not the sequencing was done thoroughly enough. Rarefaction curves express the predicted number of species in n samples randomly selected from the total number of samples in more detail. The number of OTUs or observed species does not grow with further sampling until the curves flatten, indicating that enough samples have been sequenced to correctly define the community (Fig. 1.8).

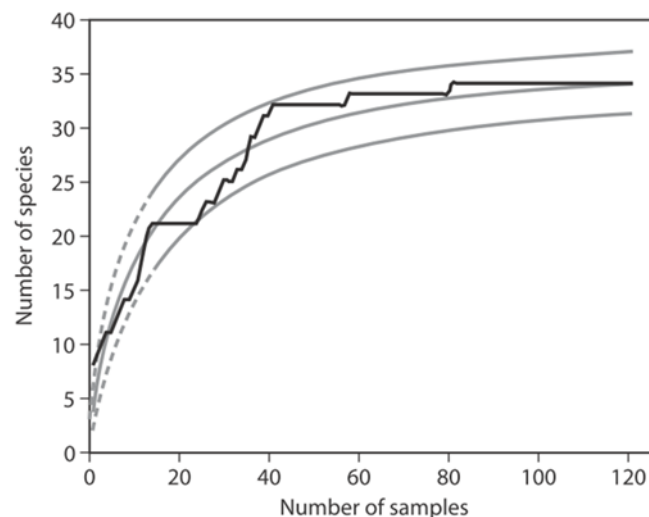


Figure 1.8 Rarefaction and accumulation curves depending on samples and individuals (Gotelli and Colwell, 2001).

Different indices, classified into three categories: species richness indices, heterogeneity indices, and phylogenetic diversity indices, can be used to conduct an alpha diversity study (Guisande et al., 2017). Heterogeneity indices use both richness and evenness in their calculations. The evolutionary history of taxa is measured using phylogenetic diversity indices.

Species richness indices: Chao1 Index

Chao1 is a unique count index that is based on the number of rare classes (OTUs) found in a given sample (Chao, 1984). The Chao1 index estimates the number of undiscovered species based on the idea that uncommon species can provide the most information about the number of undetected species. Chao1 derives it from the numbers of singletons (species with just one occurrence), doubletons (species with two occurrences), and observed species, which are calculated using the following equations:

$$S_{es} = S_{obs} + \frac{a^2}{2b}$$

where S_{obs} is the total number of observed species in a given sample, a is the number of singletons, and b is the number of doubletons. As a result, this index is particularly effective for describing data sets with low-abundance classes, such as microbial communities, where a significant percentage of species are simply transitory or pollutants and might be readily overestimated.

Heterogeneity index: Shannon's Index

Another parameter widely used to describe species diversity in a community is the Shannon diversity index (H). It considers both the abundance and evenness of the current species (Peet, 1974). H, in particular, is derived from the expression:

$$H = - \sum_{i=1}^S p_i \log_2 (p_i)$$

where S is the number of species and p_i is the proportion of the species i within the sample. The higher value of this index, the more varied the community. As both the richness and the evenness of the data grow, the Shannon index rises. In a community with only one species, the Shannon index would be 0, p_i would be 1, and $\log p_i$ would be zero. In contrast, if species are spread uniformly, the index value will be high. As a result, the H value allows researchers to determine not only the number of species present, but also how their abundance is distributed among all species in the community.

Evenness index: Pielou's index

The Pielou's index (Pielou, 1966) is a metric for determining how equally species are distributed in a population. It is computed using the formula below:

$$J' = \frac{H'}{H'_{max}}$$

where H'_{max} is the maximum possible value of H' , which is obtained from the Shannon diversity index. J' values vary from 0 to 1, with 0 indicating no evenness and 1 indicating an equal distribution of species in the sample.

1.4.2. Beta diversity

Beta diversity (β) describes the diversity among different microbial communities. A high beta diversity value indicates minimal resemblance across species from various sites/communities. There are two types of beta diversity indices: qualitative (unweighted) and quantitative (weighted). Only the availability or lack of species determines the qualitative measurements. Quantitative metrics, on the other hand, are based on the number of times each species has been observed (relative abundance).

There are two types of beta diversity metrics:

- Non-phylogenetic beta diversity metrics like Jaccard and Bray-Curtis distances;
- Phylogenetic beta-diversity metric like unweighted and weighted UniFrac distance.

Jaccard distance and Bray Curtis dissimilarity

The Jaccard distance is a measure of how different two sets of samples are. It is derived by dividing the difference between the sizes of the union and the intersection of two sets by the size of the union, as indicated in the formula below, or by subtracting the Jaccard coefficient from 1.

$$d_J(A,B) = 1 - J(A,B) = \frac{|A \cup B| - |A \cap B|}{|A \cup B|}$$

The Bray-Curtis dissimilarity (Bray and Curtis, 1957) is a method for determining how different two samples are in terms of species population. It's calculated using the formula below:

$$BC_{ij} = 1 - \frac{2C_{ij}}{S_i + S_j}$$

where i and j are the two samples, S_i and S_j are the total number of species counted at each site, and C_{ij} is the sum of only the lower count for each species found at both sites. It has two possible limit values: 0 when the samples are identical and 1 when the samples are completely separate sets with no shared members.

UniFrac Measures

UniFrac is a β -diversity index that compares different samples taking into account phylogenetic information. UniFrac considers phylogenetic distances between observed species for calculating the relatedness of community members. UniFrac variants (weighted and unweighted), are frequently employed in microbial ecology. This distance may be computed using the following formula:

$$distance = \frac{\sum unshared\ branch\ lengths}{\sum shared + unshared\ branch\ length}$$

Duplicate sequences do not contribute to the branch length since this index is unweighted. As a result, any information related to abundance is ignored. A variant of the unweighted index is the weighted UniFrac distance. It is a β -diversity quantitative measure since it assesses distances on the basis of abundance. The UniFrac value (u) is as follows:

$$u = \sum_i^n b_i \times \left| \frac{A_i}{A_T} - \frac{B_i}{B_T} \right|$$

where n represents the total number of branches in the tree, b_i represents the length of branch i , A_i and B_i represent the number of sequences from branch i in A and B samples, respectively, and

A_T and B_T represent the total number of sequences in the considered microbial communities. The average distance of members of the two communities to the tree's root may also be used to normalize the distance u . For identical communities, the final result will be 0; otherwise, it will be 1 (Lozupone and Knight, 2007).

1.5. MULTIVARIATE DATA ANALYSIS: PRINCIPAL COORDINATE ANALYSIS (PCoA)

Principal Coordinates Analysis (PCoA) plots may be generated using the distance matrices derived in QIIME2 (Bolyen et al., 2019) using the various beta-diversity measures (Legerde, 1998). PCoA is a common unconstrained ordination approach that allows to explain the majority of the data variance with fewer variables than the original ones. It is quite similar to PCA (Jolliffe, 1986), with the primary distinction being that PCoA permits visualization of data included in the distance matrix since it calculates locations in the multidimensional framework using distances between samples.

The distances between the N subjects are first determined as a $D_{N \times N}$ matrix, which is then translated into the Δ matrix:

$$\Delta = -\frac{1}{2} D$$

The Δ matrix is centered so that the new axis system origin (specified with the additional variables) is in the centroid of objects, resulting in a P matrix:

$$C_{ki} = \delta_{ki} - \frac{1}{N} \sum_{p=1}^N \delta_{kp} - \frac{1}{N} \sum_{q=1}^N \delta_{qi} + \frac{1}{N^2} \sum_{p=1}^N \sum_{q=1}^N \delta_{pq}$$

The average row and column of the Δ matrix are represented by the second and third terms, respectively, while the average of the matrix is represented by the last term. The eigenvalues λ_i ($i=1,2,\dots,m$; $m \leq N-1$) and eigenvectors u_{ki} ($k=1,2,\dots,N$; $i=1,2,\dots,m$) of the P matrix are also computed, by multiplying the eigenvectors by the square root of the respective eigenvalues, the f_{ki} main coordinates are obtained:

$$f_{ki} = \sqrt{\lambda_i} u_{ki}$$

Even in this instance, an ordination diagram with samples represented as points and axes associated with the new PCoA variables may be constructed. A distance matrix depicts the dissimilarity between samples and reflects the relationships among distinct microbial communities by calculating each distance between each pair of samples.

2. OBJECTIVES

This thesis focuses on studying complex microbial communities associated with food matrices applying conventional and metagenomics approaches. The main aim of the work proposed in this thesis was to exploit the microbiota of novel and potential food matrices than can be exploited as ingredients or as sources of bioactive molecules. Several diverse products have been analyzed, all with the common denominator of being foods or having the potential to become a resource to develop novel nutraceuticals. Microbiological survey of these products aims to assess the potential risk associated to these foods, in order to both control possible outbreaks and prevent food waste.

Sliced cooked ham packed in modified atmosphere has been a first goal of this investigation. Microbiota has been explored after opening of the packages, during the conservation in the fridge, with the aim to determine a secondary shelf life of this food, meaning the shelf life after the opening.

The microbial and pathogenic microbes of 5 different cooked ham brands purchased in a local supermarket was studied during the secondary shelf-life. The microbiota analysis was conducted using dependent and independent culture methods. The latter was based on NGS techniques, essential for having a more comprehensive analysis of the bacterial population. Through the Illumina platform, the sequencing of the 16S rRNA gene regions was obtained, each OTU was taxonomically characterized and quantified in terms of relative abundance, obtaining the composition profile of the whole microbial population. The culture-dependent method was based on the isolation of microorganisms on selective medium plates, clustering by RAPD-PCR and taxonomic designation by partial sequencing of the 16S rRNA gene or ITS1 region. The study was completed by an organoleptic analysis of the samples and a chemical investigation of organic compounds.

This project is the follow-up of another study conducted in the UNIMORE MIBI lab, aimed to study the evolution of sliced cooked ham microbiota packaged in modified atmosphere over the shelf life (before opening), during which *Leuconostoc carnosum* was identified as a major contaminant (Raimondi et al., 2019). For this reason, the PhD project was extended to the investigation of phenotypic traits and immunomodulatory properties of *Leuconostoc carnosum* isolated from meat products. This study complements a counterpart focused on genomics of

Laeconostoc carnosum, to which I contributed, not reported in this PhD thesis (Candeliere et al., 2020, 2021). *L. carnosum* is a dominant species isolated from meat and meat products packaged in modified atmosphere or vacuum, such as cooked ham and fresh sausage (Raimondi et al., 2018, 2019). *L. carnosum* plays an important role during the storage, where it reaches a concentration up to 108 cfu/g. Albeit being a species that colonized meat-based food products, little attention was dedicated to the physiological characteristics. Twelve strains of *L. carnosum* have been isolated and characterized in the UNIMORE MIBI lab. The tests were carried out regarding the metabolic features, such as the ability to produce acid lactic from carbohydrates, the resistance at different stress conditions, and the growth rate at different temperatures. In addition, the potential impact on the health of these strains was investigated. Three selected strains were challenged for the capability to colonize a mouse model and their immunomodulatory properties verified.

Genome disclosure of *L. carnosum* opened the perspective to resolve the phylogenetic relationships within the genus *Leuconostoc* through a phylogenomic approach, through ANI, dDDH, and core genome comparison. At present, the genus encompasses 16 recognized species: *L. carnosum*, *L. citreum*, *L. falkenbergense*, *L. fallax*, *L. gelidum*, *L. holzapfelii*, *L. inhae*, *L. kimchi*, *L. lactis*, *L. litchi*, *L. mesenteroides*, *L. miyukkimchii*, *L. palmae*, *L. pseudomesenteroides*, *L. rapi*, and *L. suonicum*. 216 deposited genome sequences were subjected to phylogenomic analysis based on the Average Nucleotide Identity (ANI) and the core genome alignment. The phylogenetic relationships within the genus were compared also on the analysis of 16S rRNA, pheS, and rpoA genes. Other parts of this project focused on microbial analysis of two diverse foods: larvae and prepupae of *Hermetia illucens*, and inflorescences of *Cannabis sativa*. In recent years, increasing attention has been paid to new food sources with a high protein concentration capable of replacing meat products in the future (Bruno et al., 2018), and insects represent a real perspective as a source of highly nutritious ingredients. The microbial composition of *Hermetia illucens* prepupae has been analysed. *H. illucens* is an insect that is easy to rear on large scale and characterized by a protein rich composition. The study was performed at three different growth temperatures, considering this parameter as a potential factor affecting the microbial composition of the insect, posing safety concerns for human health. Furthermore, larval biomass of *H. illucens* was processed to a meal product with stability over time was verified searching pathogens that can affect the health of laying hens.

On the other side, cannabidiol (CBD) enriched products starting from inflorescences of *Cannabis sativa* are attracting a wide interest, justified worldwide by the scientifically demonstrated health beneficial effects of CBD for the treatment of many disorders. Finally, the study of

microbiota has been extended to inflorescences of seven *Cannabis sativa* varieties, harvested for the extraction of biochemicals of medical and cosmeceutical interest. The samples analysed were cultivated and harvested for 3 years in two Italian locations. The potential hazardous microorganisms contaminating the hemp inflorescences were analysed. The analysis were carried out according to the guidelines of pharmacopoeia 9.8.

3. Project 1

Survey of microbiota of sliced cooked ham during the secondary shelf-life

3.1. INTRODUCTION

3.1.1. Secondary shelf-life

According to Food and Agriculture Organization (FAO), food production in the world is higher than the demand, resulting in an amount of 1.3 billion tons of wasted food per year, which are related to the consumption of around 3.3 billion metric tons of CO₂ and 250 km³ of water and the utilization of 1.4 billion hectares of cultivated crops (FAO, 2013). This phenomenon is also reflected in a series of economical, ethical, and social repercussions, considering that 11% of the world's population has limited access to food resources (Wohner et al., 2019).

Significant food losses may occur along the food supply chain, from producer to consumer (Salihoglu et al., 2018), precisely, if it occurs along the food chain, is recognized as 'food losses', while if it takes place in the last part of the food chain (distribution, sale, and final consumption) it is called 'food waste' (FAO, 2013). The EU aims to reduce food waste by 2030, according to the Sustainable Development Goals (SDGs) (Wohner et al., 2019; Abdelradi, 2018). The amount of waste generated differs according to the type of food, with the major amount of post-harvest losses occurring in plant-based foods. However, the highest cost in terms of wasted resource per amount of aliment is associated with animal-based foods, which have the highest value of water footprint (Mekonnen & Gerbens-Leenes, 2020). Up to 61% of this waste is produced downstream of the chain, especially at the household level (UNEP, 2021). The quantity of food waste that originated in the domestic environment is not equally distributed among countries: it is minimum in the developing countries and abundant in the rich ones, where waste reaches about 50% of losses, an amount corresponding to the food production of South Africa (Gustavsson et al., 2011). This is attributed to the diverse availability of edibles, that determines variation in consumers' behavior. The large availability of food, together with the greater awareness of health and hygiene risks

associated with the consumption of altered food, means that in the populations of the first world, food is not consumed beyond the expiration date (Ojha et al., 2020). As a solution to household food waste, the size of the packages could be reduced also if the approach will lead to an increase in the size of packaging waste materials, with a strong environmental impact (Nicosia et al., 2021).

For conserved foods, the time from opening the package until the product becomes unacceptable, generally in terms of sensorial properties, is called “secondary shelf-life” or period after opening (PAO). It is mandatorily reported on package labels of only some foods, and it indicatively informs the consumers in terms of use after opening. However, such indications do not have any scientific evidence; the consumer often tends to reject foods that are still suitable for consumption (Nicosia et al., 2021). For example, in Europe, about 27% of losses are attributed to meat waste (Gustavsson et al., 2011) and are produced by consumer rejection caused by spoilage or minimal undesirable features (Iulietto et al., 2015).

Worldwide, the changes in consumers' lifestyles have led to vast consumption of meals-ready-to-eat (Bonifacie et al., 2021); among the ready-to-eat products, cooked ham is of great importance. Sliced cooked ham in modified atmosphere packaging (MAP) is a main product of the meat processing industry. Although cooked ham undergoes cooking at least 70 °C, bacterial proliferation occurs towards the end of the shelf-life even after maintaining hygienic precautions and preservative procedures (Raimondi et al., 2019).

This study evaluated the secondary shelf-life of five Italian commercial brands of MAP cooked ham, simulating domestic storage. Detailed information on the evolution of the products, including microbiological, sensory, and chemical features, was collected for 12 d after the opening of packages to estimate the effective secondary shelf life in a domestic refrigerator.

3.1.2. Cooked ham

Cooked ham is among the most popular and consumed cured meats in many countries and is consumed as a ready-to-eat product or through gastronomy preparations. For this reason, its impact on the nutrient supply in the human diet is very significant (Válková et al., 2007; Toldrá and Reig, 2016; Ramarosan et al., 2018).

Salt, an indispensable ingredient for any cured meat, is contained in cooked ham at concentrations of approximately 1.9%. However, this recent feature results from twenty years of

activity aimed at reducing the salt content, which was present at levels above 2% in the past (Barbieri et al., 2016).

Cooked ham is obtained from the rear leg of pork, suitably boned and trimmed (possibly skinned, defatted and denervated) (Toldrá and Reig, 2016). It is subjected to salting using a wet process and a heat treatment sufficient to give it the characteristics of a cooked product or determine the coagulation of proteins (Toldrá and Reig, 2016). In general, cooked hams have to satisfy several quality features such as not having structural defects (absence of cavities, cracks, good seal of the slice), having a pink color, being soft to chew, and having a typical flavor and aroma (Desmond et al., 2002; Válková et al., 2007).

Production process

Figure 3 shows the summary diagram of the typical production process for obtaining this food product. The production process of cooked ham begins with the boning of fresh ham, removing the thigh bones, and eliminating the tendinous muscle part, soft fat, and intramuscular connective tissue. The cutting ends with the trimming of the pigskin and the storage of hams hanging from the hooks (Clement et al., 1995).

After selecting the pork legs, the most critical procedural phases are those associated with the salting meat.

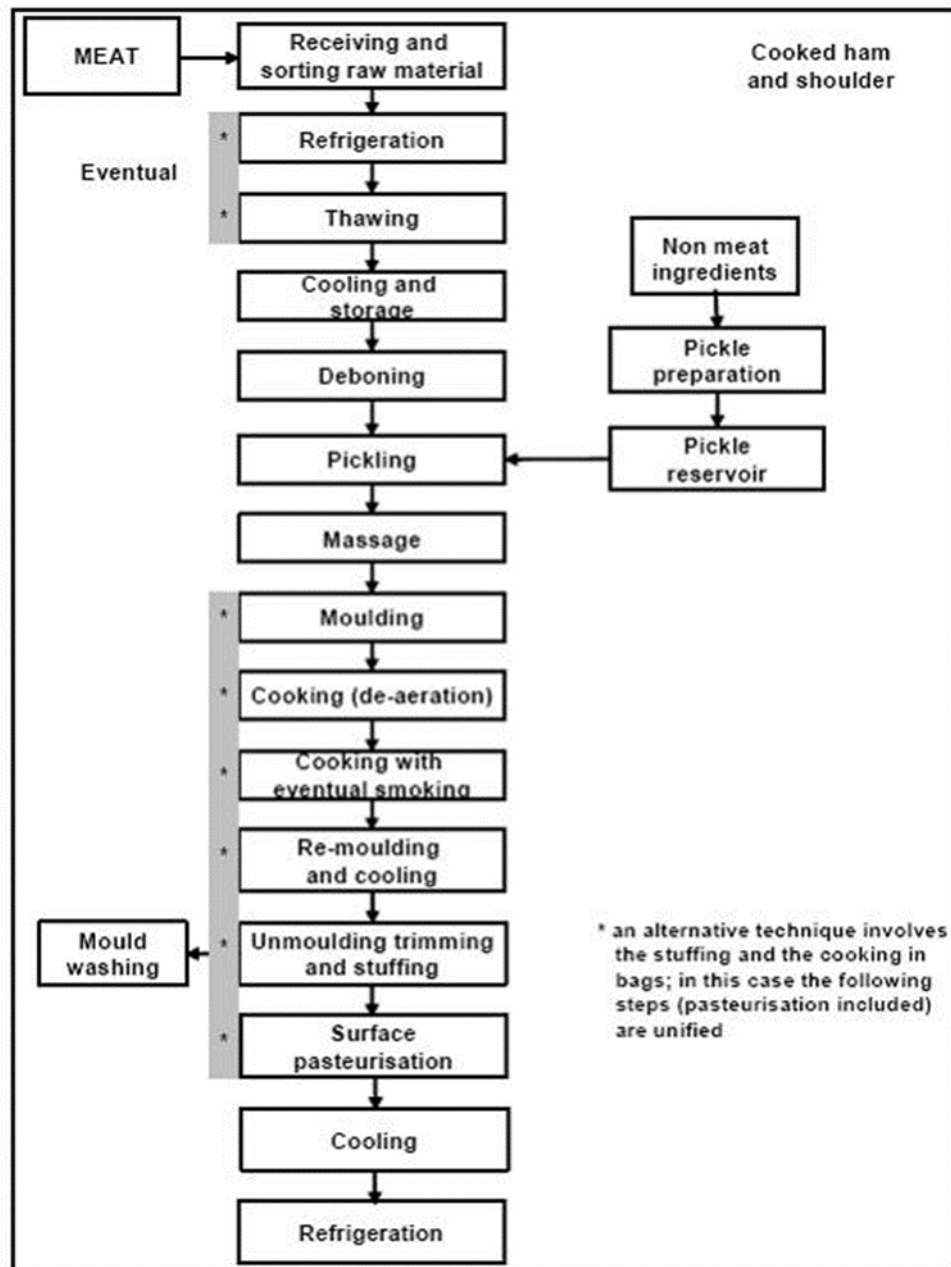


Figure 3. Production process scheme for cooked ham (Clement et al., 1995)

3.1.3. Microbiological characteristics of fresh meat

In a living and healthy animal, muscle masses are sterile until at the time of slaughter. Following slaughter and evisceration, high bacterial contamination of the carcass occurred from the skin, visceral, and the environment; it is therefore closely related to the hygienic conditions in which the process was conducted. In 1949 Ingram proposed dividing the microbial flora present on meat into two groups:

- Extrinsic bacteria occur in the surface parts of meat. They represent the predominant portion of meat bacteria and derive mainly from the environment for contamination of the atmosphere and equipment and manipulation operations (Korkeala et al., 1990; Makela et al., 1991; Samelis et al., 2000; Gill 2005; Pearce et al., 2006);

- Intrinsic bacteria occur in the deep tissues of healthy animals. They derive from the gut of slaughtered animals and reach muscle tissue through internal pathways before or after the animal's death. Several authors have challenged the hygienic importance of these bacteria, showing the absence of microbial cells in the deep muscle tissue of animals slaughtered under normal conditions (Gill, 1979).

In table 2 were reported the main parameters that affect the microbial growth in cooked ham.

Table 2. Parameters that affect microbial development. They are divided into two groups: intrinsic parameters and extrinsic parameters.

Parameters affecting microbial development	
Intrinsic parameters	Extrinsic parameters
Activity water (Aw)	Temperature of conservation
pH	relative humidity
redox potential	Presence and concentration of gas in the conservation environment
Nutrient content	
Presence of compounds	Presence and activity of other microorganisms
antimicrobials	
Biological structures	

In the case of cooked meat products, the heat treatment of raw materials, generally conducted at a minimum temperature of 65-75 °C, allows eliminating all vegetative cells; however, germ spores belong to the genus *Clostridium* and *Bacillus* may remain unchanged (Kadam et al., 2012; Ramaroson et al., 2018). Besides, if heat treatment is not carried out most appropriately, it may be insufficient to eliminate the most thermotolerant bacterial species such as Enterococci (Ramaroson et al., 2018).

3.1.3.1. Intrinsic parameters

Water, energy sources, nitrogen sources, vitamins, and minerals, as well as pH are essential for all microorganisms to develop and survive in food products. Meat products are rich in proteins, lipids, vitamins, and mineral salts but low in carbohydrates; this composition allows the growth of some bacterial (altering) species rather than others (Pond et al., 2004). Microorganisms need, for their growth, minimum values of A_w . In general, Gram-negative bacteria are more demanding than Gram-positive bacteria. For example, in cooked meat products, the value of A_w is generally greater than 0.94; this allows the growth of most microorganisms (Aymerich et al., 2000). The pH is one of the most relevant parameters in selecting bacteria within a food product. Most microorganisms grow at neutral pH values (6.6-7.5). Cooked meat products have a high pH (about 6), favoring deterioration processes due to faster growth of altering bacteria (Ray and Buhia, 2013; Aymerich et al., 2000). Moreover, for the growth of bacteria into the meat, another parameter was the redox potential (Eh). In general, to grow, aerobic microorganisms need a positive Eh (oxidizing atmosphere) while anaerobic microorganisms need an oxygen-depleted atmosphere (Eh negative) (Jay et al., 2009). Microaerophilic bacteria grow better under slightly reducing conditions. This group includes some lactobacilli whose growth is favored in cooked meat products, whose Eh is between +90mV and -50mV (Cenci-Goga, 2012).

Within food, naturally occurring antimicrobial substances or chemical additives further contribute to the selection of species that can develop. In recent years, the use of conventional antimicrobial chemical additives, such as nitrates and nitrites, has been preferentially reduced and the utilization of natural substances with antibacterial activities has attracted interest, including essential oils of plant origin, bacterial cultures, and/or metabolites (bacteriocins). Therefore, their use in cooked meat products might improve the microbiological stability and safety (Doulgerakiet al., 2012; Ercolini et al., 2010; Skandamis Nychas , 2001).

3.1.3.2. Extrinsic parameters

The storage temperature affects the duration of the latency phase, the maximum growth rate, and the final number of cells. Low storage temperatures result in a decrease in bacterial growth rate and favor the development of psychotropic species (optimal growth temperature between 4 °C and 7 °C) such as the Gram-positive lactic acid bacteria and the Gram-negative bacteria *Pseudomonas spp.* (Doulgeraki et al., 2012). Mesophilic species (optimal growth temperature 30 °C/40 °C) such

as staphylococci, micrococci, and mesophilic lactobacilli are generally unable to grow in food products since they are stored at refrigeration temperature. Still, they could develop if the other growing conditions are optimal. The thermophilic bacteria (optimal growth temperature between 55 °C/65 °C) of greater food interest belong to the genera *Bacillus* and *Clostridium* and are particularly interested in the canning industry (Jay et al., 2009). In vacuum-packed cooked meat products, the dominant bacterial population is composed by lactic acid bacteria; however, the growth rate of each genus is affected by temperature: lactobacilli prevail at temperatures between 4 °C and 7 °C while at lower temperatures, *Carnobacteriaceae* dominated (Ray and Bhunia, 2013).

The relative humidity of the storage environment is crucial as it affects both the *A_w* of food and the development of microorganisms on the surface. A food with a low *A_w* value must be stored under relative humidity conditions, such as not allowing the absorption of water from the environment since this would favor the increase of *A_w* and, therefore, microbial development. On the other hand, a food characterized by a high value of *A_w*, placed in an environment with low relative humidity, tends to lose moisture at the expense of the organoleptic qualities of the product so that, in order to limit surface contamination and deterioration of the food, without compromising its sensory characteristics, it is necessary to modify the storage atmosphere without lowering the relative moisture value (Cenci-Goga, 2012).

Moreover, the packaging conditions and consequently the gaseous composition inside the food product have a decisive influence on the microflora composition of the product. The presence of high concentrations of O₂ promotes the development of *Pseudomonas spp.*, which is considered the main cause of meat products spoilage stored in aerobic conditions. Vacuum or modified atmosphere results in a high concentration of CO₂ in the gaseous composition inside the packaging, this favors the development of facultative anaerobic species such as LAB to the detriment of aerobic species (Cenci-Goga, 2012).

3.1.4. Microbiota of MAP cooked ham

Packaging in a modified atmosphere is a method to preserve and extend the shelf life of cooked ham, reducing the O₂ content inside the trays with increasing CO₂ and nitrogen. However, the residual oxygen promotes the growth of several microorganisms that proliferate inside the packages in some cases, causing alteration of the product (Smiddy et al., 2002). The microbiota of cooked ham in MAP is characterized by an abundance of microorganisms that are found in this type of matrix the environment ideal for growth. Among the organisms that populate cooked ham, the

most representative are lactic acid bacteria with a widespread environmental diffusion and are widely present in slaughtering and meat processing environments, finding good development conditions in various meat products (Hu et al., 2009; Slongo et al., 2009; Zhang et al., 2009; Kreyenschmidt et al., 2010; Vercammen et al., 2011; Sliekers et al., 2015) (see chapter 4).

Part of the natural flora can develop at refrigeration temperature in addition to lactic acid bacteria, other psychrotrophic bacteria that colonize cooked ham are *Pseudomonas*, *Aeromonas*, *Alteromonas*, *Enterobacteriaceae*, and *Brochothrix thermospha* (Garcia-Lopez et al., 1998; Vasilopoulos et al., 2008; Piotrowska-Cyplik et al., 2017; Sliekers et al., 2017; Raimondi et al., 2019).

The proportions of these different bacterial groups depend on various factors, such as the pH of the meat, storage temperature and oxygen permeability of the packaging plastic film (Luong et al., 2020). In general, heat treatment destroys natural flora except for spores and thermophilic bacteria (André et al., 2013). In addition, salt, nitrite, and refrigeration help further inhibit the surviving flora development (Rawat, 2015). Nonetheless, *Bacillus* spp. and cold-tolerant *Clostridium* spp. survive at the high cooking temperature and become dominant species in MAP cooked ham (Piotrowska-Cyplik et al., 2017).

The cooked ham contamination occurs in the following stages of cooking and, therefore, with the operations slicing and packaging. For this reason, superficial pasteurization is always performed, which results, however, ineffective against thermophilic bacteria (Holley, 1997).

Another common microorganism found in cooked ham is *Listeria monocytogenes*. This species is considered a contaminant during the tumbling of cooked ham (Samelis et al., 1997). High-pressure treatment (HP) (approximately 100-700 MPa, 5-40 °C, for 10 minutes) in conjunction with natural preservatives has been exploited to inactivate diverse spoilage microorganisms, including pathogens, and to extend the shelf-life of cooked ham products (Vercammen et al., 2011). The HP treatment did not affect the final product's sensory properties, such as colour, and allowed for a nine-week delay in spoiling. Although this method has advantages on most pathogenic bacteria, some lactic acid bacteria can survive even at high pressures (Han et al., 2011).

The composition of the microbiota of cooked ham is highly variable depending on various factors such as variances in raw materials, meat additives, and processing procedures throughout the countries (Tomović et al., 2013).

3.1.5. Spoilage microorganisms of MAP cooked ham

Several studies demonstrated over the years the altering role of LAB in cooked ham (Vasilopoulos et al., 2008; Kreyenschmidt et al., 2010; O' Neill et al., 2018; Raimondi et al., 2019). These alterations are generally caused by enterococci and heterofermentative LAB such as *Leuconostoc*, *Weissella*, *Lactobacillus*, *Carnobacterium*, (Vasilopoulos et al., 2008; Hu et al., 2009; Pathakos et al., 2015; Raimondi et al., 2019).

The products of their metabolism trigger the alterations for which they are responsible: lactic acid, acetic acid, ethanol, propionic acid, and CO₂ that can compromise the sensory characteristics of the product until it is unacceptable for consumption (Vermeiren et al., 2004).

LAB may be responsible for the sensory alteration of the product in which they are present, especially when reached a high concentration. They produce acetic acid and lactic acid, whose accumulation can make the contaminated product unacceptable for consumption. The concentration of these compounds within the product depends on several factors, such as the availability of substrates, the species and strains peculiar behavior, the different metabolic capacities, and the charge with which they are present. Several studies have shown that LAB charges upper than 7 log₁₀ ufc/g are associated with sensory alteration of the contaminated product (Nychas et al., 2008; Vermeiren et al., 2004; Korkeala and Bjorkroth, 1997). A correlation has also been shown between the rate of acidification exercised by a certain LAB species and its relative growth rate. The species that grow most rapidly (species belonging to the genus *Leuconostoc*) have a greater capacity for acidification (Vermeiren et al., 2004). Besides, lowering pH due to the accumulation of organic acids can lead to a reduction in the ability of proteins to retain water resulting in accumulation in the packaging of a milky liquid (Diez et al., 2009).

The production of other volatile organic compounds such as butanoic acid and acetoin is also responsible for off-flavours (Krockel., 2013). *Brochothrix thermosphacta* causes alteration of both fresh and processed slices of meat on which it develops, giving rise to the formation of smelly metabolites, in particular acetoin and diacetyl, which can accumulate in the product until it is inedible (Kilcher et al., 2010; Gribble et al., 2013).

The increase in the volume of packages is among the alterations that occur most frequently in vacuum-packed cooked meat products. It is the result of CO₂ concentration rise inside vacuum packaged products attributable to metabolic by-products of heterofermentative lactobacilli such as *Lactobacillus brevis*, *Lactobacillus fermentum* and *Leuconostoc mesenteroides* (Hernández-Macedo et al., 2012, Grazia et al., 2011). During the production process, other microorganisms belonging to

the family of Enterobacteriaceae are considered as spoilage bacteria of cooked ham, indicating a poorly hygienic condition. Yeasts and molds might occur in MAP cooked ham when the product is stored at low temperatures and have a low pH and activity water. Both enterobacteriaceae and yeast and mold can alter the quality of products causing gas production that has as consequence a reduction of shelf life (Rawat, 2015). The metabolism of *Clostridium* spp. might produce H₂S, contributing to product alteration.

Another factor of quality loss consists of a mucous formation and viscous patina on the product surface. The main species capable of causing such alteration are *Leuconostoc gelidum*, *Leuconostoc carnoum*, *Latilactobacillus sakei*, and *Latilactobacillus curvatus* (Pothakos et al., 2015). The viscous patina formed by these bacteria consists of exopolysaccharides (EPS) whose production is favored under cellular stress conditions and is associated with a protection mechanism implemented by bacterial cells against dehydration, phagocytosis, the attack of bacteriophages, toxic compounds, and antibiotics or, to the biofilms formation that allow cells to the environment surfaces adhesion in which they are located (Ullrich, 2009).

Colour change is one of the most frequent and easily visible alterations. It is related to the ability of some LAB, *Leuconostoc*, *Weissella viridans*, *Latilactobacillus sakei*, enterococci, and Carnobacteria to produce and accumulate hydrogen peroxide (Doulgerakiet al., 2012). In contact with oxygen, therefore, at the time of opening the packages, hydrogen peroxide causes the oxidation of nitrogen oxide + myoglobin (nitrosomyochromogen), a pigment responsible for the pinkish color of cooked meat, giving the product in contact with O₂ greenish color (Doulgerakiet al., 2012). Among the species belonging to the genus *Carnobacterium*, which all produce hydrogen peroxide, only *Carnobacterium divergens* and *Carnobacterium viridans* were associated with the greening surface in cooked hams (Holley et al., 2002). In addition to lactic acid bacteria, other microorganisms such as Coagulase-negative staphylococci are present in a large variety of meat products (Baird-Parker, 1963) They are widespread in the environment, the main habitats being represented by the skin of mammals, and easily contaminated the product during slaughtering or the production phases characterized by human involvement. While many micrococcaceae may cause alteration of color in MAP cooked ham (Lorenzo et al., 2012), some others, belonging to the genus *Staphylococcus* (*S. xylosus*, *S. simulans*, and *S. carnosus*) are used as starter cultures in meat products (Sánchez Mainar et al., 2016).

3.2. AIM OF THE PROJECT

A considerable waste and loss of food have been documented, with relevant environmental, social, economic, and food safety impacts (Abdelradi, 2018). Consumers reject several food products, also before the expiring date, for the presence of spoilage and undesirable characteristics (Iulietto et al., 2015). Worldwide, the changes in consumers' lifestyles have led to wide consumption of meals-ready-to-eat, mainly cooked ham in MAP (Bonifacie et al., 2021). In this project, we focused on the secondary shelf life of MAP cooked ham. Several studies investigated microbiota and sensorial features over the shelf-life of MAP cooked ham or unopened packages (Vasilopoulos et al., 2008; Kreyenschmidt et al., 2010; O' Neill et al., 2018; Raimondi et al., 2019), using conventional or metagenome approaches. On the other side, the evolution of microbiota after the opening of the package, during the period of fridge conservation, has never been addressed. This study aimed to investigate the secondary shelf-life of MAP cooked ham (five Italian commercial brands) after opening the package, simulating a domestic consumption. A detailed description of the microbiota composition and diversity throughout the secondary shelf-life was assessed combining a culture-dependent method to precisely trace LAB, total aerobic, molds, enterobacteria and pathogen bacteria biotypes, and 16S rRNA gene profiling. Dominant strains were isolated on plates, genotyped by RAPD-PCR fingerprinting, and were given a taxonomic assignation by partial sequencing of the 16S rRNA or ITS genes. Cooked ham samples were analyzed at the opening of packaging and for the following 12 days, during which they were stored at 4 °C. Defects were evaluated for all the samples, and the prevalent VOCs were determined by SPME coupled with GC-MS.

3.3. MATERIALS AND METHODS

Five brands of MAP sliced cooked ham, 3 packages for each brand, were purchased in local markets (Modena, Italy). All the samples had the same expiration date ($\pm$ 3 days) and at least two weeks of shelf life left. Table 3 lists the ingredients as well as their nutritional values.

Microbiological, chemical, and sensory investigations were performed when the packages were opened (0 d), then every four days (4, 8, and 12 d). Cooked ham samples were stored in plastic bags in the same household refrigerator set at 4 °C. A probe and data logger were used to monitor and record the temperature profile (mini TH, XS Instruments, Carpi, Italy).

Culture-dependent enumerations, genomic DNA extraction, bacterial strains biotyping by RAPD-PCR, chemical analyses were carried out at UNIMORE MIBI lab. 16S rRNA and ITS genes sequencing and metagenomic 16S rRNA gene profiling were performed by Eurofins Genomics (Germany). Bioinformatic analyses were completed at UNIMORE.

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and all the media were purchased from BD Difco (Franklin Lake, NJ, USA).

Table 3. Nutritional composition and list of the ingredients corresponding to the 5 brands of sliced cooked ham in MAP retrieved from the local market.

Brand	A	B	C	D	E
Nutrition facts (g/100g)					
Total Fat	6.0	16.0	6.0	2.0	2.0
of wich Saturated	2.8	6.4	1.2	0.8	0.8
Total Carbohydrate	1.1	< 0.5	0.5	1.0	1.1
of wich Sugars	0.5	< 0.6	0.5	1.0	1.1
Protein	23.0	16.0	18.0	20.0	20.0
Salt	2.3	1.8	2.0	2.0	1.9
Ingredients					
	pork leg	pork leg	pork meat	pork leg	pork leg
	Salt	salt	Salt	salt	salt
	dextrose	dextrose	dextrose	dextrose	dextrose
	sucrose	sucrose			
		spices			
	Flavors	natural flavors	natural flavors	natural flavors	flavors
	Sodium	Sodium	Sodium	Sodium	Sodium
	ascorbate	ascorbate	ascorbate	ascorbate	ascorbate
	(E301)	(E301)	(E301)	(E301)	(E301)
	Sodium nitrate	Sodium nitrate	Sodium nitrate	Sodium nitrate	Sodium nitrate
	(E250)	(E250)	(E250)	(E250)	(E250)
Suggested Storage Temperature (°C)	0 – 4	1 - 4	0 – 4	0 - 4	0 - 4
Suggested Secondary Shelf life (days)	1	-	1	2 - 3	2 - 3

3.3.1. Sensorial evaluation and spoilage assessment

After fridge storage, at each time point, a slice of cooked ham was sampled and let to reach room temperature for 15 min; then, sensory assessments were performed by a trained sensory panel in order to determine the presence of the primary sensory defects. Descriptors of odour, texture, and colour were assigned a score between 0 and 5, the limits indicating the most and less desirable level, respectively.

3.3.2. Chemical analysis

A puncture electrode (Sension+ electrode 5233, HACH, Switzerland) was used to measure pH in 5 different points per sample. Organic acids, glucose, and ethanol were determined by HPLC, according to Vasilopoulos et al. (2008). The protocol consisted of homogenizing 10 g of cooked ham with four volumes of 4.5 mmol l⁻¹H₂SO₄. The mixture was centrifuged (4 °C) at 8000g for 15 minutes. Fats were removed by diethyl ether treatment. Subsequently, a 1:1 dilution with a 20 % (v/v) dilution of trichloroacetic acid (TCA) was prepared to remove proteins, and the mixture was centrifuged (4 °C) at 20000g for 15 min. A Jupiter 5 µm C18, 90 Å, 2 × 250 mm column (Phenomenex, Torrance, CA, USA) was used with 10 mmol l⁻¹ H₂SO₄ as mobile phase at a flow rate of 0.6 ml min⁻¹. The column temperature was kept at 60 °C.

Following the protocol of Raimondi et al. (2018), volatile organic compounds (VOCs) were monitored using solid-phase microextraction (SPME) coupled with gas chromatography-mass spectrometry analysis (GC–MS). A Carboxen-Polydimethylsiloxane fiber (Sigma Aldrich, St. Louis, MO, USA) was exposed for 1 hour at 25 °C to the headspace of a 10-mL vial containing 2 g of the material. The volatiles were desorbed in the injector of a GC–MS apparatus (7820–5975; Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-624 column by thermic desorption at 240 °C (Agilent Technologies). Separation was obtained with 1.3 mL/min helium flow and a temperature gradient of 2 min isotherm at 50 °C, 6 °C/min increase to 110 °C, 10 °C/min increase to 240 °C, 4 min isotherm at 240 °C. The volatiles tentative assignment was based on comparing their retention times and mass spectra to those from the National Institute of Standards (NIST) mass spectral library (2014).

3.3.3. Microbiological analyses

Using a Lab Blender Stomacher (Seward Medical, London, UK), 10 g of cooked ham were suspended and homogenized in saline peptone water (8.5 g/L NaCl and 1 g/L peptone) at the concentration of 10% (w/v) for 4 min. For the enumeration and isolation of bacteria and yeasts, appropriate decimal dilutions in saline peptone water were spread on agar plates: Plate Count Agar (PCA) for total aerobic bacteria, Lactobacilli MRS Agar for lactic acid bacteria (LAB), Violet Red Bile Glucose Agar (VRBGA) for enterobacteria, and Rose Bengal Chloramphenicol Agar (RBCA) for yeasts, and Baird-Parker Agar (BPA), for putative staphylococci. The plates were incubated in aerobiosis at 30 °C for 48 h, except for RBC plates incubated at 25 °C for 5-7 days and MRS plates incubated anaerobically at 30 °C for 72 h, with anaerobiosis generated by GasPaK (BD Difco). To identify the presence of *Salmonella* sp., the initial suspension was pre-enriched for 24h at 30 °C, 100 µL were seeded in 10 mL of Rappaport Vassiliadis soy peptone broth (RVS, Soy peptone 4.5 g, MgCl₂ · 6H₂O 29 g, NaCl 8 g, K₂HPO₄ 0.4 g, KH₂PO₄ 0.6 g, green malachite 0.036 g, pH adjusted at 5.2), and incubated for 24 h at 41 °C. The enrichment culture was then seeded on Xylose Lysine Deoxycholate (XLD) plates and incubated for 24 h at 37 °C.

Extraction of genomic DNA

A representative number of colonies (up to 48 colonies per sample per medium) were randomly picked up from PCA, MRSA, RBCA, VRBGA, and BPA and streaked in the same medium. Instagene Matrix (Bio-Rad Laboratories, Redmond, WA) was used to extract DNA useful as PCR template.

The procedure consists of the following steps:

- resuspended one colony of each isolate from Petri plate in a microtube containing 1 mL of sterile distilled H₂O;
- Stir for 30'' in a vortex;
- Remove the supernatant after centrifuging for 5' (13,000 rpm – 17,900 x g);
- Add 50 µl InstaGene Matrix to the pellet and vortex for another 30'' to resuspend it;
- Incubate for 30' at 56 °C;
- Stir for 2' in a vortex;
- Incubate the sample at 100 °C for 8';
- Stir for 2' in a vortex;

- Keep DNA frozen at -20 °C.

Before usage, DNA samples must be defrosted, vortexed, then centrifuged at 13,000 rpm (17,900 x g) for 5 minutes. Only the supernatant is picked up in the next RAPD process.

Bacterial strains typing by RAPD-PCR

In a 96-well plate, the RAPD-PCR reaction is performed in a final volume of 15 µl. A reaction mixture is produced for 100 samples by placing in a tube:

- 1,012.5 µl PCR Water Nuclease Free;
- 150 µl 10x Dream Taq Green Buffer (with MgCl₂ 2 mM);
- 15 µl dNTP mixture a concentration of 10 mM (dATP, dCTP, dGTP, dTTP);
- 7.5 µl M13 RAPD primer a concentration of 100 µM (M13 RAPD, which sequence is 5'-GAGGGTGGCGGT TCT-3');
- 15 µl Thermo Scientific DreamTaq DNA Polymerase (5 U/µl, 500 U).

Finally, 12 µl of the mixture were poured into the well, followed by 3 µl of the centrifuged DNA template were added.

The amplification reaction was performed with a MyCycler thermal cycler from Bio-Rad configured to alternate the standard PCR cycles as follows:

	Temperature	Time	Cycle
Denaturation	94 °C	3 min	1
Annealing	94 °C	30 sec	30
	58 °C	30 sec	
	72 °C	30 sec	
Extension	72 °C	7 min	1

Agarose gel electrophoresis (2 % in TAE Tris Acetate EDTA 1X buffer) was used to examine the final PCR results (Bio-Rad Laboratories, Redmond, WA). The electrophoretic separation was performed in TAE 1x Buffer in an electrophoresis cell (Sub-Cell® Model 96 Cell) at a constant voltage of 85 V for 75 minutes. As a molecular weight marker, Thermo Fisher Scientific's GeneRuler DNA Ladder Mix (100-10,000 bp) was used.

The gel was stained for 30' in an ethidium bromide solution (0.5 mg/L) (Bio-Rad Laboratories, Redmond, WA) after the run, and then was destained for 30 minutes in TAE 1x. Finally, the gel was examined in a ChemiDoc™ XRS+ System with Image Lab™ Software (Bio-Rad Laboratories, Redmond, WA). A UV transilluminator with a wavelength of 254 nm is used to observe the presence of the banding pattern. The Image Lab™ program scanned and stored the resulting image of the distinct biotypes and RAPD-PCR patterns.

The isolates were grouped in biotypes with > 75 percent similarity by Pearson correlation coefficient after being amplified using M13-RAPD primer (5'-GAGGGTGGCGG TTCT-3') (Quartieri et al., 2016). A single strain for each biotype was taxonomically identified by sequencing the V1–V3 region of the 16S rRNA gene for bacteria and ITS1 for fungi. To amplify the two target regions, the primer pairs 16S-500f (5'-TGG AGA GTT TGA TCC TGG CTC AG-3')/16S-500r (5'-TAC CGC GGC TGC TGG CAC-3') and ITS1(5'-TCC GTA GGT GAA CCT TGC GG-3')/ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS (Kolbert et al., 2004; White et al., 1990) were used.

The amplification process was carried out in a final volume of 50 µl with the following reagent quantities, intended per sample:

- 5 µl 10X Dream Taq Green Buffer (with MgCl₂ 2 mM);
- 5 µl Primer 16S 500 Forward (2 µM);
- 5 µl Primer 16S 500 Reverse (2 µM);
- 5 µl dNTP mixture a concentration of 10 mM (dATP, dCTP, dGTP, dTTP);
- 0.5 µl DreamTaq DNA Polymerase (5 U/µl, 500 U) from Thermo Scientific;
- 24.5 µl of sterile ddH₂O;
- 5 µl of template DNA (from InstaGene matrix).

The thermocycler was utilized in the following program:

	Temperature	Time	Cycle
Denaturation	94 °C	2 min	1
Annealing	94 °C	1 min	45
	34 °C	1 min	
	72 °C	2 min	
Extension	72 °C	7 min	1

Purification of DNA from an agarose gel

The PCR products were separated on an agarose gel (TAE 1%) after amplification. Thermo Fisher Scientific's (Waltham, MA, USA) GeneRuler DNA Ladder Mix (100-10,000 bp) was used as a molecular weight marker. The run was set up at 85 V for 75'. After the run, the gel was stained in an ethidium bromide solution (0.5 mg/L) for 30' before being destained in TAE 1x for another 30 minutes. ChemiDoc™ XRS+ System with Image Lab™ Software was used to check for the presence of the band of interest (Bio-Rad Laboratories, Redmond, WA). A clean, sharp scalpel was used to remove the DNA fragments.

Using the QIAquick Gel Extraction kit, the bands were cut and purified (Qiagen, Hilden, Germany). Up to 10 µg of DNA may be purified using this method (70 bp to 10 Kb).

The extraction was done according to the manufacturer's protocol, which may be found in the handbook:

- For each mg of agarose gel, add 3 µl of QG buffer to each sample;
- Incubate for 10' at 50 °C. Further to dissolve the gel, vortex the tube every 2-3'. Check that the liquid is yellow after the slice has completely dissolved (similar to Buffer QG without dissolved agarose). The yellow color suggests a pH of 7.5, which is a good pH for DNA adsorption on the membrane. If the mixture is orange or violet add 10 µl 3 M sodium acetate, pH 5.0, and stir well. The mixture should develop a bright yellow color;
- For each weighed milligram, add 1 µl of isopropanol and stir thoroughly;
- In a 2 mL tube or a vacuum collector, place a QIAquick spin column (included in the package). To bind the DNA, apply the material to the spin column and centrifuge for 1'. Remove the flow-through and re-insert the column into the same tube;
- 500 µl Buffer QG must be added to the QIAquick column and centrifuged for 1'. Flow-through must also be discarded, and the QIAquick column must be reinserted into the same tube;
- For washing, add 750 µl Buffer PE to the QIAquick column and centrifuge for 1'. Before using Buffer PE, ethanol (96-100%) must be added;
- Centrifuge the QIAquick column for 1' in a 2 mL collection tube to remove any remaining wash buffer;- Fill a clean 1.5 mL microcentrifuge tube halfway with QIAquick column. To the center of the QIAquick membrane, add 30 µl Buffer EB (10 mM Tris-Cl, pH 8.5) or water, and centrifuge the column for 1'. The goal of this step is to elute DNA.

All centrifugation stages were carried out at 17,900 x g (13,000 rpm), in a traditional tabletop microcentrifuge.

The extraction yield was calculated using a nanophotometer (Implen, Westlake Village, CA, USA) to measure the DNA concentration and calculate the number of ng of DNA extracted. In a 15 µl volume, the collected samples were diluted to a final concentration of 10 ng/l of DNA. 2 µl of 16S 500 forward primer (10 M) was added after that. Finally, the NCBI BLAST program (<https://blast.ncbi.nlm.nih.gov>) was used to compare the sequences to the reference sequences in the GenBank database.

16S rRNA gene profiling

When packages were opened (0 days) and when sensory characteristics were still acceptable after 8 days, metataxonomic analysis was undertaken. The manufacturer's standard technique was used to extract the whole DNA (DNeasy Mericon Food Kit; Qiagen, Hilden, Germany):

- Fill a 50 mL centrifuge tube with 2 g of homogenized material;
- Vortex together 10 mL Food Lysis Buffer and 25 µl Proteinase K solution;
- Incubate at 60°C for 30', stirring constantly. After incubation, chill the sample to 15 to 25 °C on ice to maximize inhibitor precipitation;
- Spin for 5' at 2500 x g in a centrifuge;
- Fill a 2 ml microcentrifuge tube with 500 µl chloroform. Fill a microcentrifuge tube with 700 µl of the clear supernatant produced after centrifugation. Then, to get a homogenous solution, combine all of the ingredients;
- Centrifuge for 15' at 14,000 x g after vortexing the microcentrifuge tube vigorously for 15 seconds;
- Pipet 350 µl Buffer PB into a new 2 mL microcentrifuge tube, then add 350 µl of the upper, aqueous phase and vortex;
- Transfer the solution to a 2 mL tube containing a QIAquick spin column. Remove the flow-through and centrifuge for 1' at 17,900 x g;
- Fill the QIAquick spin column halfway with 500 µl Buffer AW2, centrifuge for 1' at 17,900 x g, and discard the flow-through. Dry the membrane by centrifuging it for 1' at 17,900 x g. Pipet

150 µl Buffer EB directly onto the QIAquick membrane in a 1.5 mL or 2 mL microcentrifuge tube. To elute, incubate for 1' at 15 to 25 °C, then centrifuge for 1' at 17,900 x g.

DNA concentration was adjusted to 5 ng/µL after quantification with a Qubit 3.0 fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA). A DNA sequencing service provider (Eurofins Genomics, Ebersberg, Germany) amplified and sequenced the V3-V4 region of the 16S rRNA gene using an Illumina MiSeq device to produce partial 16S rRNA gene sequences (Illumina, San Diego, CA, USA). With the BioProject ID, the sequences are available in the NCBI repository.

Raw sequences were analyzed with the QIIME 2.0 pipeline, version 2021.4 (Bolyen et al., 2019), with appropriate plugins for trimming (CUTADAPT) and denoising (DADA2) into amplicon sequence variants (ASVs) (Martin 2011; Callahan 2016). Taxonomy assignment was carried out with the feature classifier VSEARCH (Rognes et al., 2016), with SILVA SSU database release 138 (<https://www.arb-silva.de/download/arb-files/>) as a reference, and similarity threshold set at 0.97. The appropriate QIIME2 plugins were utilized to compute the alpha diversity (Chao1 and Pielou's evenness) and beta diversity (Bray-Curtis dissimilarity) and to compare them within and between 0 and 8 d samples (i.e., the Kruskal-Wallis test for alpha diversity; PERMANOVA for beta diversity). Differences were considered significant for $P < 0.05$. Principal Coordinate Analysis (PCoA) was computed with QIIME2, based on the beta-diversity distance matrix.

Linear discriminant analysis Effect Size (LEfSe, <http://huttenhower.sph.harvard.edu/galaxy>) was utilized to discover distinctive taxonomic features characterizing samples at 0 and 8 d (Segata et al., 2011). Taxa presenting a significant differential abundance ($P < 0.05$) and logarithmic LDA (linear discriminant analysis) score > 2 were considered as microbial biomarkers of the corresponding timepoint.

3.3.4. Statistical analysis

Unless otherwise specified, values were reported as means $\pm$ SD of triplicate samples. The comparison was performed according to Student's t-test or one-way ANOVA followed by Tukey post hoc comparisons, using the MaxStat software (MaxStat Pro, version 3.6). The Kruskal-Wallis test was performed on sensory properties, followed by Dunn's nonparametric post-comparison test. Differences were considered statistically significant for $P < 0.05$. Principal component analysis (PCA) was used to explore the data matrix of VOCs with size {60, 157}, including the 157 relative areas of VOCs, determined for the three replicates of each brand at the 4-time points. The number of significant principal components (PCs) was defined using the screen plot, which reports the

percentage of variance explained by each PC vs the PC number. The PCA model was calculated using Past version 3.14 (Hammer et al., 2001).

3.4. RESULTS

Trays of MAP sliced cooked ham from 5 commercial brands were stored for 12 days in a domestic refrigerator, and the temperature profile was monitored via a data logger. Values ranged between 4.1 °C and 6.5 °C (Figure 3.1), with an average temperature of 5.2 ± 0.6 °C (mean $\pm$ SD). The temperature fluctuated in a narrow range, representing possible domestic storage without extreme temperature abuse. It should be noted that the domestic storage temperature suggested on the labels is usually 0-4 °C: even if the measured temperature falls outside this range, it is to be considered a realistic condition, based on previous data collected in various domestic refrigerators, which ranged from 4.6 to 10 °C (Nicosia et al., 2021).

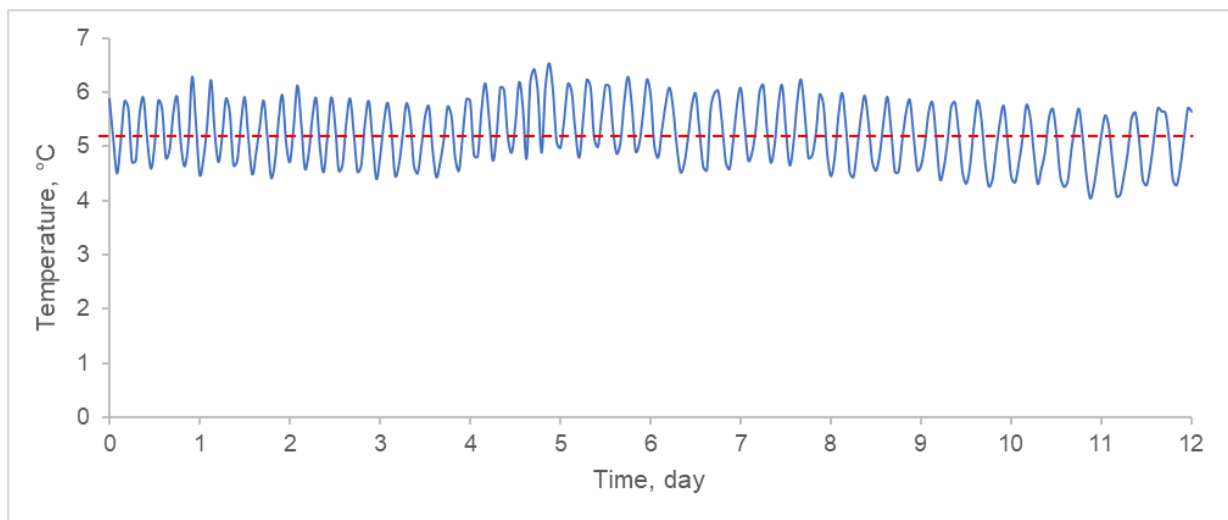


Figure 3.1 Temperature profile recorded in the domestic refrigerator during the 12 days-storage of cooked ham samples. The red dashed line represents the mean value calculated across the whole monitoring period.

3.4.1. Spoilage assessment

The sensory properties of the cooked ham samples were evaluated upon first opening and after 4, 8 and 12 days of secondary shelf life. Typically, negative changes were logged after 8 days, and products became unacceptable after 12 days. The most notable changes were the emergence of sour taste, rotten smell and discolouration (Figure 3.2). Brands behaved differently during this time frame. At 8 days, the discolouration of samples D was significantly more pronounced than in C and E ones ($P < 0.05$), and the overall extent of deterioration was more remarkable in D than in other samples. At 12 days, significantly higher rotten odour scores were recorded for samples C, D, and E than for samples A and B ($P < 0.05$), furthermore an overall deterioration was higher in D than for A and B samples ($P < 0.05$). In addition, samples C and D exhibited decreased drip and increased discolouration, rotten smell, and acidity flavour ($P < 0.05$; Table 4).

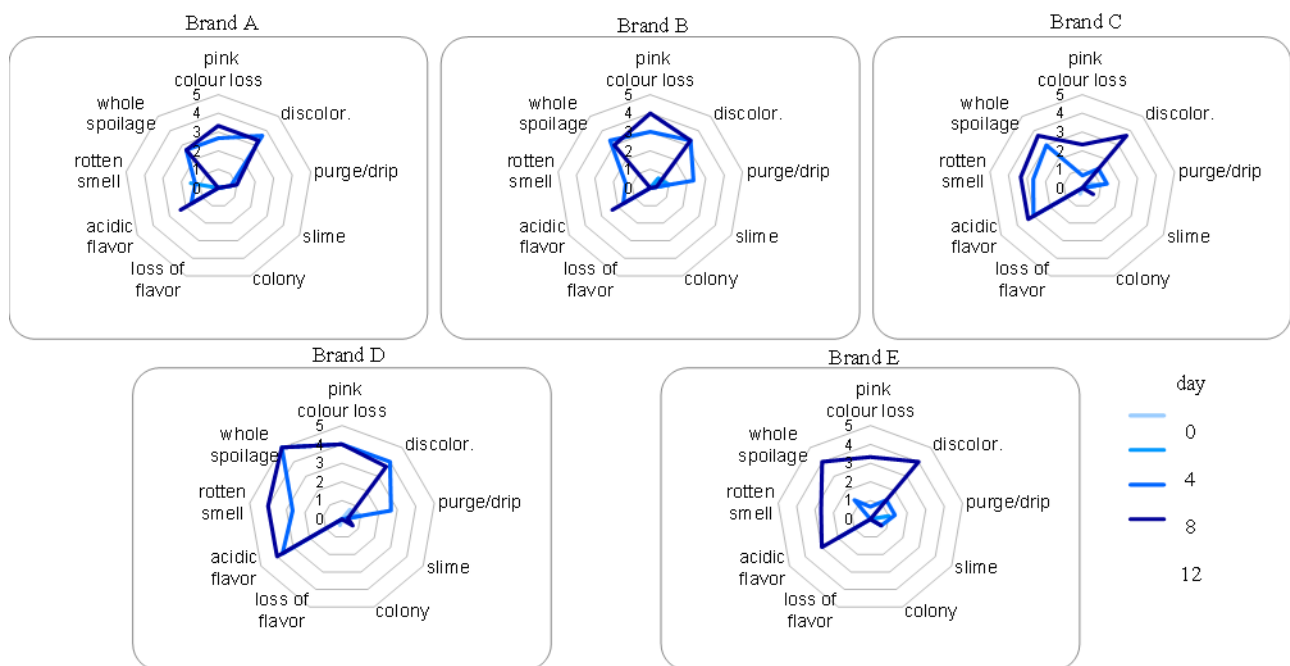


Figure 3.2 Spider charts for scores (0-5) of the sensory defects detected in A, B, C, D and E samples, with different colour lines for each timepoint.

Table 4. Sensory defects detected at the time of opening (T0) and after 4, 8 and 12 d (T4, T8, T12) in the 5 tested brands of cooked ham. Each value is given as mean of three replicates. At each time point, common lowercase letters in the same line indicate non-significant differences between the brands; common uppercase letters in the same line indicate non-significant differences of a single brand comparing all timepoints ($p < 0.05$). If letters are not provided, no significant difference was observed.

	T0					T4					T8					T12				
Brand	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
Pink colour loss	0	0	0	0	0	0	0	0	0	0	2.7	3	0.7	4	0.7	3.3	4	2.3	4	3.3
Discolor.	0	0.7	0.3 ^{AB}	0.7 ^A	0	0	0.7	0 ^B	0 ^A	0	3.7 ^{ab}	3.3 ^{ab}	1.3 ^{aAB}	4 ^{bB}	1.3 ^a	3.3	3.3	3.7 ^A	3.7 ^B	4
Purge/drip	1	1	1 ^A	1 ^A	1	0.3	1	1 ^A	1 ^A	1	0.7	2.3	1.3 ^A	2.7 ^B	1.3	1	0.3	0 ^B	0.3 ^A	0
Slime	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.7	0	0	0.7	0.7	0.7
Colony	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Loss of flavor	0	0	0.3	0.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acidic flavor	0	0	0 ^A	0 ^A	0	0	0	0 ^A	0.3 ^A	0	1.7	1.7	3 ^B	3.7 ^B	2	2.3	2.3	3.3 ^B	4 ^B	3
Rotten smell	0	0	0	0 ^A	0	1.5	0	0	0 ^A	0	1.3	1.3	2.7	2.7 ^B	0	0 ^a	0 ^a	3.3 ^b	4 ^{bB}	2.7 ^b
Whole spoilage	0	0	0	0	0	0	0	0	0	0	2.7 ^a	3.3 ^a	3 ^a	5 ^b	1.3 ^a	2.7 ^a	3 ^a	3.7 ^{ab}	5 ^b	4 ^{ab}

3.4.2. Culture dependent enumeration

Viable counts on PCA, MRSA, VRBGA, and RBCA plates were measured to monitor total aerobic bacteria, LAB, putative enterobacteria, staphylococci, and fungi, respectively, throughout the secondary shelf life (Figure 3.3).

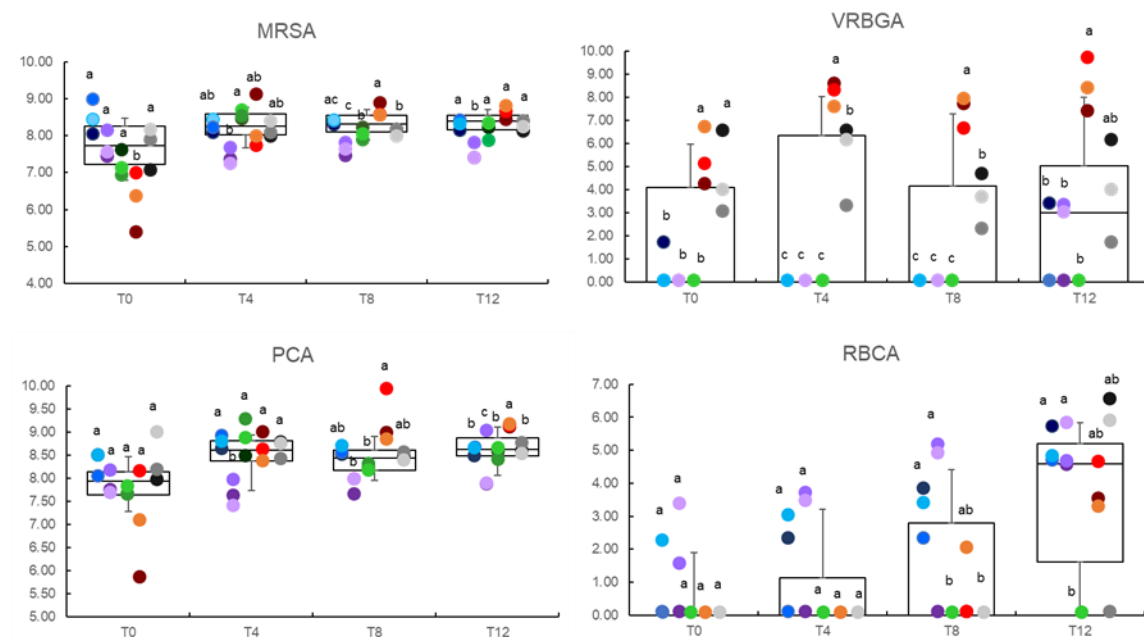


Figure 3.3 Viable counts in PCA, MRSA, VRBGA, and RBCA plates of A (blue), B (violet), C (green), D (red), and E (grey) samples. Boxes indicate the 25th, 50th, and 75th percentiles; whiskers indicate the 10th and 90th percentiles. Within each time point, brands sharing the same letter did not significant difference ($p > 0.05$).

Total aerobic bacteria ranged from 5.8 to 9.0 Log cfu/g (7.8 ± 0.7 Log cfu/g, mean $\pm$ SD) at 0 d, with no significant differences among brands ($P > 0.05$). However, after 4, 8 and 12 days of refrigerated storage, total aerobic bacteria increased significantly in packages of brand D, which progressively became the most contaminated ($P < 0.05$). A significant increase was also observed for brands A and C, while brands B and E did not show any significant changes.

Initial LAB charge differed widely between samples ranging from 5.7 to 9.1 Log cfu/g (7.7 ± 0.9 Log cfu/g, mean $\pm$ SD), with Brand D samples exhibiting the lowest values ($P < 0.01$). After 4, 8 and 12 days, the spread narrowed, with LAB charges between 7 and 9 Log cfu/g (mean = 8.3 Log

cfu/g). During the secondary shelf life, LAB increased significantly in the C and D Brands samples, with the latter progressively becoming the richest ($P < 0.05$). In the other Brands, LAB has remained unchanged.

Initially, putative enterobacteria were below the detection limit in most Brands A, B, and C samples. In contrast, all brands D and E samples had contamination in the range of 3.0 to 6.7. Log cfu/g, with no significant differences between them ($P > 0.05$). Throughout the secondary shelf life, presumed enterobacteria increased up to 9.7 Log cfu/g in the D samples ($P < 0.05$), while they remained unchanged in the E samples ($P > 0.05$). After 12 days, Enterobacteriaceae appeared in a few brands A and B samples, which had a load up to 3.3 Log cfu/g, while they were never found in Brand C.

Fungi were initially found in only three samples (A3, B2 and B3). In samples A, the fungal load increased over time, reaching 5.0 ± 0.6 at T12. After 12 d, fungi were isolated from most of the samples (11 of 15), with a mean load of 4.9 ± 1.0 Log cfu/g.

Putative staphylococci and *Salmonella* sp. were analysed after 12 d of secondary shelf life. Putative staphylococci were found in samples A, D and E, with counts ranging from 2.5 to 6.9 Log cfu/g (Table 4). *Salmonella* sp. was absent in all samples.

Table 4. Enumeration of putative Staphylococci on BPA plate at 12 d.

Brand	A	B	C	D	E
BPA, Log cfu/g					
Sample 1	2.5	< 2	< 2	6.9	2.8
Sample 2	3.0	< 2	< 2	6.2	< 2
Sample 3	2.8	< 2	< 2	6.4	< 2

3.4.2.1. Identification of the isolates

Five hundred sixty isolates were selected from plates at 0 and 12 d and were clustered into 68 dominant biotypes, according to RAPD-PCR fingerprinting. One representative of each biotype was subjected to sequencing the 16S rRNA gene or ITS to obtain species designation.

The strains isolated in PCA encompassed 25 biotypes belonging to 11 species: *Carnobacterium divergens* (5), *Latilactobacillus sakei* (5), *Leuconostoc mesenteroides* (5), *Leuconostoc carnosum* (2), *Bacillus nealsonii* (1), *Candida zeylanoides* (1), *Carnobacterium*

gallinarum (1), *Carnobacterium maltaromaticum* (1), *Carnobacterium* sp. (1), *Massilia aurea* (1), *Moellerella wisconsensis* (1), and *Proteus hauseri* (1).

The strains isolated in MRSA belonged to 15 biotypes, attributed to the species *Latilactobacillus sakei* (7), *Leuconostoc mesenteroides* (3), *Leuconostoc carnosum* (2), *Carnobacterium divergens* (1), *Latilactobacillus curvatus* (1), and *Weissella viridescens* (1).

In addition, 6 strains were isolated from VRBGA plates, belonging to the species *Erwinia billingiae* (2), *Massilia arvi* (1), *Moellerella wisconsensis* (1), *Proteus hauseri* (1), and *Pseudomonas* sp. (1).

Thirteen different biotypes were found on RBCA plates, belonging to the species *Candida zeylanoides* (5), *Kazachstania servazzii* (3), *Candida glabrosa* (1), *Candida novergica* (1), *Candida sakei* (1), *Debaryomyces hansenii* (1), and *Debaryomyces* spp. (1).

The 9 biotypes isolated on BPA plates at T12 belonged to the species *Proteus hauseri* (5), *Staphylococcus xylosus* (2), *Corynebacterium stationis* (1), and *Kocuria* spp. (1).

L. mesenteroides and/or *L. carnosum* abundantly contaminated the samples belonging to brands A, B, C, and E, occurring already at 0 d and after 12 d in the magnitude of 7–8 Log cfu/g in PCA or MRSA plates (Table 5). In brand D, a high load of *L. carnosum* (up to 8.9 Log cfu/g) appeared only after 12 d. *Latilactobacillus sakei* was found only in brands A, B, and E samples, generally found in the magnitude of 6–8 Log cfu/g MRSA in plates at 0 and 12 d. In brand C, *L. sakei* occurred only at 0 d. *C. divergens* characterized only samples of brands D and E, being present already at 0 d, generally in the magnitude of 7 Log cfu/g, and reaching up 9 Log cfu/g after 12 d. *C. gallinarum* and other *Carnobacterium* sp. occurred only in samples of brand C, at 0 and 12 d, respectively. Interestingly, for C samples only, all the biotypes of the isolates at both 0 and 12 d were LAB. *W. viridescens* occurred only after 12 d in 2 samples of brand D, where it reached the remarkable concentration of 9.1 Log cfu/g.

Contamination with *M. wisconsensis* was observed only in the D and E brands, both upon opening the packages and after 12 d. The *M. wisconsensis* load was highly variable at both times, ranging from 2.0 to 9.7 Log cfu/g, the highest value observed in a sample of brand D after 12 d. Other Gram-negative species (e.g., *E. billingiae*, *M. arvi*, *M. aurea*, *P. hauseri* and *Pseudomonas* sp.) appeared sporadically in a single sample of different brands and generally in low quantities (2–3 Log cfu/g).

Most of the yeast strains were isolated at 12 d, belonging to different species of the genus *Candida* (*C. glabrosa*, *C. norvegica*, *C. sake*, and *C. zeylanoides*). An exception was represented by *K. servazzii* that was detected in sample B since T0 reached 5.8 Log cfu/g at the last time point.

Table 5. Dominant microbial contaminants isolated from PCA (P), MRSA (M), VRBGA (V), RBCA (R), and BPA (B) plates at 0 and 12 d of secondary shelf life. Isolates were genotyped by RAPD-PCR fingerprinting and subjected to the 16S rRNA gene or ITS sequencing to obtain a taxonomic designation. Concentration, inferred from plate counts, is reported as Log cfu/g.

Brand	Package	Sampling time (d)	LAB							Proteobacteria				Other bacteria		Fungi												
			<i>Carnobacterium divergens</i> (P, M)	<i>Carnobacterium gallinarum</i> (P)	<i>Carnobacterium maltaromaticum</i> (P)	<i>Carnobacterium</i> sp. (P)	<i>Lactobacillus sakei</i> (M, P)	<i>Latilactobacillus curvatus</i> (M)	<i>Leuconostoc carnosum</i> (M, P)	<i>Leuconostoc mesenteroides</i> (M, P)	<i>Weissella viridescens</i> (M)	<i>Erwinia billingiae</i> (V)	<i>Massilia arvi</i> (V)	<i>Massilia aurea</i> (P)	<i>Moellerella wisconsensis</i> (V, P)	<i>Proteus hauseri</i> (V, B, P)	<i>Pseudomonas</i> sp. (V)	<i>Bacillus nealsonii</i> (P)	<i>Corynebacterium stationis</i> (B)	<i>Kocuria</i> sp. (B)	<i>Staphylococcus xylosus</i> (B)	<i>Candida glabrosa</i> (R)	<i>Candida norvegica</i> (R)	<i>Candida sake</i> (R)	<i>Candida zeylanoides</i> (R, P)	<i>Debaryomyces hansenii</i> (R)	<i>Debaryomyces</i> sp. (R)	<i>Kazachstania servazzii</i> (R)
A	1	0				8.2	7.2	8.4																				
		12						8.1	8.5		2.5	2.5			2.5	3.1			3.1	3.3					5.7			
	2	0				7.0	7.8	8.0																				
		12						8.4	8.6												3.0				4.6			
	3	0				8.0		8.5																		2.2		
		12							8.6						2.2						2.6				4.8			
B	1	0				7.5	6.9	7.5					7.2															
		12				6.8	7.4	7.7					7.2											3.9		4.4		
	2	0				8.3		8.1																		1.5		
		12				7.2		8.0		3.3																4.6		
	3	0				7.4	7.2	7.7																		3.3		
		12				7.2		7.8		3.0																5.8		
C	1	0	6.9			7.5	7.8																					
		12			8.2		8.4																					
	2	0	7.3				7.1																					
		12			8.4		8.0																					
	3	0				7.3	7.3																					
		12			8.5		8.5												2.0									
D	1	0	5.5										5.8	5.5														
		12	8.9				7.8	9.5					7.4	6.9						3.5								
	2	0	7.8	7.8										5.1														
		12	9.1				8.7	8.2					9.7	6.1						4.1	4.4							
	3	0					6.6																					
		12	8.9				8.9						8.4	6.4						2.6	3.1							
E	1	0	7.8			6.3	7.1	6.6					6.6															
		12	7.9			8.2	8.2	7.2					6.2	2.8							6.5							
	2	0	7.7				8.0	7.3					2.9	2.3														
		12	8.4			8.1	8.1	7.6	7.9				2.0	2.0	8.4													
	3	0	8.7			8.3	8.3						4.0															
		12			7.7	7.7	8.0	7.4					4.0									5.7			5.2			

3.4.3. Analysis of microbial population by 16s rRNA gene profiling

Metataxonomic analysis was performed at opening and after 8 d of secondary shelf life when samples were not yet altered. Metagenomic investigation of 16s rRNA gene profiling yielded from 52,964 to 148,150 reads per sample, with a total of 3,442,935 in 30 samples. The reads were dereplicated into 822 ASVs which hit a reference sequence in the Silva database and were collapsed at the 7th level of taxonomic annotation in 326 OTUs. The cooked ham microbiota was dominated mainly by Proteobacteria and Firmicutes phyla (Figure 3.4). Chao1 richness differed among the diverse brands; it was highest for C samples and lowest for A and D (Figure 3.5A). The richness decreased significantly after 8 d of storage in the D samples. Pielou evenness presented significant differences among the brands and showed similar levels of diversity within the same brand at different time point, excluding relevant variations during the secondary shelf-life (Figure 3.5B). Brand D was quite heterogeneous in terms of evenness, with significant variability among samples at both time points.

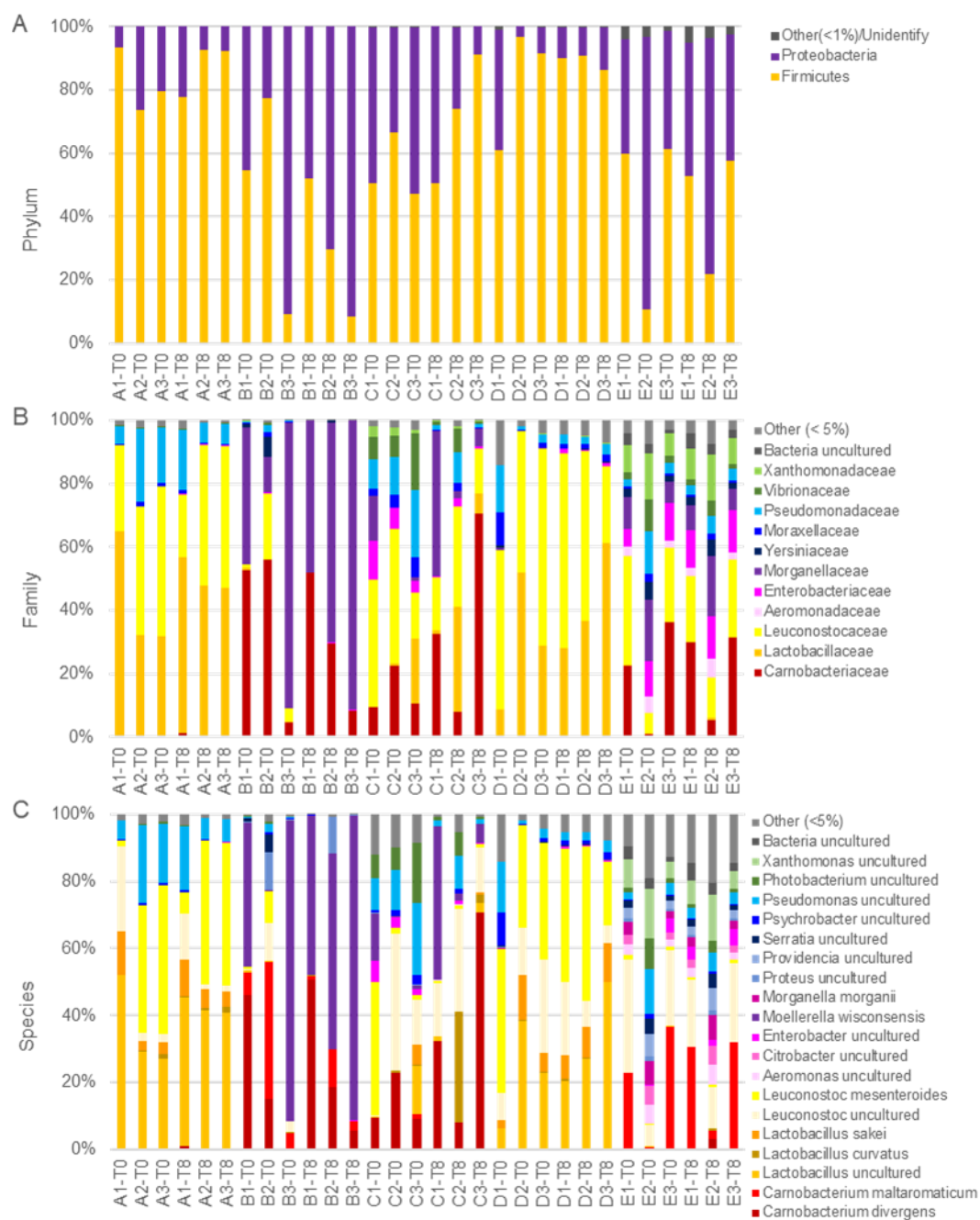


Figure 3.4 Relative abundance of the main phyla (A), families (B), and species (C) in slices of cooked ham of 5 different brands at packages opening and after 8 d.

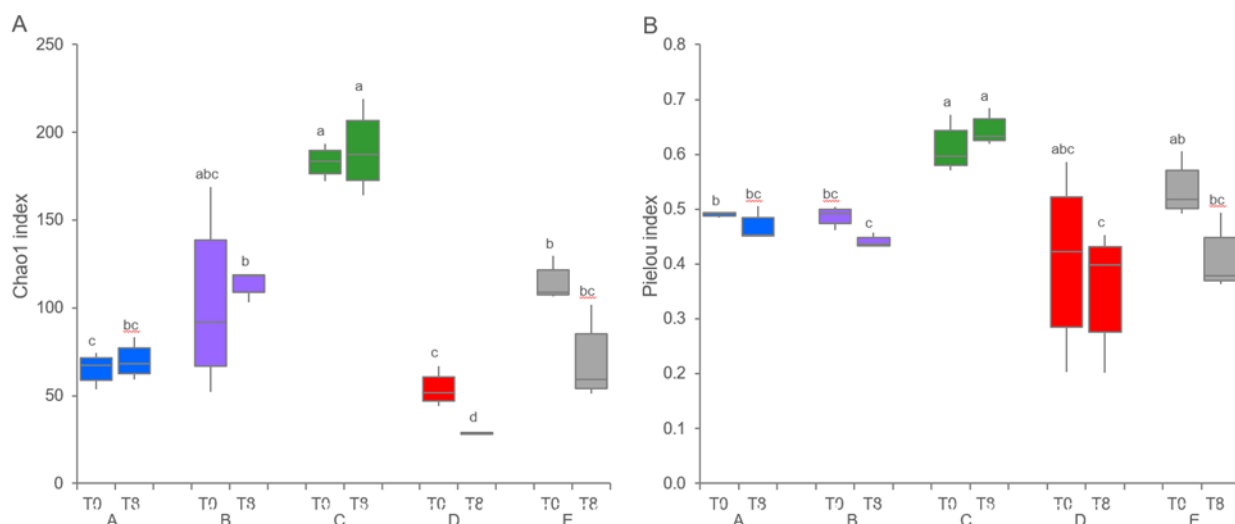


Figure 3.5 Indexes of α diversity based on ASV identification in the cooked ham samples; Chao1, richness; Pielou, evenness. Common lowercase letters indicate non-significant differences between values

In the PCoA plot calculated from the Bray-Curtis beta diversity distance matrix (Figure 3.6A), initial samples could not be clearly separated from those with 8 d of secondary shelf life. On the other hand, samples were grouped according to the brand, regardless of the timepoint. For example, samples of brands A and D clustered together and shared some biomarkers such as *Lactobacillus* and *L. mesenteroides* that mainly contributed to Pco1 (Figure 3.6B). Accordingly, Firmicutes was the most abundant phylum in A and D samples, ascribed primarily for the families of Leuconostocaceae and Lactobacillaceae (Figure 3.4).

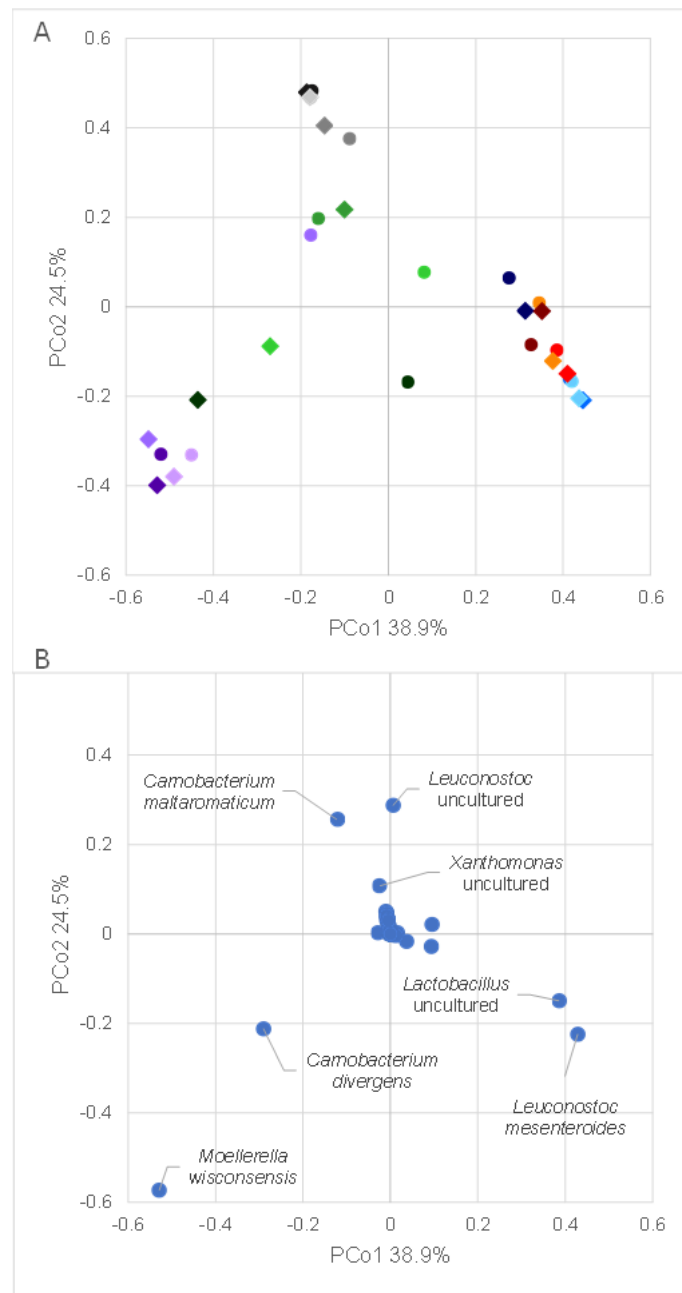


Figure 3.6 (A) PcoA plot of beta diversity based on Bray-Curtis dissimilarity index of cooked ham microbiome. Different colours indicate brands, A (blue), B (violet), C (green), D (red), and E (grey). Circles mark samples at the opening, diamond after 8 days. (B) PcoA plot of the species contribution to samples differentiation.

In general, the evolution of the 15 samples during the first 8 days of secondary shelf life did not determine relevant variations, with few exceptions (B2, C1, C3). The microbiota composition of A, D and E brands was minimally altered during the first 8 days of secondary shelf life.

Samples B lay contiguous at low Pco1 and Pco2 values, characterized by *M. wisconsensis* and *C. divergens*. Consistently, in 5 out of 6 B samples, *M. wisconsensis* outnumbered other bacteria.

E samples grouped together (high Pco1 and low Pco2 values), with *C. maltoaromaticum* and *Leuconostoc* as the main biomarkers. Consequently, the Leuconostocaceae, Carnobacteriaceae, and Morganellaceae families dominated the E samples (Figure 3.4). Other common bacteria identified in all E samples were *Lactobacillus*, *Pseudomonas*, *Psychrobacter*, *Photobacterium*, *Xanthomonas*, *Proteus*, *Serratia*, *Providencia*, *Citrobacter*, *Aeromonas*, *Shewanella*, *Acinetobacter*, *Vibrio*, *Hafnia-Obesumbacterium*, *Klebsiella*, *Brochothrix*, and *Halomonas*.

LefSe algorithm was used to discover different taxonomic features that characterize samples at 0 or 8 d. Only Bacillaceae, *Geobacillus*, and *Parageobacillus thermoglucosidasius* showed significantly higher abundances in samples at 0 d (Figure 3.7). The same analysis, applied to identify specific biomarkers for each brand, indicated that brand B was characterized by *L. mesenteroides*, brand D by Enterobacterales and Morganellaceae, and brand E by *Photobacterium*.

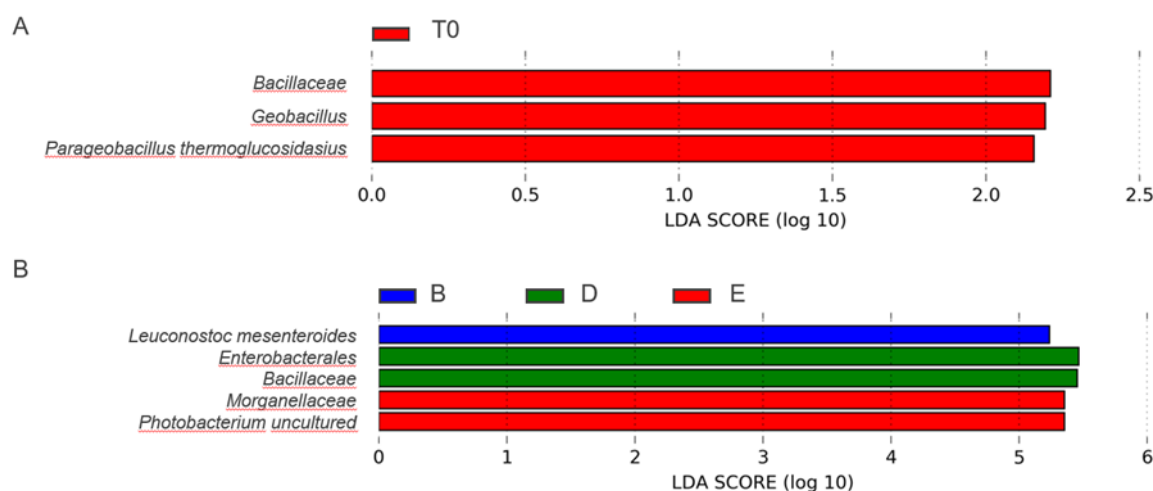


Figure 3.7 Linear discriminant analysis Effect Size (LefSe) of taxonomic features characterizing T0 samples or appearing as specific features of the distinct brand.

3.4.4. Glucose and organic acids

Glucose levels initially varied from 0.09 to 0.68 w% (g per 100 g of ham) (Figure 3.8). Its concentration was significantly higher in D and E samples than in A, B, and C. Generally, it significantly dropped during the secondary shelf life ($P < 0.05$). Still, after 12 days of refrigeration at 5 °C and relevant metabolic activity, it was still present utmost of the samples (11 out of 15) although in negligible quantities (0.01 to 0.17%).

Lactic acid was the main metabolite, monitoring in all the samples and at all timepoints throughout the secondary shelf life. The initial concentration varied from 0.15 to 0.62 w%, with the highest levels in the samples of brand A ($P < 0.05$). However, lactic acid significantly increased during the secondary shelf life in B, C, and E samples ($P < 0.05$), where its concentration almost doubled (from 0.30 ± 0.14 to 0.71 ± 0.01 ; from 0.26 ± 0.07 to 0.70 ± 0.06 ; from 0.38 ± 0.02 to 0.68 ± 0.02 in B, C, and E, respectively), while it did not significantly increase in A and D samples.

Acetic acid was another major metabolite detected in cooked ham over time. Its concentration was shallow in all the samples at 0 and 4 d, whereas it earned toward the end of the secondary shelf-life. Ethanol was accumulated in minor amounts, without significant differences among the brands, generally tending to amass over time.

The pH of cooked ham was progressively reduced during the secondary shelf-life (5.7 ± 0.3 at T0; 5.5 ± 0.4 at T4; 5.4 ± 0.3 at T8; 5.3 ± 0.3 at T12; overall means $\pm$ SD) ($P < 0.01$), with different kinetics depending by the brand.

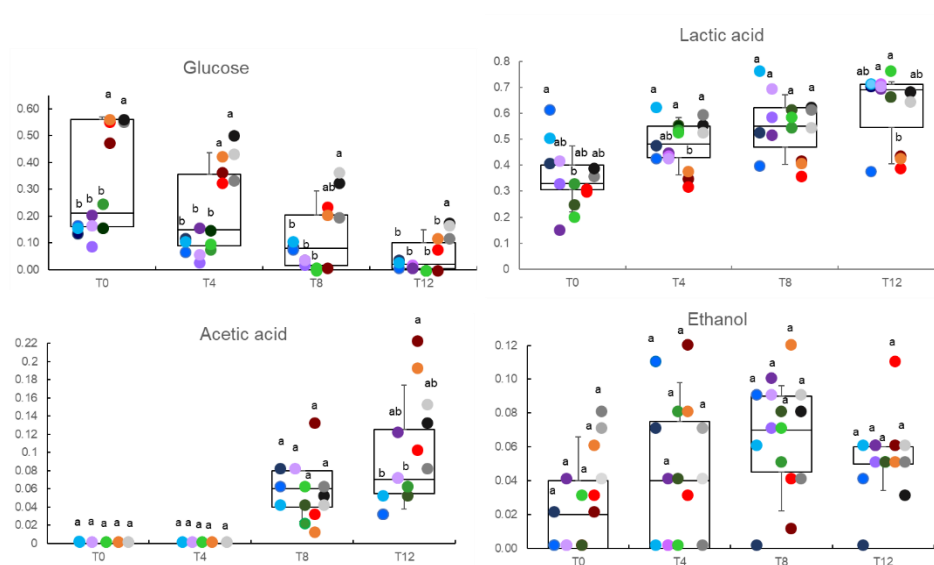


Figure 3.8 Concentration of Glucose, Acetic acid, Lactic acid, Ethanol, and pH variation in A (blue), B (violet), C (green), D (red), and E (grey) samples. Boxes indicate the 25th, 50th, and 75th percentiles; whiskers indicate the 10th and 90th percentiles. Within each time point, brands sharing the same letter did not significant difference ($p > 0.05$).

3.4.5. VOCs

A total of 157 volatile organic compounds (VOCs) were recognized by SPME-GC analysis in the headspace of cooked ham samples, including organic acids, ethanol and other alcohols, ketones, fatty aldehydes, esters, phenols and a variety of aromatic compounds, furans, linear and branched aliphatic hydrocarbons, terpenoids, and sulfides (Figure 3.9).

In the PCA model of VOCs profiles, the 2 most informative dimensions, PC1 and PC2, accounted for 47% and 31% of data variability (Figure 3.10A). A major loading in determining differences among samples during the secondary shelf life was presented by ethanol, acetic acid, and acetoin. Acetoin, 3-methyl-1-butanol, and 2-3 butanediol showed a positive contribution to PC1, while ethanol and acetic acid presented a negative one (Figure 3.10B). Interestingly, only brand D was marked by higher values of PC1 compared to other brands but, differences became evident only throughout the secondary shelf life, with increasing PC1 values over time. Acetic acid was the main positive contributor to PC2. All samples at 0 d were clustered at low values of PC1 and PC2, regardless of the brand. A typical evolution of all brands was the accumulation of acetic acid: the distribution of samples along the PC2 clearly showed a correlation between increasing time from the opening of the trays and rising values of PC2.

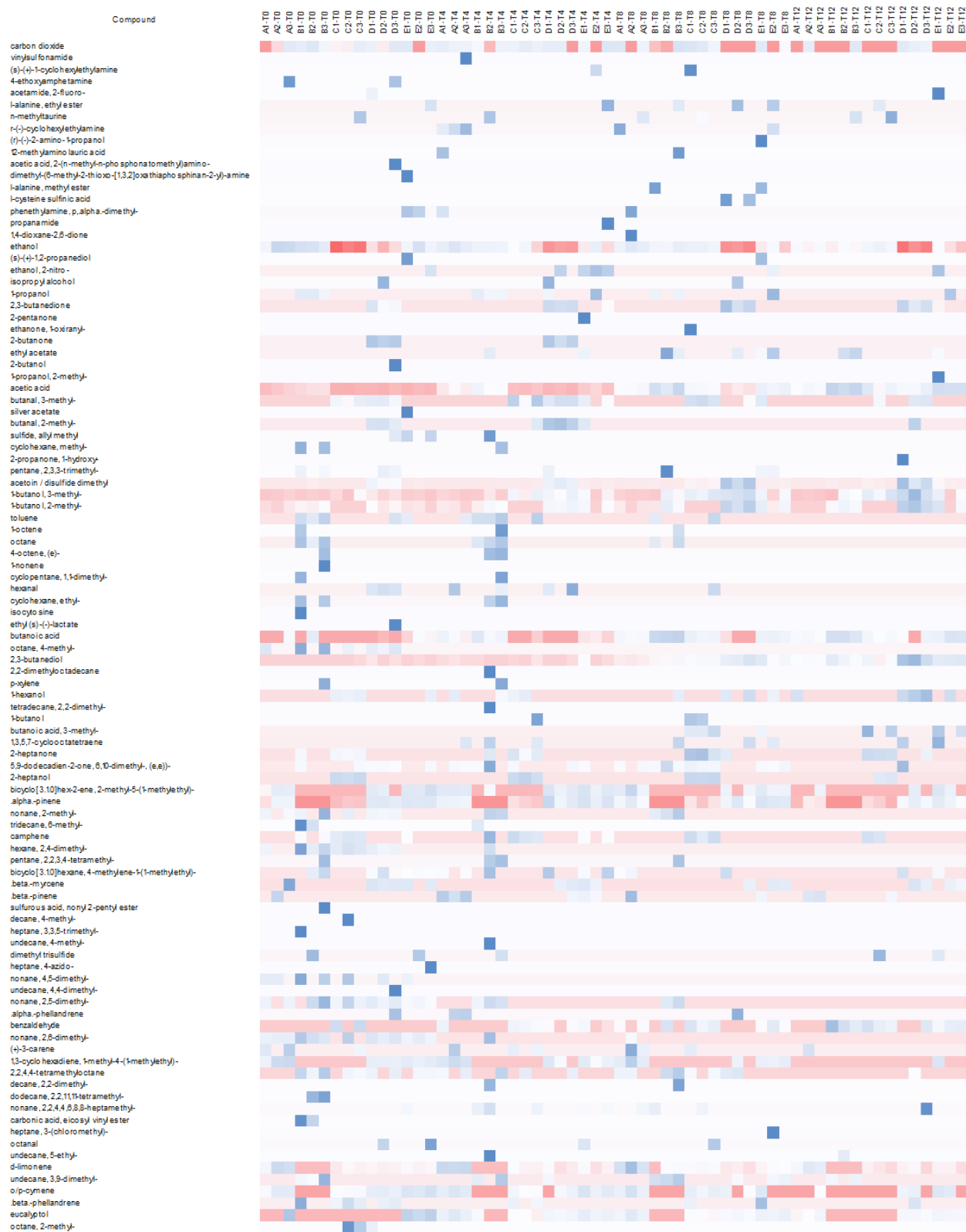




Figure 3.9 Distribution among cooked ham samples of the 157 volatile organic compounds (VOCs), identified in the headspace of cooked ham samples during the secondary shelf life. The area of each VOC was meancentered and normalized by the standard deviation. Values are reported as colours ranging from the lowest (deepest blue) to the highest (deepest red).

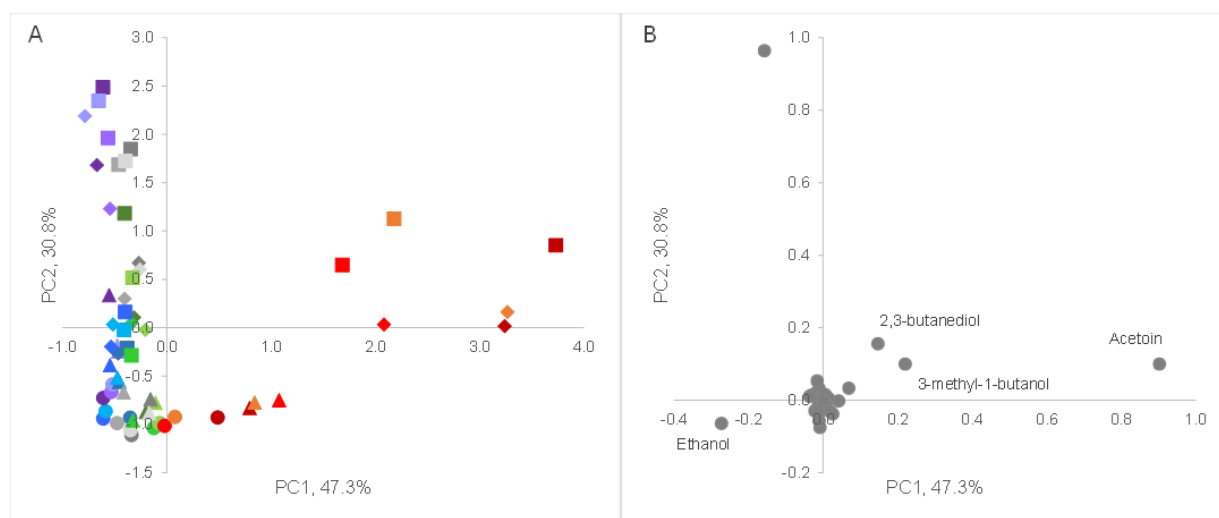


Figure 3.10 PCA plot (PC1vs PC2) of the VOCs profiles in A (blue), B (violet), C (green), D (red), and E (grey) samples. (A) scores plot of cooked ham samples. (B) loading plot of the volatile molecules. The compounds with the highest loading value in PC1vs PC2 are labelled.

3.5. DISCUSSION

MAP sliced cooked ham is one of the most widely used ready-to-eat products, particularly subject of numerous studies aimed at defining its microbial communities and the resulting impact on safety, deterioration, and preservation (Vasilopoulos et al., 2010; Raimondi et al., 2019; Candeliere et al. 2021; Zagdoun et al. 2020). In this study, the microbial charge and chemical-physical features of cooked ham were checked for 12 d after the opening of packages, during which products were preserved in a domestic refrigerator. All products were purchased, opened, and analysed in a range of 10–15 days before the expiring date and were packed around 1–2 weeks before the experiment. The sensory analyses of 5 brands of MAP sliced cooked ham, during the secondary shelf life at an average temperature of 5.2 ± 0.6 °C, showed a significant deterioration after 8 d. Still, products became unacceptable only after 12 d, comparing acid flavour, rotten smell, and discolouration.

The organoleptic properties of samples suggested the possibility of extending the secondary shelf life of cooked ham well beyond the 1-3 days indicated by the producers (Table 3). Metagenomic analysis and the identification of isolates pointed out that, even at the opening of packages, all the products presented high microbial charge and a rich microbiota, generally dominated by LAB. According to data reported in the literature (Candeliere et al. 2021; Iskandar et al. 2017; Raimondi et al. 2019; Vasilopoulos et al. 2010), the genera of *Leuconostoc*,

Carnobacterium, and *Lactobacillus* were among the greatest colonizers of MAP and vacuum-packed cooked ham.

When the packages were opened and after 8 d of storage, before samples deteriorated, the 16S rRNA gene profiles were analysed. The differences among microbiota were evident and mainly attributed to the brand, with LAB representing the dominant bacteria in samples A and D. In the others, more complex communities were identified, also encompassing several Proteobacteria. For example, a facultative anaerobic Gram-negative rod, *Moellerella wisconsensis* belonging to the Enterobacteriaceae, was the primary colonizer of B samples as early as at 0 d. This species is probably considered an environmental contaminant since literature reports a variety of isolation sources, including water, foods, and samples of human origin (Leroy et al., 2016). Furthermore, *M. wisconsensis* can be involved in disseminating antibiotic resistance, and its pathogenic role is addressed (Stock et al., 2003; Leroy et al., 2016; Ahmad et al., 2020).

Culture-based identification revealed the presence of another Enterobacteriaceae contaminant, *Proteus auseri*. It is a Gram-negative motile bacterium, associated with the human pathogens *Proteus vulgaris* and *Proteus mirabilis*, recurrent in packages of the brands A and D, where it increased to substantially high levels of contamination at 12 d. Other Proteobacteria, belonging to the genera *Pseudomonas*, *Erwinia*, and *Massilia*, occurred less frequently and abundantly. Facultative anaerobic Gram-negatives can contaminate the product at a certain level during the processing and before the packaging and, likewise LAB, can grow in the ham unimpeded by the modified atmosphere (Stock et al., 2003; Kreyenschmidt et al., 2010; Drzewiecka, 2016). *S. xylosus* only occurred in brand A. It is used as a biocontrol agent to preserve meat products from mycotoxin produced by fungi, convert nitrates to nitrites, and is responsible for flavour components (Stahnke et al., 1994; Cebrián et al., 2020).

The metagenomic analysis revealed the occurrence of other bacterial contaminants that were not recovered with the culture-dependent method, and that could represent a potential hazard for sensorial properties and/or food safety if they could grow too much. Among them, *B. thermosphacta* gives cooked ham sour off-flavours, discolouration, and slime (Dušková et al., 2016; Raimondi et al., 2019; Borch et al., 1996; Vasilopoulos et al., 2015). This species was detected in almost all samples already at the beginning of the secondary shelf-life, although in modest quantities (up to 1.1%). Opportunistic pathogenic bacteria have also been identified, albeit in small numbers. *E. coli* and *Vibrio* sp. were other frequent contaminants found in most samples, reaching up to 3.0% and 1.8% of the microbiota, respectively (means 0.5% and 0.2%, respectively). At the

same time, *Salmonella enterica*, *Klebsiella pneumoniae* were sporadically expressed in a sole or few samples.

The presence of *K. servazzii* and *D. hansenii* as early as at 0 d suggests that these yeast species, occasional contaminants of cooked meat products (Hernández et al., 2018), colonized the products before the packaging and continued to thrive during the secondary shelf life. Once the packages were opened, the protective effect of the modified atmosphere was lost, with only a slight residual inhibitory activity of the CO₂ solubilized in the matrix. Yeasts strains belonging to the genus *Candida* (*C. zeylanoides*, *C. sake*, *C. norvegica*, and *C. glabrosa*) remained below the detection limit upon opening the packages but were abundantly detected after 12 d. *C. sake*, *C. glabrosa*, and *C. zeylanoides* appeared to be associated with specific brands and may derive from the manufacturing. Frequent sources of yeast contamination are the surfaces of machinery that come to contact with meat during processing (Hernández et al., 2018).

The high charge of bacteria already present at the opening of cooked ham packages prevented the onset of other species and led to the outnumber of these dominant bacteria. For instance, a further increase of LAB was pointed out in the samples where they were already abundant at the opening of packages, leading *Leuconostoc*, *Carnobacterium*, *Lactobacillus*, and, to a lesser extent, *Weissella* to dominate these microbial communities. On the other hand, the growth of Gram-negatives and other bacteria occurred in samples initially characterized by a complex community, where LAB were not dominant. The high LAB load likely protected the samples from abundant contamination by other bacteria, allowing for the evolution of safer products. In these samples, a similar role was performed by several *Lactobacillus*, *Leuconostoc*, and *Carnobacterium* species, among which the most frequently identified were *L. sakei*, *L. carnosum*, *L. mesenteroides*, and *C. divergens*. Generally, the low storage temperatures made *L. carnosum* and *L. mesenteroides* the most suitable species for cooked ham (Vasilopoulos et al., 2008; Raimondi et al. 2021). In addition to their preservative effect, LAB can also be concerned in spoilage (Comi et al., 2016; Raimondi et al., 2019). *L. mesenteroides*, *L. carnosum*, and *C. divergens* were declared as responsible for greenish colour around the cooked ham slices, discolouration, and off-odour, with *L. carnosum* and *L. mesenteroides* mainly responsible for sour odour (Björkroth et al., 1998; Diez et al., 2008; Schirmer et al., 2009; Comi et al., 2016; Leisner et al., 2007).

Molecules obtained by thermally induced lipid oxidation, such as straight-chain fatty acids, aldehydes, ketones, and alcohols (1-hexanol), are responsible for the aroma of cooked ham (Leroy et al., 2009). The spices added to the product also contributed with terpenoids and sulfides such as 3-carene, gamma-terpinene, humulene, d-limonene, beta-phellandrene, and alpha-pinene (Rux et al.,

2019). Moreover, during shelf life, from the catabolism of sugars, various VOCs, amino acids, and lipids by the growing microorganisms were produced (Casaburi et al., 2015; Estevez et al., 2003; Holm et al., 2013; Rivas-Canedo et al., 2009; Raimondi et al. 2019). Lactic acid, ethanol, acetic acid, acetoin, 3-methyl-1-butanol, and 2-3 butanediol were the primary metabolites that characterized the evolution of the analysed cooked ham during the secondary shelf life, impacting their sensory properties. LAB and other bacteria produced via saccharolytic metabolism lactic and acetic acid, and ethanol (Casaburi et al., 2015). The increase of acetic acid as a function of sampling time was justified by the widespread presence of heterofermenting bacteria such as *L. mesenteroides* and *L. carnosum* that dominated the spoilage microbiota of all samples. The lower amount of acetic acid compared to lactic acid was consistent with the results obtained by Vasilopoulos et al. (2008). LAB and enterobacteria produced other compounds such as acetoin, 3-methyl-1-butanol, 2-3 butanediol, 3-methyl-butanol, 2-butanone, 2-methyl-1-propanol, 1-hexanol. In particular, the alcohols 2-methyl-1-propanol and 3-methyl-1-butanol derive from the catabolism of valine and leucine amino acids (Ardö, 2006). The esters were principally produced by enterobacteria and microorganisms able to form esterase involved in creating ethyl bonds (Argyri et al., 2015). The presence of *Weissella viridescens* and *Leuconostoc carnosum* was already correlated with acetoin concentration in fermented foods (Diez et al. 2009; Candelieri et al. 2021).

3.6. CONCLUSION

Incorrect cooking of meat, inappropriate sanitization techniques, and recontamination during slicing and packaging are the primary drivers of microbial contamination in cooked ham. The microbiota of MAP cooked ham was rich even before the packages were opened. The modified atmosphere packaging conditions, which are typically of 70% N₂ and 30% CO₂, help to prevent chemical and microbial spoilage during primary shelf life. However, opening the package causes a sudden change in the headspace gas composition and a loss of protection, resulting in a secondary shelf life that is significantly shorter than the primary shelf life. Microbial contamination likely did not originate from conservation during the secondary shelf life but was already present when the packages were opened, two weeks before the expiring date of the products, indicating the phases of production up to the packaging as those crucial in managing the safety risk. The presence of a high charge of LAB when opening the packs of cooked ham impeded the emergence of other species and probably prevented the advent of harmful bacteria. The exploitation of specially selected strains belonging to the genera *Lactobacillus* and *Leuconostoc* with reduced impact on the product's

organoleptic properties could be an effective strategy to control these microbial communities and extend the primary and secondary shelf life of the product, reducing domestic food losses.

The results of this study are accepted in:



Microbiota Survey of Sliced Cooked Ham During the Secondary Shelf Life

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4. Project 2

Phenotypic traits and immunomodulatory properties of *Leuconostoc carnosum* isolated from meat products

4.1. INTRODUCTION

4.1.1. Lactic acid bacteria

Lactic acid bacteria (LAB) are microorganisms that mainly produce lactic acid from the fermentation of sugars. LAB belong to the phylum Firmicutes, class Bacilli, and order Lactobacillales. They do not represent a phylogenetically defined taxon encompassing species belonging to 14 genera: *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Symbiobacterium*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Giraffa, 2012).

These bacteria are ubiquitous and usually present in food products, are involved in numerous natural fermentation processes, and are widely used on the industrial level. They reside in a variety of niches where carbohydrate-based substrates are available, such as plants, plant-derived matrices, grass silage, fermented foods (e.g. dairy products, fermented dough, milk, vegetables, and meats), spoiled foods, organic matrices, and sewage (Hammes and Hertel, 2006).

They are generally auxotrophic for amino acids, vitamins, and nucleotides, being evolved in very rich nutritional niches (Kandler and Weiss, 1986). Certain species are components of the commensal microbiota, naturally colonizing diverse traits within the body of humans and animals,

and represent, with *Bifidobacteria*, the most popular probiotics (Rossi et al., 2016). In some instances, some species of *Enterococcus* and all those of *Streptococcus* genera, except for *S. thermophilus*, can be responsible for human pathologies (Makarova and Konin, 2007).

LAB can be used in food processing, contributing to the safety of the final products and the development of positive organoleptic proprieties. Lactic acid resulting from carbohydrate degradation reduces pH hindering the growth of pathogens and spoilage bacteria. Several LAB can produce H_2O_2 and/or bacteriocins, limiting the growth of competing microorganisms. In some cases, they can inhibit the growth of *L. monocytogenes* and other dangerous foodborne pathogens (De Vuyst and Leroy, 2007). The use of selected LAB strains as starters can extend the shelf-life of food products through the inhibition of microbes responsible for spoilage.

The numerous information acquired thanks to the knowledge of the genome of these microorganisms allow us to carry out advanced studies of phylogeny and evolutionary genomics. LAB genome presents a GC-content < 55%. The order Lactobacillales includes several families, among which Enterococcaceae, Streptococcaceae, and Lactobacillaceae. The sole genus *Lactobacillus* encompasses more than 215 species with diverse morphological and functional qualities.

4.1.1.1. Classification of lactic acid bacteria by fermentation capacity

Lactic acid bacteria can metabolize hexose sugars such as glucose or pentoses such as ribose, arabinose, xylose, rhamnose. The metabolism of hexoses follows two enzymatic pathways, the homofermentative and the heterofermentative path depending on the bacterial species, which lead to different final products and have an extra energy yield. The metabolism of pentoses follows the metabolic pathway of pentose phosphates.

Homofermentative metabolism

Homofermentative bacteria degrade glucose through the glycolytic pathway with the formation of pyruvate, which is then reduced to lactic acid by lactate dehydrogenase, with the simultaneous re-oxidation of the coenzyme NADH (Fig. 4). With this metabolic pathway, 2 moles of lactic acid are produced for each mole of glucose, and an energy yield of 2 moles of ATP is obtained (Khalid, 2011).

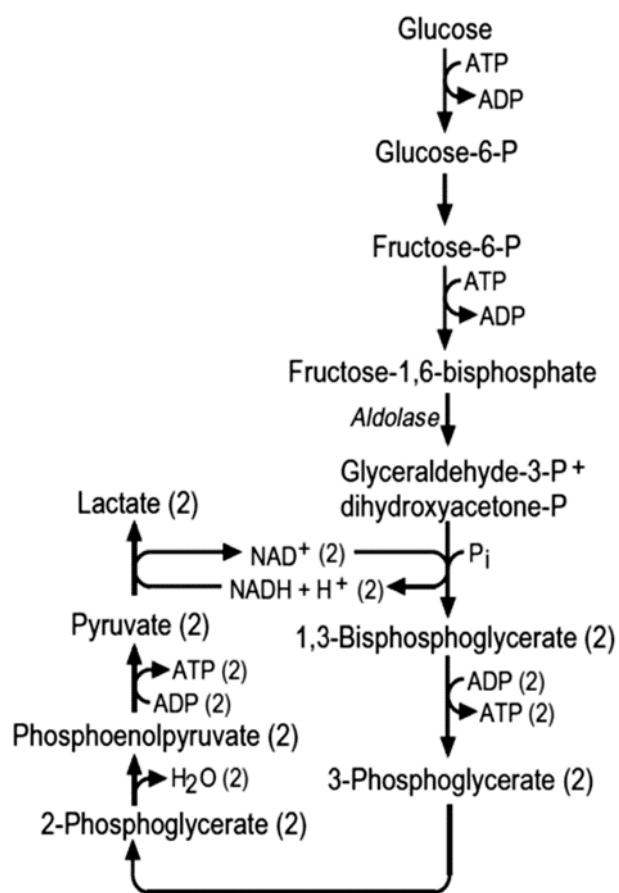


Figure 4. Glucose degradation in homofermentative bacteria (Bisen, 2015)

Heterofermentative metabolism

In heterofermentative bacteria, the fermentation of hexoses does not follow glycolysis but the pentose phosphate pathway, since they lack the enzyme aldolase that catalyzes the splitting of fructose diphosphate into the two triose phosphates. In the pentose phosphate pathway, the key reaction is the cleavage of xylulose into glyceraldehyde 3-phosphate and acetyl phosphate, catalyzed by the phosphoketolase enzyme. 3-phosphate glyceraldehyde is metabolized to lactic acid following the homofermentative path, and acetyl-phosphate is reduced to ethanol with simultaneous re-oxidation of the coenzyme NADH (Figure 4.1). In some conditions, acetyl-phosphate can be converted to acetate while the coenzyme NADH is reoxidized by reducing fructose to mannitol. Hetero-fermenting bacteria, from a mole of glucose form an equimolar mixture of lactic acid, carbon dioxide, and ethanol or acetate, with an energy yield in ATP of a single mole (Carr et al., 2002).

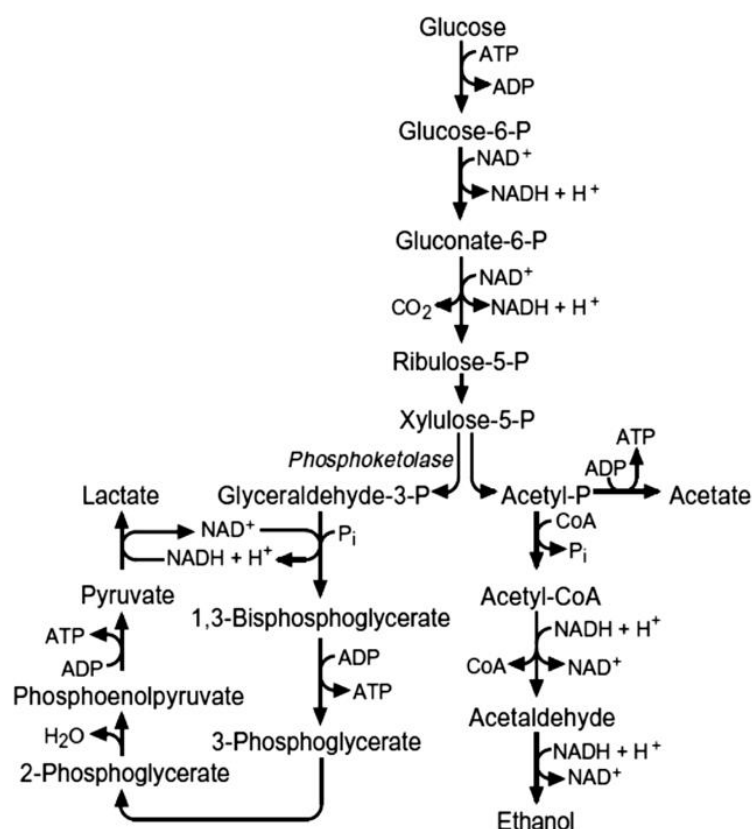


Figure 4.1 Glucose degradation in heterofermentative bacteria (Bisen, 2015)

4.1.2. *Leuconostoc carnosum*

Leuconostoc carnosum is a heterofermentative lactic acid bacterium belonging to the order of Lactobacillales, which frequently occurs in the microbiota of foods, mostly meat-based products where it was isolated for the first time (Shaw and Harding, 1989; Raimondi et al., 2019; Raimondi et al., 2018; Goto et al., 2004; Samelis et al., 2000). For its peculiarity, it is highly adapted to grow in meat environments where it can outcompete undesired microorganisms and reach high levels of viable bacteria (Raimondi et al., 2019; Raimondi et al., 2018). *L. carnosum* is a facultative anaerobe, aerotolerant. It tends to dominate the microbiota of vacuum-packaged or modified atmosphere packaged (MAP) meat products, emerging in the first days after packaging and reaching substantial-high charges (in the order of 10^8 cells/g) towards the end of the shelf life, when they

prevail over the microbiota (Björkroth et al. 1998; Vasilopoulos et al. 2010; Raimondi et al., 2019). Other meat products such as sausages, vacuum-packaged smoked bacon, and sliced cooked poultry are colonized by *L. carnosum* (Li et al., 2019; Geeraerts et al., 2018), albeit this bacterium has also been isolated from processed vegetables such as kimchi (Hong et al., 2014; Jung et al., 2014).

L. carnosum has been deemed responsible for meat deterioration by souring, discoloration, gas production, and slime formation (Shaw and Harding, 1989; Björkroth et al., 1998; Samelis et al., 2006; Raimondi et al., 2019). Based on genomic annotation (Candeliere et al., 2021), most strains can produce and secrete bacteriocins, including leucocin B, a class Iic bacteriocin effective against *L. monocytogenes* (Felix et al., 1994).

According to comparative genomics, *L. carnosum* encompasses a compact group of closely related heterofermentative bacteria that endorse most metabolic features (Candeliere et al., 2021). Many peptidase genes in the core genome and auxotrophy for nitrogen molecules, including many amino acids, vitamins, and cofactors, are compatible with adaptation to a nitrogen-rich environment like meat. Interestingly, it does not encode genes for biogenic amine production nor genetic determinants for antibiotic resistance.

L. carnosum presents high adaptability to adverse environmental conditions such as cold, oxidative, and high salt stresses that favor growth in adverse conditions, giving them a competitive advantage against other microbes. Although genomics of *L. carnosum* has been investigated, the metabolic activities of this species can be only partially inferred from the genome and additional biochemical efforts are claimed to evaluate the phenotypic potential of strains that potentially impact sensory features of foods, with potential industrial interest.

4.2. AIM OF THE PROJECT

L. carnosum frequently occurs in the microbiota of foods, mostly meat-based products where it can reach high loads 10^8 cfu/g. Their capacity to adapt to different environmental conditions, playing a double role in meat or meat products: spoilage and preservation. In this study, information on the metabolic potential of 12 strains of *L. carnosum* has been made available. The tested strains were isolated from the cooked ham in MAP or fresh sausages and had the genome sequenced (BioProject accession number PRJNA542256) and annotated (Candeliere et al., 2020, Candeliere et al., 2021). Growth kinetics were determined at different temperatures, as well as growth in oxidative and osmotic stress conditions. Biochemical characterization was performed to pinpoint the substrates that can be fermented, evaluate proteolytic and lipolytic capabilities, assess the

production of EPS and biofilm, and analyze the susceptibility to antibiotics. *In vitro* and animal models have been exploited to determine the potentiality of *L. carnosum* to reach and colonize the gut and modulate the immune response.

4.3. MATERIALS AND METHODS

4.3.1. Strains, media, and culture conditions

Twelve strains of *L. carnosum*, all originating from MAP sausage or cooked ham, were previously taxonomically characterized through 16S rRNA sequencing and collected from MIBI laboratory. The strains stored at -80 °C were spread onto DeMan, Rogosa agar plates (Lactobacilli MRS agar; BD Difco, Sparks, MD, United States) anaerobically for 48 h at 30 °C. A single colony was picked from each strain to inoculate fresh and static tubes of MRS broth and incubated at 30 °C for 48h.

The effect of temperature on growth kinetic and biomass yield was determined, cultures were incubated at 4, 8, 15, 23, 30, 37 and 42 °C. The growth of strains was evaluated by measuring the turbidity at 600 nm (OD₆₀₀) every 90 min. The specific growth rate (μ_{max}) was calculated through OD₆₀₀ data from the exponential tract of the growth curve. Optimum, minimal and maximal growth temperature (T_{opt} , T_{min} and T_{max}) were inferred with the Sym'Previus software (<http://symprevius.eu>) fitting in a secondary growth model the μ_{max} data as a function of temperature (Rosso et al., 1993).

4.3.2. Tolerance to osmotic and oxidative stress

Stationary phase cells grown overnight in MRS broth were seeded (5 % v/v) in fresh MRS broth supplemented with increasing concentrations of NaCl (0, 20, 40, 60, 80, and 100 g/L) or H₂O₂ (0, 0.0625, 0.125, 0.25, 0.5, and 0.75 g/L), to determine the tolerance to osmotic and oxidative stress (Montanari et al., 2018; Fessard and Remize, 2019). OD₆₀₀ was measured after the MRS tubes were incubated at 30 °C for 48 h.

4.3.3. Biochemical characterization

The biochemical profile of the strains was assessed for the fermentation of 49 substrates utilizing API 50 CH test strips (bioMerieux, Marcy-l'Etoile, France), according to the manufacturer's instructions. Briefly, the bacterial biomass from MRS-agar plates incubated for 24 h anaerobically at 30 °C was utilized to create a suspension with a turbidity equivalent to 2 McFarland in 10 mL of API 50 CHL Medium (Polypeptone 10 g/L, Yeast extract 5g/L, Tween 80 1 mL, Dipotassium phosphate 2 g/L, Sodium acetate 5 g/L, Diammonium citrate 2 g/L, Magnesium sulfate 0.2 g/L, Manganese sulfate 0.05 g/L, Bromocresol purple 0.17 g/L). The suspension was used for inoculating each tube of the strip. Anaerobiosis was obtained in the inoculated cupules by using sterile paraffin oil. The results were read after 48 h of incubation at 30 °C.

A modified MRS medium, in which glucose was replaced by 5 g/L of arginine (hereinafter referred to as MRS-arg) was used for evaluating the arginine utilization by *L. carnosum* strains. MRS-arg cultures, firstly seeded (5% v/v) with grown MRS tubes, were incubated for 24 h and propagated six times in the same medium. Then, OD₆₀₀ was measured and compared with that achieved by control MRS cultures.

Citrate fermentation was evaluated in citrate medium (Milliere et al., 2008), composed of: 4.5 g/L citric acid, 10 g/L proteose peptone (no. 3, BD Difco), 5 g/L yeast extract, 2 g/L K₂HPO₄, 5 g/L sodium acetate · 3H₂O, 1 g/L Tween 80, 0.2 g/L MgSO₄ · 7H₂O. Cultures in citrate medium, firstly seeded (5% v/v) with grown MRS tubes, were incubated for 48 h and propagated six times in the same medium. Then, OD₆₀₀ was measured and compared with that achieved by control cultures containing glucose.

4.3.4. Proteolytic and lipolytic activity

Proteolytic activity was assayed in MRS-agar plates supplemented with 30 g/L of gelatin (BD Difco) (Smith and Goodner, 1958). 5 µL of grown cultures of *L. carnosum* were spotted onto the surface of the plates. After 72h of incubation at 30 °C, plates were placed at 4 °C for 5h, then the diameter of hydrolysis halo around the spots was measured.

Lipolytic activity was evaluated in an MRS-agar medium containing triglyceride emulsions. It consisted of 900 mL of MRS, supplemented with 20 mL of 1 g/L rhodamine solution and an emulsion of 50 mL ddH₂O, 30 mL of seed oil, and 150 µL of Tween 80. Rhodamine solution and the oil emulsion were sterilized separately (i.e., filtered at 0.22 µm and autoclaved, respectively)

and mixed to the sterile MRS. Spots of 5 µL of grown cultures of *L. carnosum* were deposited onto the surface of the plates. After 72h of incubation at 30 °C, the plates were placed under a UV lamp, and the diameter of the hydrolysis halo around the spots was measured.

4.3.5. Antibiotic susceptibility

The antibiotic susceptibility of *L. carnosum* strains was tested accordingly to the EUCAST protocol (Matuschek et al., 2013). Grown MRS cultures were diluted in sterile saline (NaCl 9 g/L) to create suspensions with a turbidity of 0.5 McFarland and spread onto MRS-agar plates. On the surface of the medium, the antibiotic paper disks were applied. The following antibiotics were assayed: amoxicillin/clavulanic acid, AMC (30 µg); ampicillin, AMP (10 µg); azithromycin, AZM (15 µg); bacitracin, BAC (10 UI); cefixime, CFM (5 µg); ciprofloxacin, CIP (5 µg); clarithromycin, CLR (15 µg); kanamycin, KAN (30 µg); neomycin, NEO (30 µg); tetracycline, TET (5 µg); vancomycin VAN (5 µg). The inhibition halo was measured after 48 h of incubation at 30 °C. Strains were considered as sensitive (≥ 20 mm), intermediate (15–19 mm), and resistant (≤ 14 mm), as previously described (Szutowska and Gwiazdowska, 2021).

4.3.6. Anti-Listeria activity and cross-inhibition of *L. carnosum* strains

The disc diffusion method was used to analyze *in vitro* the activities of *L. carnosum* strains supernatants against *Listeria monocytogenes*. Cell-free supernatants of *L. carnosum* strains were harvested by centrifugation (5000 × g for 10 min) and split in aliquots, treated with one of the following: i) no treatment; ii) the pH was adjusted to 6.0 with 1 M NaOH; iii) addition of 0.1 mg/mL catalase, incubation at 25 °C for 30 min, then at 80 °C for 10 min; iv) addition of 0.2 mg/mL proteinase K, incubation at 37 °C for 1 h, then at 65 °C for 10 min. The supernatant aliquots were filter-sterilized at 0.22 µm and used for agar disk diffusion tests. Twenty µL of the sample (i.e., *L. carnosum* culture or supernatant aliquots) was spotted on 6-µm-diameter filter paper disks, placed over Brain Heart Infusion agar plates (BHI, Brain heart, infusion from (Solids) 6 g/L, Peptic digest of animal tissue 6 g/L, Sodium chloride 5 g/L, Dextrose 3 g/L, Pancreatic digest of gelatin 14.5 g/L, Disodium phosphate 2.5 g/L, DB Difco) seeded with 100 µl of *L. monocytogenes*. The diameter of the zone of inhibition was measured after 16 h of incubation at 37 °C.

For the cross-inhibition test, the samples of *Leuconostoc carnosum* were grown anaerobically at 30 °C for 48 h in MRS broth. First, 1 mL of culture was placed in sterile Petri dishes and covered

with 25 mL of agarized MRS. Once the plates were solidified, holes were made to form wells where 50 µL of cell-free supernatant were added. Next, the diameter of the inhibition halos was measured after 48 h of incubation anaerobically at 30 °C.

4.3.7. EPS and biofilm production

Exopolysaccharide (EPS) production was performed in MRS agar plates supplemented with 40 g/L of sucrose. The mucoid aspect of *L. carnosum* colonies was evaluated after 14 d of incubation at 4 °C or 48 h at 25, 30, and 37 °C (Fessard et al., 2016).

Biofilm formation was assayed by crystal violet staining in a microtiter plate (Landeta et al., 2013). *L. carnosum* was inoculated in MRS broth supplemented with 0.1 g/L Tween 80 and incubated for 24 h at 30 °C. Grown cultures were 10-fold diluted in the same medium and poured in a 96-well microtiter plate, 150 µL each well. After 14 d incubation at 4 °C or 48 h at 30 °C, the supernatants were removed and the biofilms adhering the wells were washed twice with phosphate-buffered saline (PBS, NaCl 8 g/L, KCl 0.2 g/L, Na₂HPO₄ 1.42 g/L, KH₂PO₄ 0.24 g/L) and stained with 150 µL of 1% Crystal Violet dye for 15 min. Stained biofilms were rinsed six times with PBS and extracted with ethanol/acetone solution (8:2 v/v) for 15 min. Biofilm formation index was calculated as the ratio between the absorbance of crystal violet measured at 570 nm and culture turbidity measured at OD₆₀₀. Strains with values > 1 were positive to biofilm formation (Amaretti et al., 2020).

4.3.8. Tolerance to simulated gastric and intestinal juices

Simulated gastric juice consisted of 3.2 g/L pepsin, 0.084 M HCl, 0.03 M NaCl, with the pH adjusted to 1.4 (Vecchione et al., 2018). Simulated intestinal fluid consisted of 8.5 g/L NaCl, 3.0 g/L bile salts (Oxgall, BD Difco), and 1.0 g/L pancreatin, with the pH adjusted to 7.4 (Castro-

Rodríguez et al., 2015). Both the gastric and the intestinal fluid were filter-sterilized at 0.22 µm. The biomass of the strains from 24-h MRS cultures was harvested by centrifugation (13000 × g at 4 °C for 5 min) and was washed in PBS (pH 5.5), then was resuspended at the concentration of 10⁶ cfu/ml in the gastric or the intestinal juice and incubated at 37 °C for 1 or 2 h, respectively. Viable counts were determined with MRS-agar plates. The resistance to sequential treatment with simulated gastric and intestinal fluids was performed. The biomass was harvested by centrifugation

(13000 × g at 4 °C for 5 min) after the gastric incubation, washed in PBS, then resuspended and incubated in simulated intestinal fluid.

4.3.9. Mice colonization

The biomass of three strains (*L. carnosum* WC 0318, *L. carnosum* WC 0319, and *L. carnosum* WC 0321), grown for 48 h in MRS broth was collected by centrifugation, suspended in PBS containing 15% v/v glycerol to obtain single-dose aliquots of 5×10^8 cfu in 150 µL stored at -80 °C. Aliquots were thawed day by day through the animal trial. The study has been performed in collaboration with the Department of Molecular Medicine, University of Padova (UNIPD).

Six-weeks-old Balb/c male mice were obtained from Envigo Laboratories (Oderzo, Italy) and housed in groups of 2 *per* individually ventilated cage, at 22 ± 2 °C under a 12-h light-dark cycle. Chow food and water were provided *ad libitum*. Mice were allowed to acclimate to the laboratory for 1 week before experimentation. Three groups of 6 mice were established, each group being assigned a *L. carnosum* strain. Each mouse received a daily dose of *L. carnosum* biomass via gastric gavage for 10 d. A control group of 6 mice daily received 150 µL of PBS containing 15% v/v glycerol. Faecal pellets were collected at the end of the treatment and after 10 d of washout from each mouse and immediately stored at -80 °C in pre-weighted tubes containing 50% glycerol. Viable *L. carnosum* in mice feces were enumerated with pour plate method within MRS-agar supplemented with 2 µg/mL vancomycin, 25 µg/mL trimethoprim, and 12.5 µg/mL cycloheximide. The plates were incubated at 30 °C for 48 h.

All experimental protocols involving mice were approved by the Animal Care and Use Ethics Committee of the University of Padova under license from the Italian Ministry of Health. In addition, they complied with the national and European guidelines for handling and use of experimental animals.

4.3.10. In-vivo effect on immune system

Male Balb/c mice were supplemented daily for 10 days with 5×10^8 cfu of *L. carnosum* WC 0318, *L. carnosum* WC 0319, or *L. carnosum* WC 0321 or sterile PBS as control. Then animals were sacrificed. The whole ileum was collected, washed in cold PBS, and the Peyer's Patches were isolated under a dissecting microscope. The mucosa was scraped off the underlying muscle layer of distal ~5 cm of ileum and immediately frozen in liquid nitrogen and stored at -80 °C.

The Peyer's patches were placed in DMEM containing dithiothreitol for 30 min to remove the mucus and then incubated for 90 minutes at 37 °C in Hanks' balanced salt solution without calcium but added with EDTA to remove epithelial cells. Peyer's patches were extensively washed with Hanks' balanced salt solution without calcium and then digested with collagenase D (2 mg/ml) and Dnase (10 µg/mL) for 5 minutes at 37 °C. Mononuclear cells were incubated for 30 min in ice-cold PBS containing 2% BSA or murine serum (blocking buffer) to block unspecific bindings. Cells were washed by centrifugation in PBS (1600 rpm, 6 min, twice) and incubated with the appropriate antibodies (Table 6). Finally, cells were washed with blocking buffer and analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA).

Frozen mucosal specimens were weighed and placed in ice-cold PBS buffer (1:10 w/vol ratio) supplemented with protease inhibitors (0.1 mM PMSF, 1 µM leupeptin, 150 nM aprotinin) and homogenized for 30 seconds. Cellular debris was removed by centrifugation (10,000 $\times g$ for 10 min at 4 °C). Levels of interleukin (IL)-1 β and IL-10 were determined in the cleared supernatants by enzyme-linked immunosorbent assay (ELISA), using commercially available kits (eBioscience, Prodotti Gianni, Milano, Italy). The assays were conducted following the manufacturer's recommended protocols.

Table 6. Antibodies used in the study

Antigen (host)	Clone	Supplier
CD3 (rat)	17A2	eBioscience (Thermo Fisher Scientific, Waltham, MA, USA)
CD4 (rabbit) Cy7 conjugate	50134-R001	Sino Biological Inc. (Wayne, PA, USA)
CD8 (rat) Cy7 conjugate	5H10	Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA)
CD25	PC61.5	EBioscience
FoxP3	FJK-16s	Invitrogen
CD11c (mouse) Cy7 conjugate	N418	EBioscience
TLR2	D-17	Abcam (Cambridge, UK)
TLR4	HTA125	Santa Cruz Biotechnology (Dallas, TX, USA)
CD80	16-10A1	Immuno Tools
MHC I	FJK-16s	EBioscience

4.3.11. Statistical analysis

Comparisons were performed with Student's t-test or one-way ANOVA followed by Tukey *post-hoc* test. Statistical analysis was done using SPSS Statistics 21 (IBM Corp., Armonk, NY, USA).

Jaccard's similarity scores among the strains were calculated with the software Past ver. 4.05 (Hammer et al., 2001) considering their metabolic profile, antibiotic susceptibility, and other physiological properties (Table 7) and were utilized to compute a UPGMA (unweighted pair group method with arithmetic mean) cladogram.

Table 7. Table of the phenotypic properties utilized to compute the UPGMA cladogram.

STRAIN	glycerol	erythritol	D-arabinose	L-arabinose	D-ribose	D-xylose	L-xylose	D-adonitol	methyl-L-β-D-xylopyranoside	D-galactose	D-glucose	D-fructose	D-mannose	L-sorbose	L-rhamnose	dulcitol	inositol	D-mannitol	D-sorbitol	methyl-L-α-D-mannopyranoside	methyl-L-α-D-glucopyranoside	N-acetyl-L-glucosamine	amygdalin	arbutin	esculin	salicin	D-cellobiose	D-maltose	D-lactose	D-melibiose	D-saccharose	D-trehalose	inulin	D-melezitose	D-raffinose
WC0318	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	1	0	0	0
WC0319	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	0	0	0	0
WC0320	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0	1	0	1	1	0	0	1	1	0	0	0
WC0321	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	1	1	0	0	0
WC0322	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	1	0	0	0
WC0323	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0	1	0	1	1	0	0	1	1	0	0	0
WC0324	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	1	0	0	0
WC0325	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	1	0	0	0
WC0326	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0	1	0	1	1	0	0	1	1	0	0	0
WC0327	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0	1	0	1	1	0	0	1	1	0	0	0
WC0328	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	1	1	0	0	0
WC0329	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0

	amidon	glycogen	xylitol	gentiobiose	D-turanose	D-lyxose	D-tagatose	D-fucose	L-arabitol	L-arabitol	potassium gluconate	potassium 2-keto-gluconate	potassium 5-keto-gluconate	citrate	arginine	AMC	AMP	AZM	BAC	CFM	CIP	CLR	KAN	NEO	TET	VAN	Anti- <i>Listeria</i>	Biofilm at 30 °C	EPS at 30 °C	GJ survival	IJ survival	Growth at 42°C	Growth with 60g/L NaCl	Growth with 6.25 mg/L H ₂ O ₂
WC0318	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	1	0	0	0	0	1	1	1	1
WC0319	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	1	0	1	1	0	0
WC0320	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	0	0	1	1	1	1
WC0321	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1	1	0	1	0	0	1
WC0322	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	0	0	1	0	1	1	0	1
WC0323	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1	1	0	1	0	0	1
WC0324	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	1	0	1	1	0	1
WC0325	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	1	1
WC0326	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	0	0	1	1	1	1
WC0327	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	0	0	1	1	0	1
WC0328	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	1	1	0	1	0	0	0	0	1	0	0	1
WC0329	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	1	1	0	1	0	0	1	0	0	0	0	1	1	0	1

4.4. RESULTS

4.4.1. Growth kinetics and temperature optimum

The growth kinetic of 12 *L. carnosum* strains was studied in MRS broth. All the strains grew well in the temperature ranging from 4 to 37 °C, showing some difficulty at 42 °C. The optimum temperature was observed in the range of 30.6 – 34.2 °C (median = 32.5 °C), while minimum and maximum ones were between 0.3–3.0 °C (median = 1.4 °C) and 42.6 – 44.8 (median = 44.0 °C), respectively (Figure 4.2). All strains presented the highest maximum specific growth rate ($\mu_{\max}$) at 30 °C ($P < 0.05$), ranging from 0.23 to 0.34 h⁻¹ with a median of 0.30 h⁻¹ (Figure 4.3). On the other hand, the highest biomass yields for all the strains were observed at 23 °C ($P < 0.05$), with OD₆₀₀ ranging from 1.30 to 3.39, with a median value of 2.56 (Figure 4.3). At the end of the growth phase, the medium generally reached a pH between 4 and 5, regardless of the growth temperature (data not shown).

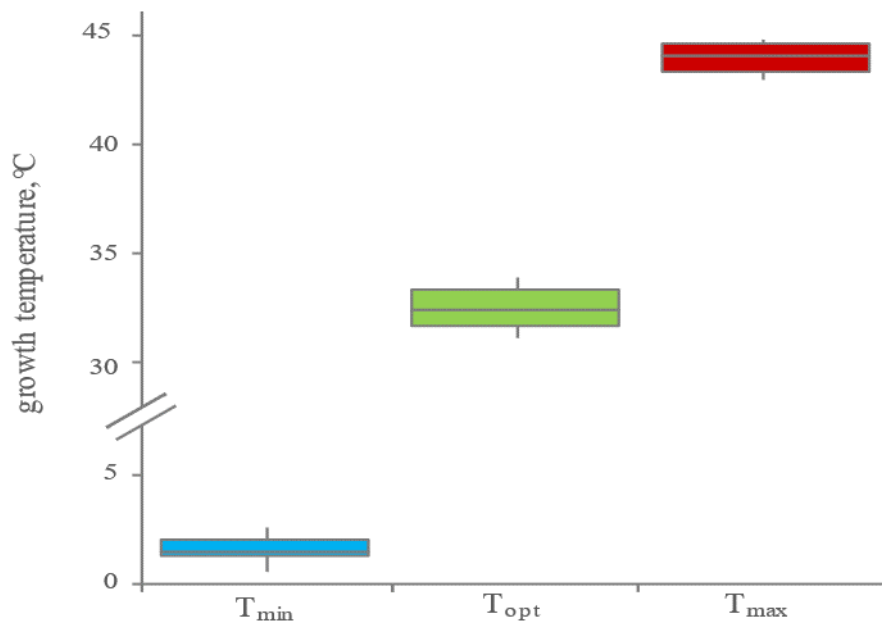


Figure 4.2 Distribution of minimum, optimum, and maximum growth temperature for 12 *L. carnosum* strains, cultured in MRS broth for 48 h. Boxes indicate the 25th, 50th, and 75th percentiles; whiskers indicate the 10th and 90th percentiles.

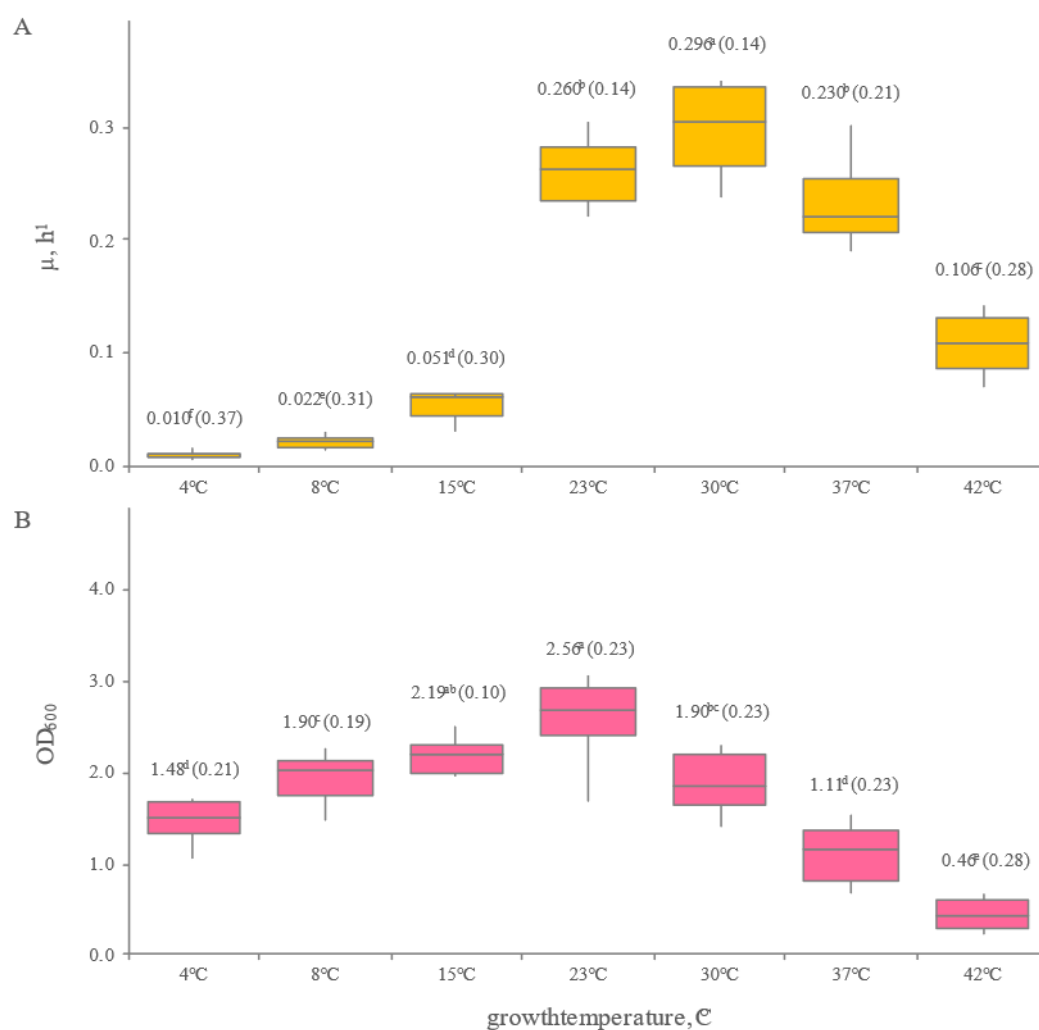


Figure 4.3 Effect of temperature on $\mu_{\max}$ (A) and the biomass yield (B) of 12 *L. carnosum* strains, cultured in MRS broth for 48 h. (B) Boxes indicate the 25th, 50th, and 75th percentiles; whiskers indicate the 10th and 90th percentiles. Mean values are reported in the labels, with the coefficient of variation in brackets. Within each panel, means with different letters significantly differ ($P < 0.05$, ANOVA with Tukey's post hoc).

4.4.2. Resistance to oxidative and osmotic stress

L. carnosum strains were cultured in MRS broth containing increasing concentrations of H₂O₂ and NaCl, to evaluate their adaptability to oxidative and osmotic stress. The concentration of 0.0625 g/L of H₂O₂ limited the biomass yield by at least 1 magnitude ($P < 0.05$), while higher concentrations entirely repressed growth (Figure 4.4). At 0.0625 g/L H₂O₂, differences among the strains were observed: *L. carnosum* WC319 did not survive, most strains reached an OD₆₀₀ < 0.2, while *L. carnosum* WC0329 grew better, yielding an OD₆₀₀ of 0.36.

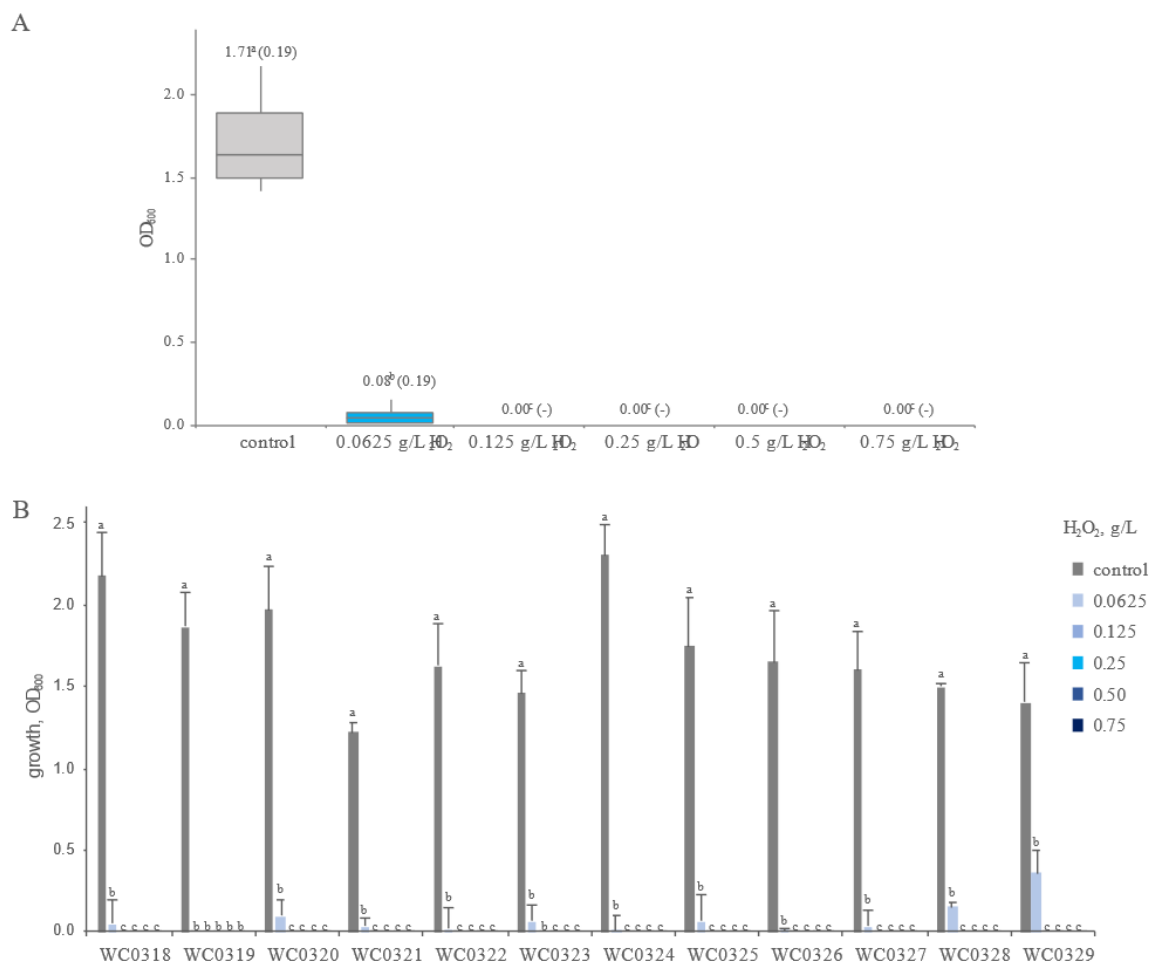


Figure 4.4 Distribution of the growth performance of 12 *L. carnosum* strains in presence of an increasing concentration of H₂O₂. (A) Boxes indicate the 25th, 50th, and 75th percentiles; whiskers indicate the 10th and 90th percentiles. The labels report the mean and, in brackets, the coefficient of variation; different letter superscripts indicate means that significantly differ ($P < 0.05$, ANOVA with Tukey's post hoc). (B) Growth yield of the single strains. Values are means $\pm$ SD, $n = 3$. Within each strain, means with different superscripts significantly differed ($P < 0.05$, ANOVA with Tukey's post hoc).

On the other side, *L. carnosum* strains were able to grow in presence of an increasing concentration of NaCl up to 60 g/L of NaCl, although with an impaired yield (Figure 4.4A). Pronounced differences were observed among the strains (Figure 4.4B). At 60 g/L NaCl, *L. carnosum* WC0327 showed the lowest growth yield and *L. carnosum* WC0318 exhibited the highest one, with OD₆₀₀ values of 0.03 and 0.38, respectively. Only *L. carnosum* WC0328 survive to some extent with 80 g/L NaCl, even though with an OD₆₀₀ < 0.1.

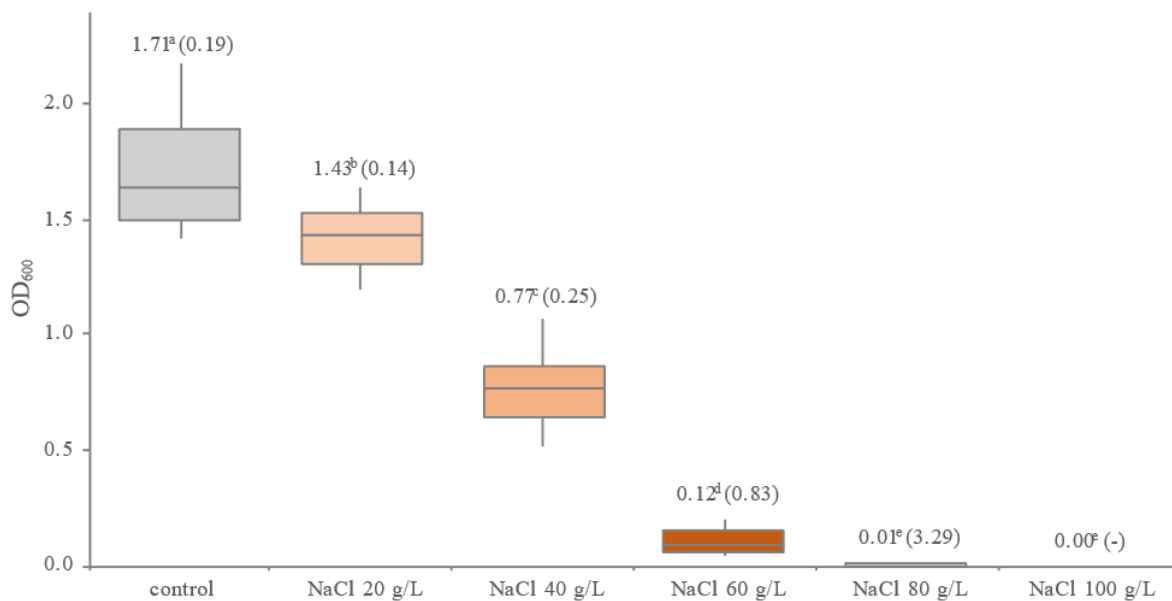


Figure 4.4A Distribution of the growth performance of 12 *L. carnosum* strains in presence of an increasing concentration of NaCl. Boxes indicate the 25th, 50th, and 75th percentiles; whiskers indicate the 10th and 90th percentiles. The labels report the mean and, in brackets, the coefficient of variation; different letter superscripts indicate means that significantly differ ($P < 0.05$, ANOVA with Tukey's post hoc).

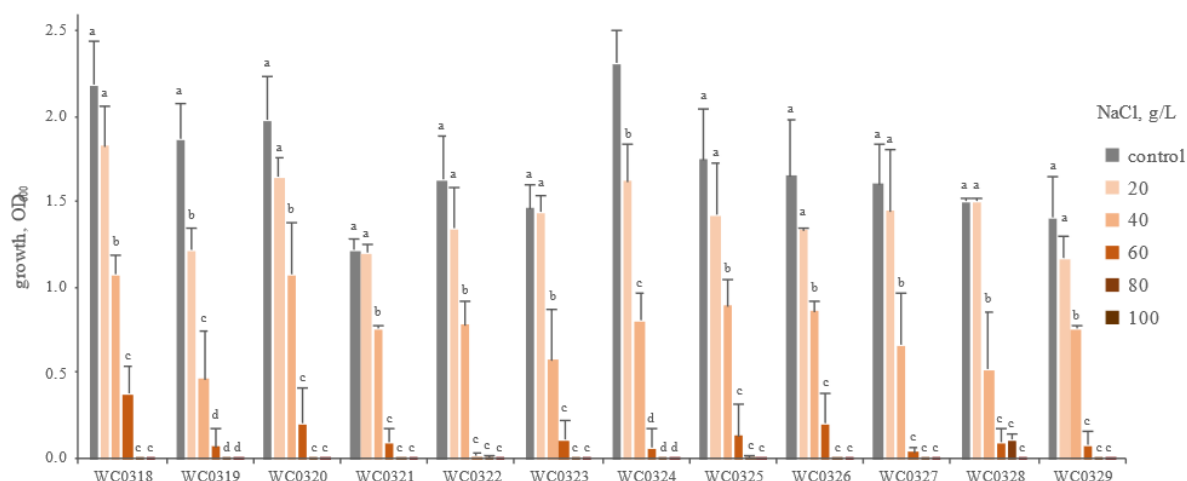


Figure 4.4B Growth of *L. carnosum* strains in presence of an increasing concentration of NaCl. Growth yield of the single strains. Values are means $\pm$ SD, n = 3. Within each strain, means with different superscripts significantly differed ($P < 0.05$, ANOVA with Tukey's *post hoc*).

4.4.3. Biochemical characterization

The 12 *L. carnosum* strains were evaluated for their ability to ferment the substrates available on API 50 CH strips. Moreover, modified MRS media were tested in further growth experiments (Figure 4.5, Table 8).

Table 8. Substrate utilization by the 12 *L. carnosum* strains assessed with API 50 CH. Only positive tests were shown, reporting the number and the % of strains able to grow.

Substrate	No. of positive test (%)
D-cellobiose	5 (42)
D-fructose	10 (83)
D-glucose	12 (100)
D-maltose	5 (42)
D-mannose	10 (83)
D-ribose	12 (100)
D-saccharose	12 (100)
D-trehalose	10 (83)
D-turanose	11 (92)
esculin	12 (100)
gentiobiose	6 (50)
methyL- α -D-glucopyranoside	11 (92)
N-acetyl-L-glucosamine	10 (83)
potassium gluconate	7 (58)

All the strains successfully utilized glucose, ribose, sucrose, and esculin. Ten out of 12 strains fermented fructose, mannose, methyl- α -glucopyranoside, N-acetyl-glucosamine, trehalose, and turanose, while cellobiose, maltose, gentiobiose, and gluconate were fermented by 5–7 strains. On the other hand, 37 substrates were not utilized by all the strains, among which glycerol, erythritol, D- and L-arabinose, D- and L-xylose, galactose, L-sorbose, L-rhamnose, inositol, mannitol, sorbitol, lactose, melibiose, inulin, melezitose, raffinose, amidon, glycogen, xylitol, tagatose, fucose, citrate, and arginine. *L. carnosum* WC0329 utilized the least number of substrates (6, i.e., glucose, fructose, ribose, sucrose, gluconate, and esculin). On the contrary, *L. carnosum* WC0323 is the most versatile fermenter of substrates (14, i.e., glucose, fructose, ribose, mannose, methyl- α -glucopyranoside, N-acetyl-glucosamine, cellobiose, maltose, esculin, sucrose, trehalose, gentiobiose, turanose, and gluconate).

All the strains showed a negative response to gelatin and triglyceride hydrolysis when they were assessed for proteolytic and lipolytic activity.



Figure 4.5 Metabolic characterization of 12 *L. carnosum* strains. The utilization of substrates was assayed with API 50 CH, except for arginine and citrate, assayed with specific media. Positive and negative assays are displayed in green and red, respectively.

4.4.4. Antibiotic resistance

The susceptibility to 11 antibiotics was screened for the 12 *L. carnosum* strains (Figure 4.6). All the strains showed resistance to cefixime and vancomycin, and most of them were resistant or gave an intermediate response to ciprofloxacin and kanamycin. On the other hand, all the strains were susceptible to amoxicillin/clavulanic acid, azithromycin, clarithromycin, and tetracycline. They also were sensitive or gave an intermediate response to neomycin. In addition, most strains were susceptible to ampicillin and bacitracin. *L. carnosum* WC0329 was resistant to ampicillin, while *L. carnosum* WC0319 and *L. carnosum* WC0322 were resistant to bacitracin.

4.4.6. EPS and biofilm production

The EPS production was assessed by observing the colonies grown at 4, 25, 30, and 37 °C (Figure 6.6). When cultured at 4 and 25 °C, only six of the screened strains showed the ability to produce EPS (i.e., *L. carnosum* WC0319, *L. carnosum* WC0321, *L. carnosum* WC0322, *L. carnosum* WC0323, *L. carnosum* WC0324, and *L. carnosum* WC0325), generating mucoid colonies. EPS production was progressively reduced with the increase in temperature. In the colonies of *L. carnosum* WC0325 at 25 °C and those of *L. carnosum* WC0321 and *L. carnosum* WC0323 at 30 °C, a reduction of mucosa was observed. No strains presented mucoid colonies at 37 °C.

L. carnosum WC0321 and *L. carnosum* WC0323 produced evident biofilm both at 4 and 30 °C. Conversely, a slight biofilm formation was observed only at 4 and not at 30 °C for *L. carnosum* WC0324, *L. carnosum* WC0326, *L. carnosum* WC0327, and *L. carnosum* WC0329 (Figure 4.7).

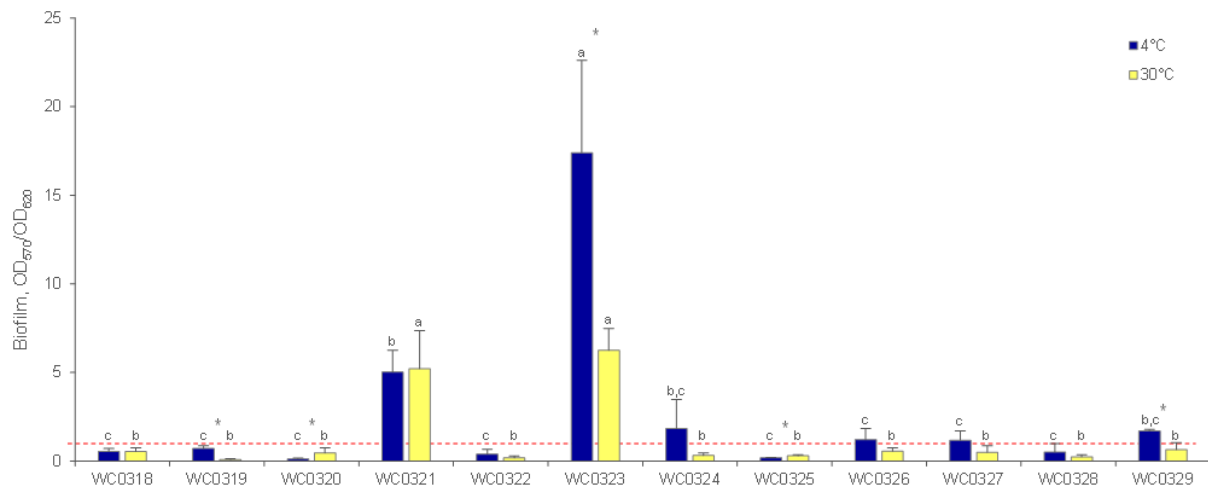


Figure 4.7 Biofilm production in *L. carnosum* strains at 4 and 30 °C, quantified with crystal violet. Values are means $\pm$ SD, n = 3. Within each strain, * indicates a significant difference between the temperatures ($P < 0.05$, t-test). Within temperature, means with different superscript significantly differed ($P < 0.05$, ANOVA with Tukey's *post hoc*). The red dashed line is the threshold of 1.0, above which biofilm formation was considered positive according to Amaretti et al., 2020.

4.4.7. Resistance to simulated gastric and intestinal juices

All the strains did not survive the treatment with simulated gastric juice (Figure 4.6). On the other hand, all the strains tolerated exposure to the simulated intestinal fluid, showing residual viability from 16 to 100% (Figure 4.6, Table 9). Coherently, no strains survived the sequential incubation, first in the gastric and then intestinal juice.

Table 9. Resistance at the simulated intestinal juice. Strains were treated for 2 h at 37 °C. The residual viability (%) was reported.

Strain	Residual viability
WC0318	100%
WC0319	100%
WC0320	16%
WC0321	100%
WC0322	28%
WC0323	69%
WC0324	100%
WC0325	42%
WC0326	25%
WC0327	20%
WC0328	100%
WC0329	48%

4.4.8. Hierarchical clustering of phenotypic diversity

Based on Jaccard's similarity of the phenotypic traits, the hierarchical clustering of the 12 *L. carnosum* strains identified three clades (Figure 4.8). One clade included *L. carnosum* WC0319, *L. carnosum* WC0324, *L. carnosum* WC0325, and *L. carnosum* WC0329, which were all unable to utilize the disaccharides cellobiose, maltose, and gentiobiose and did not produce bacteriocins and biofilm, at least at 30 °C. The second clade encompassed *L. carnosum* WC0321, *L. carnosum* WC0322, *L. carnosum* WC0323, which were the most susceptible to kanamycin and neomycin. In

addition, they produced EPS, with *L. carnosum* WC0321 and *L. carnosum* WC0323 that also performed anti-*Listeria* activity and were the leading biofilm producers. The third clade included *L. carnosum* WC0318, *L. carnosum* WC0320, *L. carnosum* WC0326, *L. carnosum* WC0327, *L. carnosum* WC0328, which were characterized by the broader spectrum of substrates utilization, particularly disaccharides.

The strains *L. carnosum* WC0318, *L. carnosum* WC0319, and *L. carnosum* WC0321, each belonging to a different clade, were selected to investigate their potential in colonizing the intestine of mice.



Figure 4.8 UPGMA cladogram of the 12 strains *L. carnosum* according to their phenotypic traits. The strains selected for the mice colonization study are labeled in red.

4.4.9. Colonization of mice gut

After 10 d of daily administration at the dose of 5×10^8 cells, *L. carnosum* WC0318, *L. carnosum* WC0319, and *L. carnosum* WC0321 failed to colonize the intestine of Balb/c mice. No colonies were observed onto *Leuconostoc* selective plates inoculated with fecal samples from the three mice groups, each receiving a single strain.

4.4.10. Effect on the intestinal mucosa-associated immune system

The phenotype of dendritic cells (DC) and lymphocytes in Peyer's patches were analyzed to evaluate the impact of *L. carnosum* on the immune system. Following 10 d of supplementation, *L. carnosum* WC0321 improved all the measured markers of DC maturation (namely CD-80, MHC-II, TLR2, and TLR4), while the effects of *L. carnosum* WC0318 and *L. carnosum* WC0319 were limited (Figure 4.9). *L. carnosum* effects were limited to DC, since no variation on polarization of CD3⁺ lymphocytes toward regulatory (CD4⁺ CD25⁺ FoxP3⁺) or inflammatory (CD3⁺ CD8⁺ IL17⁺) phenotype were archived.

The expression level of IL-1 β and IL-10 was measured after 10 days of supplementation to evaluate the impact of *L. carnosum* on mucosal immune system balance. No significant effects on mucosal cytokine levels were observed ($P > 0.05$), as reported in Figure 4.10.

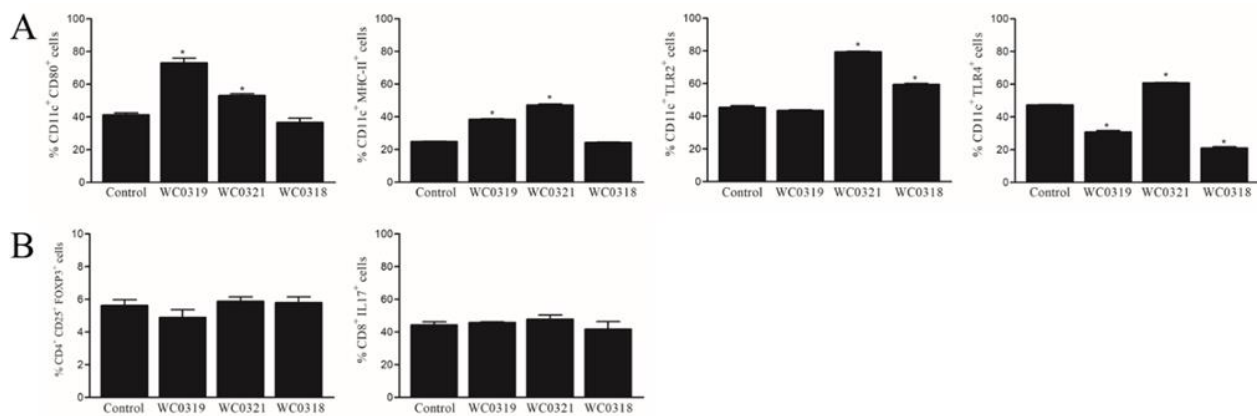


Figure 4.9 Effect of daily oral supplementation with 5×10^8 cfu of *L. carnosum* WC0318, *L. carnosum* WC0319, and *L. carnosum* WC0321 on intestinal mucosa immune cells: A) DC (CD11c⁺); B) CD3⁺ lymphocytes. * Indicates significant difference versus the control ($P < 0.01$, t-test).

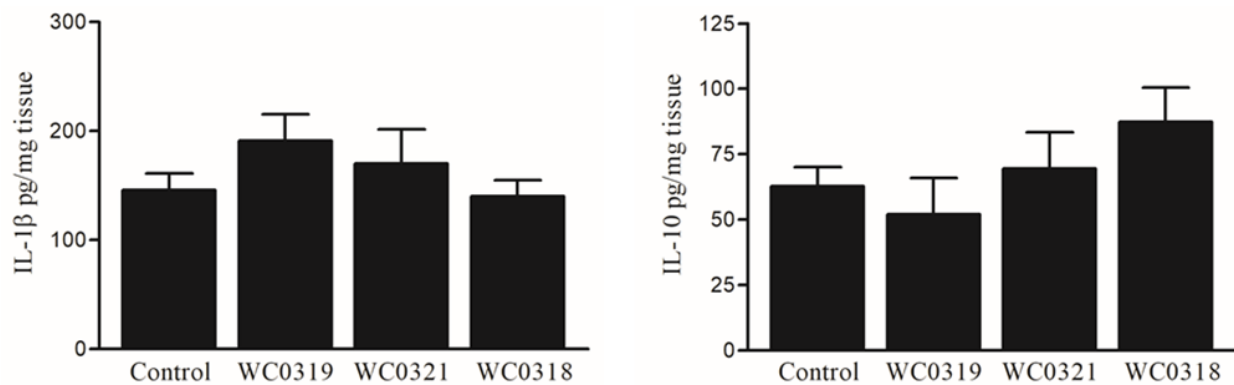


Figure 4.10 Effect of daily oral supplementation with 5×10^8 cfu of *L. carnosum* WC0318, *L. carnosum* WC0319, and *L. carnosum* WC0321 on intestinal mucosa IL-1β and IL-10, quantified by ELISA. Cytokine levels were expressed as pg/mL of tissue lysate. Means did not significantly differ from the control ($P > 0.05$, t-test).

4.5. DISCUSSION

The genome of twelve strains of *L. carnosum* has been subjected to comparative analysis (Candeliere et al., 2021). The same 12 strains in terms of biochemical, physiological, and functional properties were studied. The range of sugars fermented by the strains was narrowed to a few mono- and disaccharides in agreement with the carbohydrates present in the main isolation sources of the species, i.e. meat products and fermented vegetables (Jung et al. 2014; Ishii et al. 2017; Raimondi et al. 2018).

All the strains fermented D-ribose, D-glucose, D-saccharose and esculin. Almost of them fermented trehalose, D-fructose, D-mannose, methyl- α -D-glucopyranoside and N-acetyl-glucosamine. Only few strains fermented D-cellobiose, D-maltose, gentiobiose and potassium gluconate.

Glucose and sucrose enter the bacterium through specific PTS-phosphotransferase systems and are fermented via the pentose phosphate and phosphoketolase pathway (Candeliere et al., 2021). In meat matrices, glucose is the result of the hydrolysis of glycogen, whereas sucrose is found in plants or in fermented meat products, where it may be added to accelerate the fermentation rate. Eight out of twelve strains fermented mannose and/or fructose. The genome of all the strains also harbours the genes of complete ABC transporters for ribose uptake. Ribose is present in certain vegetables and in meat, properties of meat products (Meinert et al. 2009). All the strains presented the ability to ferment trehalose that, mainly in conjugated forms, actively participates in signalling in plants, and esculin, a plant glucoside. Moreover, cellobiose fermentation was limited to a few strains, according to previous indications (Shaw and Harding, 1989). However, all the *L. carnosum* strains contain the genes for a complete cellobiose PTS transport system. The ability to ferment substrates such as trehalose, esculin, and cellobiose indicates a remote and efficient association of *L. carnosum* with plants (Jung et al. 2014; Ishii et al. 2017). *L. carnosum* did not catabolize arginine, unlike other *Lactobacillus sakei* and other LAB which thrive in meat products achieving energy from arginine through the arginine deiminase pathway. Unlike other *Leuconostoc* species, *L. carnosum* did not catabolize citrate, lacking the genes encoding citrate transporter, citrate lyase, and oxaloacetate decarboxylase, and thus based only on carbohydrate fermentation for energy supply (Candeliere et al. 2021; Özcan et al. 2019).

During processing and conservation, meat products are subjected to factors that hamper the growth of microorganisms, such as temperature (generally low), pH variations, osmotic and

oxidative stress. Therefore, efficient colonizers are expected to show some adaptation to respond to such hostile conditions (D'Angelo et al., 2017). According to the results obtained by Fessard and Remize (2019), *L. carnosum* revealed that the species is mesophilic and versatile, growing from 4 to 37 °C with an optimum in the range of 30.5 – 34.2 °C. The ability to survive at temperatures that are compatible with refrigeration explains the bloom of *L. carnosum* described in many meat-based products (Samelis et al., 2000). Although the growth rate is much lower at 4 °C than at mesophilic temperatures, long shelf-life periods enable *L. carnosum* to reach remarkably high charges. Furthermore, *L. carnosum* may take advantage of any temperature abuse occurring in refrigerators and in the cold chain since an increase from 4 to 8 °C causes a two-fold rise of the specific growth rate, even if the optimum temperature is normally never reached.

The ability of *L. carnosum* to efficiently grow up to 60 g/L NaCl, albeit with reduced yields in the presence of increasing the amount of salt, is consistent with his adaptability to high-salt food matrices. On the other side, according to the absence of catalase, all the strains are susceptible to hydrogen peroxide.

EPS can protect bacterial cells against biotic and/or abiotic stress, including dehydration, high temperature, acid and osmotic stress. They participate in biofilm formation and cell adhesion mechanisms, exerting a role in the interaction with substrates and other organisms. EPS producing bacteria take part in the deterioration of meat-based foods, such as MAP cooked ham, where they cause the appearance of a ropy slime, occasionally before the sell-by date, being this phenomenon unacceptable for consumers. Genes putatively involved in EPS production were found in the genomes of all the strains herein studied (Candeliere et al., 2021), but only 6 out of 12 strains were positive to the ropy phenotype, with a stronger tendency to form dense colonies at the lower growth temperatures. This observation confirms that some strains of *L. carnosum* could be involved in the appearance of slime in certain foods and may be responsible for the quality decline.

Nowadays, an increasing demand for healthy, fresh, and natural foods devoid of added chemical preservatives and stabilizers has fostered the search for bioprotective starters, particularly among LAB. Various species belonging to the genus *Leuconostoc*, including *L. carnosum*, produce bacteriocin of different classes, which affect *Listeria* (e.g. leucocins A and C) and/or other LAB such as *Leuconostoc* and *Weissella* (e.g. leucocin B) (Felix et al. 1994; Wan et al. 2015, Pérez-Sánchez et al. 2011; Parente et al., 1996). This study gives evidence of the protective role of *L. carnosum* WC0321 and *L. carnosum* WC0323 against *L. monocytogenes*, consistently with the presence in these strains of the genes that encode for leucocin B and the ABC-like transporter LanT, the former included in plasmids and the latter in the genome (Candeliere et al. 2021). Interestingly,

these strains exerted antimicrobial activity also against other *L. carnosum* strains, and it should be investigated if the same bacteriocin mediated such effect. Consistently, *L. carnosum* WC0329, which includes in the plasmid only genes for leucocin B but does not have the gene encoding for the transporter, did not exert any anti-*Listeria* activity. The ability to inhibit *L. monocytogenes* and likely other LAB involved in spoilage is encouraging for the possible utilization of specific *L. carnosum* strains as bioprotective starters to contain the growth of both spoilage and pathogenic microorganisms. The exploitation of starters that do not produce EPS and biogenic amines, and lack lipolytic and proteolytic activities, may extend the shelf-life of the products, particularly if the growth of these strains is accompanied by the absence of adverse effects on visual, olfactory, and gustative properties (Ammor and Mayo 2007; Singh et al. 2012; Zarour et al. 2012).

The potential impact of *L. carnosum* on health is relevant since consuming conserved meats at retail offers great microbial exposure, with loads of 10^7 – 10^8 cfu/g. The fate of these high amounts of live bacteria depends on genetic and functional traits that can confer the ability to resist the stress conditions that they encounter, firstly in the stomach, then in the intestine. *L. carnosum* challenged with simulated gastric juice did not survive, whereas it resisted the stress conditions likely encountered in the intestine such as pH, redox conditions, intestinal motility, nutrients and host secretions (gastric acid, bile, digestive enzymes and mucus) (Gerritsen et al., 2011). This result was consistent with the unsuccessful recovery of *L. carnosum* from feces of mice boosted with a high dosage of 3 different strains of *L. carnosum*.

The oral supplementation of different *L. carnosum* strains did not affect the maturation of intestinal lymphocytes and mucosal levels of IL-1 β and IL-10 in mice but influenced the maturation of surface markers of intestinal DC. The response of DC was higher with *L. carnosum* WC0321, compared to WC0318 and WC0319, confirming significant genetic variability and phenotypic diversity among strains. This is not surprising since the existence of strain-specific effects on host mucosal-associated immune system is recognized in the probiotic field (McFarland et al. 2018; Luyer et al. 2005; Kekkonen et al. 2008; Sagheddu et al. 2020). Although viable probiotics are more effective than non-viable probiotics, non-viable probiotics may exert significant beneficial effects on human health (Gueimonde et al. 2004; Taverniti et al. 2011; Lahtinen, 2012), thus supporting the immune-modulatory activity of *L. carnosum* that, following the ingestion with the food, is not able to survive to the harsh gastrointestinal environment. Indeed, various probiotic cell components, including capsular structures, cell wall extracts, DNA, S-layers proteins, can activate DCs through Toll-like receptor signaling, which plays a critical role in generating protective immune responses (Lahtinen, 2012; Engevik et al. 2021).

As a whole, ingestion of *L. carnosum* is expected not to impact health status, or at least not to determine any colonization, even transient. However, it is still possible that *L. carnosum* is part of the wide and understudied biodiversity of LAB identified in human feces (Rossi et al., 2016). This aspect deserves a deeper investigation through whole-genome sequencing data-mining.

Intriguingly, *L. carnosum* WC0321, exhibiting the strongest immunomodulatory activity *in vivo*, produces both EPS and biofilms to a great extent and its genome encodes several specific genes involved in EPS biosynthesis that are missing in *L. carnosum* WC0318 and WC0319. In particular, the genome of *L. carnosum* WC0321 encodes protein-tyrosine kinases and protein phosphatases that regulate the formation of exopolysaccharides (EPS), EPS biosynthetic genes referred to the *ywq* group, and EPS glycosyltransferases, nowadays recognized to mediate plenty of beneficial properties in probiotic strains (Angelin and Kavitha, 2020). Overall, these data support the possibility that certain strains of *L. carnosum* might exert favourable effects on the host mucosal-associated immune system.

Some antibiotic resistance was highlighted, although it was not predicted by comparative genomics. The shared resistance to the β -lactam cefixime and the fluoroquinolone ciprofloxacin is likely due to some intrinsic mechanism, as it happens for vancomycin (Flórez et al. 2016). On the other hand, the resistance to ampicillin or bacitracin showed by a few strains can be caused by some mechanism that eluded the detection by genome scanning.

Overall, many of the features evaluated here should assure the safety of *Leuconostoc carnosum* and provide significant opportunity for the exploitation of chosen starters in preserved meats, where the species is highly competitive in response to low temperatures and the presence of salt. Bacteriocin producers *L. carnosum* WC0321 and *L. carnosum* WC0323 are potential candidates for such technological use. These strains should be tested in a real food matrix to confirm their biopreservative activity and observe whether they have any negative impacts on food sensory qualities, such as slime production.

These results of this study are published in:



Phenotypic Traits and Immunomodulatory Properties of *Leuconostoc carnosum* Isolated From Meat Products

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5. Project 3

Phylogenomic analysis of the genus *Leuconostoc*

5.1. INTRODUCTION

5.1.1. Genus *Leuconostoc*

The name *Leuconostoc* derives from “leucos” light and “nostoc” an algal genus (Euzéby, 2009). With the genera *Convivina*, *Fructobacillus*, *Leuconostoc*, *Oenococcus*, and *Weissella*, it encompasses a group of LAB belonging to the family of Leuconostocaceae (Nieminen et al., 2014). Recently, a taxonomical reclassification of these genera was performed. Based on the average amino acid identity of core proteins (cAAI), they were included in the order Lactobacillales (Zheng et al., 2020). In particular, the Leuconostocaceae presented a common root with the heterofermentative *Lactobacillus spp.*, the genus *Pediococcus*, and the *Lactobacillus plantarum* group, resulting in a monophyletic group within the Lactobacillaceae.

According to the List of Prokaryotic names with Standing in Nomenclature (<https://lpsn.dsmz.de/genus/leuconostoc>), the genus *Leuconostoc* encompasses 16 validly named species (Parte et al., 2020). They are saccharolytic bacteria that catabolize carbohydrates to lactic acid through heterolactic fermentation. They are facultative anaerobe, with an optimum temperature between 20-30 °C (Dellaglio et al., 1995). Some species grow well at low temperatures and do not grow beyond 37 °C (Raimondi et al., 2021). They prefer a pH of around 6-7 to develop (Dellaglio et al., 1995). Some species can produce dextran from sucrose, such as *L. carnosum*, *L. citreum*, *L.*

gelidum, and *L. gasicomitatum* (Shaw&Harding, 1989; Björkroth et al., 2000; Ogier et al., 2008; Holzapfel et al., 2009).

They are present where carbohydrate-based substrates are available, such as plants, plant-derived matrices, silage, fermented foods (e.g. dairy products, fermented dough, milk, vegetables, and meats), spoiled foods, and sewage (Dellaglio et al., 1995). In some cases, species are associated with a specific habitat, such as vegetables (Yu et al., 2020), meats (Candeliere et al., 2021), or other foods (Vedamuthu, 1994).

Whole-genome sequencing, physiological, biochemical, and chemotaxonomic approaches are required to define new species taxonomy (Ramasamy et al., 2014), along with genetic relatedness studies compare housekeeping gene sequences targeted in different microbial entities that confer precise genetic fingerprints. For example, in addition to the 16S rRNA gene, two housekeeping genes that code for the phenylalanyl-tRNA synthase alpha-subunit (*pheS*), and RNA polymerase alpha-subunit (*rpoA*) are genetic targets for microbial identification of bacteria (Das et al., 2014). For the identification of *L. falkenbergense* strains at the species level, *rpoA* has been exploited (Wu and Gu, 2021), while *pheS* is one of the targets in the Multilocus Sequence Analysis (MLSA), successfully applied for differentiation of species of the genus *Leuconostoc* (Rahkila et al., 2014).

5.2. AIM OF THE PROJECT

New bioinformatics tools are available for taxonomic studies, that allow the exploitation of the increasing number of accessible genomes. Phylogenomics is a novel genome-based approach of taxonomy that complements and overtakes the comparison of the 16S rRNA gene (Patané et al., 2018). At present, the use of genome sequences is recommended for taxonomic purposes instead of conventional DNA-DNA hybridization and 16S rRNA phylogeny (Chun and Rainey, 2014; Chun et al., 2018; Kim et al., 2021). Phylogenomics is inferred using a set of core genes rather than a single gene (Eisen and Fraser, 2003), providing a relevant improvement in terms of reliability as evidenced by high bootstrap values support (Ahrenfeldt et al., 2017). ANI, which provides an average measure of similarity across homologous regions shared by a pair of genomes, is among the major metrics used for this purpose (Palmer et al., 2020).

The characterization of most *Leuconostoc* species is based on comparing the new identified isolated with the existing ones and a comprehensive phylogenomic study that analyzes the relationships within the genus is still lacking. To fill this gap, this project investigated the evolutionary relationship among the species through a phylogenomic approach and redefined the species boundaries based on genome similarity. Phylogenetic relatedness of 221 *Leuconostoc* genome sequences belonging to 13 species has been explored through ANI, dDDH, and core genome comparison. Furthermore, phylogenetic relationships were reconstructed using the single genes encoding 16S rRNA, the phenylalanyl-tRNA synthase alpha-subunit (*pheS*), and RNA polymerase alpha-subunit (*rpoA*), and the consistency of these outputs with the ANI phylogenetic tree was assessed.

5.3. METHODS

Genome sequences of 221 *Leuconostoc* available in September 2021 were selected from the NCBI database, and their accession numbers are reported in Table 10. Only genomes from pure culture sequencing were used, while metagenome-assembled genomes were not considered. To check the completeness and contamination of genomes, CheckM has been utilized (Parks et al., 2015).

Genomes annotation was performed with Prokka (Parks et al., 2015) with the minimum percentage identity parameter set at 80%. The pangenome was calculate with Roary (Page et al., 2015).

The 16S rRNA, *rpoA*, and *pheS* genes were extracted from each genome and aligned with Clustal Omega on EMBL-EBI website (Sievers et al., 2011; Madeira et al., 2019).

The Average Nucleotide Identity (ANI) between all the genomes was calculated with ANI Calculator (<http://enve-omics.ce.gatech.edu/ani/>) by all-against-all approach (Rodriguez-R and Konstantinidis, 2016).

Phylogenetic trees were generated with the maximum likelihood method using RAxML tool starting from the alignments of 16S rRNA, *rpoA*, and *pheS* genes and the core genes identified by Roary (Stamatakis, 2014). The ANI distance matrix was used to compute an Unweighted Pair Group Method with Arithmetic mean (UPGMA) unrooted phylogenetic tree with DendroUPGMA tool (Garcia-Vallvé et al., 1999). The ANI tree was compared with those constructed from the alignment of 16S rRNA gene, *pheS*, *rpoA*, or the core genome, utilizing the following ‘generalized’

Robinson-Foulds metrics: the Jaccard–Robinson–Foulds (JRF), computed with $k = 1$ (Nye et al., 2006), and the information-based measures of Mutual Clustering Information (MCI) and Shared Phylogenetic Information (SPI) (Smith, 2020). JRF, MCI, and SPI were computed with the R package ‘TreeDist’, archived at <https://dx.doi.org/10.5281/zenodo.3528123>. Comparison relied on matching each informative split within a tree (i.e. an internal branch of the tree, with at least 2 leaves at each extremity) with an informative split within the other tree. For each metric, the comparison yielded a normalized similarity score in the range 0–1 between two trees and a score for each match of paired tree splits.

Table 10. List of the 221 *Leuconostoc* strains corresponding to the available genome assemblies on NCBI database in September 2021. The assignment to groups according to the ANI tree is reported.

Group	Genus	Species	Subspecies	Strain	Type strain	Size (Mb)	GC%	Assembly	Isolation source	Completeness (%)	Contamination (%)	16S Genbank accession	16S rRNA sequence identity (%)
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		AtHG050		2.03	37.7	GCA_004368765.1	Vegetable/Fruit/Corn/Plant	100.00	1.05		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		CECT_9268		2.08	37.7	GCA_900290155.1	Vegetable/Fruit/Corn/Plant	99.83	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		CECT_9266		2.11	37.7	GCA_900312985.1	Vegetable/Fruit/Corn/Plant	99.83	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	KMB_608		1.96	37.7	GCA_003346165.1	Dairy	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	LN32		1.85	37.8	GCA_002092705.1	Dairy	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		GL1		1.82	38.1	GCA_001541125.1	Dairy	100.00	1.31		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	KMB_611		2.1	37.5	GCA_003346345.1	Dairy	100.00	1.05		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>dextranicum</i>	NBRC_100495		1.72	37.9	GCA_007990145.1	Collection strain	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		NCTC12954		2.08	37.7	GCA_900461615.1	Collection strain	100.00	1.05		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		LK-151		2.14	37.7	GCA_002370415.1	Vegetable/Fruit/Corn/Plant	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	DRC0211		2.12	37.6	GCA_002009375.1	Vegetable/Fruit/Corn/Plant	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		OG02		1.72	37.9	GCA_002276945.1	Dairy	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	LN27		2.22	37.5	GCA_002092745.1	Dairy	100.00	1.57		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		SRCM102733		2.06	37.7	GCA_009913915.1	Vegetable/Fruit/Corn/Plant	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		WC0331		2.02	37.6	GCA_008690745.1	Meat	100.00	1.05		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	FM06		1.95	38	GCA_002148235.1	Dairy	100.00	0.52		
G01	<i>Leuconostoc</i>	sp.		DB-1		2.07	37.7	GCA_013414845.1	Vegetable/Fruit/Corn/Plant	98.95	1.05		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		M11		1.98	37.8	GCA_009497095.1	Fish	100.00	1.57		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	LbE16		2.04	37.5	GCA_001184255.1	Dairy	100.00	1.05		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	BD1710		2.12	37.6	GCA_002117185.1	Dairy	100.00	1.05		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	ATCC_8293	T	2.08	37.7	GCA_000014445.1	Vegetable/Fruit/Corn/Plant	100.00	1.05	AB023242	100
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	LN34		1.88	37.7	GCA_002092775.1	Dairy	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		WC0330		1.77	37.9	GCA_008690805.1	Meat	100.00	0.79		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	J18		2.02	37.7	GCA_000234825.3	Vegetable/Fruit/Corn/Plant	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LbT16		1.91	37.8	GCA_001184265.1	Dairy	99.48	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	KMB_609		2.01	37.7	GCA_003346155.1	Dairy	100.00	1.05		

Group	Genus	Species	Subspecies	Strain	Type strain	Size (Mb)	GC%	Assembly	Isolation source	Completeness (%)	Contamination (%)	16S Genbank accession	16S rRNA sequence identity (%)
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	LN25		2.28	37.5	GCA_002092735.1	Dairy	100.00	1.57		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		DPC_7261		3.35	40.3	GCA_014854895.1	Vegetable/Fruit/Corn/Plant	100.00	100.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	LN08		1.97	37.8	GCA_002092625.1	Dairy	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		YL48		2.06	37.6	GCA_002886025.1	Vegetable/Fruit/Corn/Plant	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		NCTC10817		1.97	37.8	GCA_900452955.1	Collection strain	100.00	1.05		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>sake</i>	NBRC_102481		1.7	37.8	GCA_007989505.1	Vegetable/Fruit/Corn/Plant	98.43	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	NBRC_100496		2.04	37.5	GCA_007990165.1	Vegetable/Fruit/Corn/Plant	100.00	1.05		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		FDAARGOS_1033		2.08	37.7	GCA_016127295.1	Vegetable/Fruit/Corn/Plant	100.00	1.05		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		CECT_9217		2.02	37.7	GCA_900290445.1	Vegetable/Fruit/Corn/Plant	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		213M0		2.03	37.7	GCA_001570665.1	Dairy	99.93	1.05		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	NBRC_3832		1.91	37.6	GCA_006538765.1	Collection strain	100.00	1.05		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		WC0332		1.86	37.7	GCA_008689715.1	Meat	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		Wikim17		1.86	37.8	GCA_000787735.1	Vegetable/Fruit/Corn/Plant	100.00	0.79		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		B5		2.13	37.5	GCA_004745755.1	Food	100.00	0.70		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		FAM_18356		1.87	37.8	GCA_005864365.1	-	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>dextranicum</i>	DSM_20484	T	1.85	38	GCA_001047695.1	Dairy	100.00	1.31	NR_113911	100
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>dextranicum</i>	LbE15		2.01	37.6	GCA_001184245.1	Dairy	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		P45		1.87	37.5	GCA_000756355.1	Vegetable/Fruit/Corn/Plant	100.00	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	LN05		2	38	GCA_002092565.1	Dairy	98.54	3.45		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF01		1.64	38	GCA_002092045.1	Dairy	99.83	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF02		1.72	37.9	GCA_002092135.1	Dairy	100.00	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	T26		1.83	38.4	GCA_000686485.1	Dairy	100.00	10.65		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LN07		1.6	38.1	GCA_002092615.1	Dairy	100.00	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF04		1.64	38	GCA_002092155.1	Dairy	100.00	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF05		1.64	38	GCA_002092085.1	Dairy	100.00	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	TIFN8		1.71	38.2	GCA_000447945.1	Dairy	99.21	0.26		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF03		1.67	38	GCA_002092145.1	Dairy	100.00	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF17		1.63	38	GCA_002092425.1	Dairy	100.00	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF19		1.65	38	GCA_002092415.1	Dairy	100.00	0.00		

Group	Genus	Species	Subspecies	Strain	Type strain	Size (Mb)	GC%	Assembly	Isolation source	Completeness (%)	Contamination (%)	16S Genbank accession	16S rRNA sequence identity (%)
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF14		1.63	38	GCA_002092335.1	Dairy	100.00	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF20		1.63	38	GCA_002092455.1	Dairy	99.74	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	NBRC_107766		1.63	37.9	GCA_007992255.1	Collection strain	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF16		1.61	38	GCA_002092515.1	Dairy	100.00	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF11		1.67	38.2	GCA_002092315.1	Dairy	99.87	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF10		1.62	38	GCA_002092245.1	Dairy	100.00	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF12		1.66	38.1	GCA_002092385.1	Dairy	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF13		1.64	38.3	GCA_002092465.1	Dairy	100.00	1.05		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		MGYG-HGUT-01341		1.74	38.6	GCA_902374135.1	Human sample	100.00	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	ATCC_19254	T	1.74	38.5	GCA_000160595.1	Dairy	100.00	0.00	NR_040818	100
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF18		1.62	38	GCA_002092495.1	Dairy	100.00	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF07		1.64	38.1	GCA_002092215.1	Dairy	100.00	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>cremoris</i>	LMGCF09		1.63	38.1	GCA_002092105.1	Dairy	100.00	0.00		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		TR154		1.86	37.8	GCA_006406185.1	Vegetable/Fruit/Corn/Plant	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		CBA7131		2.09	37.6	GCA_003255835.1	Human sample	99.48	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	BD3749		1.99	37.8	GCA_001583825.1	Vegetable/Fruit/Corn/Plant	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		SRCM103453		2.18	37.6	GCA_004103675.1	Food	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		SRCM102735		2.06	37.6	GCA_009913935.1	Vegetable/Fruit/Corn/Plant	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		SRCM103356		2.04	37.6	GCA_004102585.1	Food	100.00	0.70		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>jonggajibkimchii</i>	DRC1506	T	1.98	37.6	GCA_001886915.1	Vegetable/Fruit/Corn/Plant	99.90	1.05	NR_157602	100
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	KIBGE-IB22		1.95	37.5	GCA_005049065.1	Vegetable/Fruit/Corn/Plant	100.00	0.70		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		KFRI-MG		1.9	37.7	GCA_000512955.1	Vegetable/Fruit/Corn/Plant	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		WiKim33		1.97	37.6	GCA_003433375.1	Vegetable/Fruit/Corn/Plant	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		SG0315		2.1	37.5	GCA_014060975.1	Vegetable/Fruit/Corn/Plant	100.00	1.05		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		SRCM103460		2.09	37.6	GCA_004194375.1	Food	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		WC0333		2.09	37.5	GCA_008689685.1	Meat	100.00	0.52		
G01	<i>Leuconostoc</i>	<i>mesenteroides</i>		406		2	37.7	GCA_001515385.1	Dairy	100.00	1.57		
G02	<i>Leuconostoc</i>	<i>suionicum</i>		CECT_8484		2.07	37.3	GCA_900289205.1	Vegetable/Fruit/Corn/Plant	100.00	0.52		
G02	<i>Leuconostoc</i>	<i>suionicum</i>		CECT_9216		2.07	37.3	GCA_900290145.1	Vegetable/Fruit/Corn/Plant	100.00	0.52		

Group	Genus	Species	Subspecies	Strain	Type strain	Size (Mb)	GC%	Assembly	Isolation source	Completeness (%)	Contamination (%)	16S Genbank accession	16S rRNA sequence identity (%)
G02	<i>Leuconostoc</i>	<i>suionicum</i>		CECT_8486		2.07	37.3	GCA_900289255.1	Vegetable/Fruit/Corn/Plant	100.00	0.52		
G02	<i>Leuconostoc</i>	<i>suionicum</i>		DmW_98		2.06	37.3	GCA_014927295.1	Insect	100.00	0.00		
G02	<i>Leuconostoc</i>	<i>suionicum</i>		LT-38		2.02	37.6	GCA_002370395.1	Collection strain	100.00	0.00		
G02	<i>Leuconostoc</i>	<i>suionicum</i>		DSM_20241	T	2.05	37.6	GCA_001891125.1	-	100.00	0.00	NR_109003	100
G03	<i>Leuconostoc</i>	<i>mesenteroides</i>	<i>mesenteroides</i>	CBA3607		1.85	37	GCA_009676745.1	Vegetable/Fruit/Corn/Plant	100.00	0.00		
G03	<i>Leuconostoc</i>	<i>mesenteroides</i>		CBA3628		1.86	37	GCA_007954745.1	Vegetable/Fruit/Corn/Plant	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>falkenbergense</i>		LMG_18969		1.94	39	GCA_014634865.1	Vegetable/Fruit/Corn/Plant	99.83	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMGTW8		1.98	39	GCA_002092295.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		1159		2.04	39	GCA_000686465.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		PS12		1.93	39.1	GCA_000686505.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMGTW1		1.97	39	GCA_002092235.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	sp.		BM2		2.05	39	GCA_002095565.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		FDAARGOS_1004		2.1	39.1	GCA_016127035.1	-	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMGH97		1.99	39	GCA_002072585.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		BM2		2.02	39	GCA_002092535.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMGH280		1.98	39.1	GCA_002072555.1	Dairy	100.00	0.70		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMGH95		1.99	39	GCA_002072565.1	Dairy	100.00	0.17		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMGH83		2.06	39.1	GCA_002072475.1	Dairy	98.41	1.40		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMGH284		2.07	39	GCA_002072575.1	Dairy	99.21	2.01		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMGCF15		1.92	39.1	GCA_002092375.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMGH278		1.94	39.1	GCA_002072495.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>falkenbergense</i>		C		2.29	38.6	GCA_014634785.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		KMB_610		1.98	39	GCA_003346375.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		4882		2.01	39.1	GCA_000297375.1	Dairy	93.19	0.17		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMGTW3		1.9	39.1	GCA_002092355.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMGH61		2.01	39.1	GCA_002072515.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LN23		1.99	39.1	GCA_002092645.1	Dairy	100.00	0.17		
G04	<i>Leuconostoc</i>	<i>falkenbergense</i>		LMG_10779	T	1.96	39.1	GCA_014634805.1	Collection strain	100.00	0.17	HM443956	100
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMGTW6		1.88	39.1	GCA_002092255.1	Dairy	100.00	0.00		

Group	Genus	Species	Subspecies	Strain	Type strain	Size (Mb)	GC%	Assembly	Isolation source	Completeness (%)	Contamination (%)	16S Genbank accession	16S rRNA sequence identity (%)
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		AMBR10		2.3	39.2	GCA_901830415.1	Human sample	100.00	0.70		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMGH100		2.06	39.1	GCA_002072505.1	Dairy	98.72	2.58		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		HPK01		1.95	39.1	GCA_002092075.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMGCF06		1.95	39.1	GCA_002092035.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LN02		1.91	39.1	GCA_002092555.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LN12		1.86	39.1	GCA_002092635.1	Dairy	100.00	0.00		
G04	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMGCF08		2.19	39.1	GCA_002092165.1	Dairy	100.00	11.08		
G05	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMG_11483		2.02	39	GCA_014634765.1	-	99.30	0.00		
G05	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		LMG_11482		2.1	38.9	GCA_014634745.1	Vegetable/Fruit/Corn/Plant	99.83	0.00		
G05	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		NCDO_768		2.13	38.9	GCA_012396745.1	Vegetable/Fruit/Corn/Plant	99.83	0.00		
G05	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		FDAARGOS_1003	T	2.11	39	GCA_016127255.1	Vegetable/Fruit/Corn/Plant	99.83	0.00	NR_040814	99.6
G05	<i>Leuconostoc</i>	sp.		S51		2.25	38.8	GCA_016517085.1	Insect	99.83	0.00		
G05	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		17_2		2.27	38.5	GCA_014634725.1	Vegetable/Fruit/Corn/Plant	99.83	0.00		
G05	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		CBA3630		2.32	39	GCA_008033175.1	Vegetable/Fruit/Corn/Plant	99.83	0.17		
G05	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		TR070		2.23	38.8	GCA_006382035.1	Vegetable/Fruit/Corn/Plant	99.83	0.00		
G05	<i>Leuconostoc</i>	<i>pseudomesenteroides</i>		KCTC_3652		3.24	38.3	GCA_000185065.2	Vegetable/Fruit/Corn/Plant	99.14	46.38		
G06	<i>Leuconostoc</i>	<i>lactis</i>		KCTC_3528		2.01	42.6	GCA_000185085.2	-	89.56	17.78		
G06	<i>Leuconostoc</i>	<i>lactis</i>		AV1n		1.74	43.2	GCA_009795665.1	Vegetable/Fruit/Corn/Plant	85.71	0.00		
G06	<i>Leuconostoc</i>	<i>lactis</i>		CBA3626		1.84	43.1	GCA_007954665.1	Vegetable/Fruit/Corn/Plant	99.83	0.52		
G06	<i>Leuconostoc</i>	<i>lactis</i>		WiKim40		1.79	43.1	GCA_001698145.1	Vegetable/Fruit/Corn/Plant	99.83	0.00		
G06	<i>Leuconostoc</i>	<i>lactis</i>		SBC001		1.84	43.1	GCA_014050705.1	Vegetable/Fruit/Corn/Plant	99.83	0.00		
G06	<i>Leuconostoc</i>	<i>lactis</i>		KACC_91922		1.69	43.4	GCA_000709265.1	Vegetable/Fruit/Corn/Plant	99.83	0.00		
G06	<i>Leuconostoc</i>	<i>lactis</i>		JCM_6123	T	1.65	43.3	GCA_014651235.1	Collection strain	99.83	0.00	NR_040823	100
G06	<i>Leuconostoc</i>	<i>lactis</i>		aa_143		1.74	43.2	GCA_004167235.1	Human sample	99.83	0.00		
G06	<i>Leuconostoc</i>	<i>lactis</i>		NBRC_12455		1.64	43.5	GCA_006539105.1	Collection strain	99.83	0.00		
G06	<i>Leuconostoc</i>	<i>lactis</i>		CCK940		1.74	43.3	GCA_002287365.1	Vegetable/Fruit/Corn/Plant	99.83	0.00		
G06	<i>Leuconostoc</i>	<i>citreum</i>		1300_LCIT		1.81	43.1	GCA_001062635.1	Human sample	99.83	0.00		
G06	<i>Leuconostoc</i>	<i>lactis</i>		1001262B_160229_C9		1.66	43.6	GCA_015551285.1	Human sample	99.83	0.00		
G06	<i>Leuconostoc</i>	<i>garlicum</i>		KFRI01		1.72	43.3	GCA_001998805.1	Vegetable/Fruit/Corn/Plant	99.83	0.00		

Group	Genus	Species	Subspecies	Strain	Type strain	Size (Mb)	GC%	Assembly	Isolation source	Completeness (%)	Contamination (%)	16S Genbank accession	16S rRNA sequence identity (%)
G06	<i>Leuconostoc</i>	<i>lactis</i>		BIOML-A1		1.75	43.1	GCA_009678855.1	Human sample	99.83	0.00		
G06	<i>Leuconostoc</i>	<i>lactis</i>		CBA3625		1.79	43.3	GCA_007954605.1	Vegetable/Fruit/Corn/Plant	99.83	0.00		
G07	<i>Leuconostoc</i>	<i>lactis (argentinum)</i>		KCTC_3773		1.72	42.9	GCA_000179875.1	Dairy	99.30	0.00	AF175403	99.86
G07	<i>Leuconostoc</i>	<i>lactis</i>		1001095IJ_161003_G05		1.68	43.3	GCA_015553465.1	Human sample	99.83	0.00		
G07	<i>Leuconostoc</i>	<i>lactis</i>		LN24		1.72	42.9	GCA_002092695.1	Dairy	99.30	0.00		
G07	<i>Leuconostoc</i>	<i>lactis</i>		LN19		1.72	42.9	GCA_002092595.1	Dairy	99.30	0.00		
G07	<i>Leuconostoc</i>	<i>lactis</i>		CBA3622		1.79	42.9	GCA_007954625.1	Vegetable/Fruit/Corn/Plant	99.83	0.00		
G07	<i>Leuconostoc</i>	<i>lactis</i>		WIKIM21		1.76	43.1	GCA_001411775.1	Vegetable/Fruit/Corn/Plant	99.83	0.00		
G08	<i>Leuconostoc</i>	<i>holzapfelii</i>		CCUG_54536	T	1.76	44	GCA_012396485.1	Vegetable/Fruit/Corn/Plant	99.37	0.28	AM600682	99.9
G09	<i>Leuconostoc</i>	<i>kimchii</i>		IMSNU_11154	T	2.1	37.9	GCA_000092505.1	Vegetable/Fruit/Corn/Plant	100.00	0.00	NR_075014	100
G09	<i>Leuconostoc</i>	sp.		C2		1.88	37.9	GCA_000219785.1	Vegetable/Fruit/Corn/Plant	100.00	0.00		
G09	<i>Leuconostoc</i>	<i>kimchii</i>		NKJ218		1.99	37.8	GCA_004551615.1	Vegetable/Fruit/Corn/Plant	100.00	0.00		
G10	<i>Leuconostoc</i>	<i>rapi</i>		DSM 27776	T	1.92	38	GCF_016908715.1	-	100.00	1.24	NR_136799	100
G11	<i>Leuconostoc</i>	<i>citreum</i>		DmW_111		1.83	38.8	GCA_002115685.1	Insect	99.33	0.75		
G11	<i>Leuconostoc</i>	<i>citreum</i>		LBAE_C10		1.93	38.8	GCA_000239895.2	Vegetable/Fruit/Corn/Plant	99.89	0.08		
G11	<i>Leuconostoc</i>	<i>citreum</i>		CBA3627		1.9	38.9	GCA_008033195.1	Vegetable/Fruit/Corn/Plant	99.89	0.56		
G11	<i>Leuconostoc</i>	<i>citreum</i>		CBA3621		1.9	38.9	GCA_007954785.1	Vegetable/Fruit/Corn/Plant	99.89	0.56		
G11	<i>Leuconostoc</i>	<i>citreum</i>		G04		1.84	38.8	GCA_006381855.1	Vegetable/Fruit/Corn/Plant	99.89	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		37		1.98	38.9	GCA_015277835.1	Dairy	99.89	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		1301_LGAS		1.82	39	GCA_001077275.1	Human sample	99.89	0.19		
G11	<i>Leuconostoc</i>	<i>citreum</i>		NBRC_102476		1.79	38.9	GCA_007989545.1	Collection strain	99.89	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		DmW_137		1.87	38.9	GCA_014927285.1	Insect	99.89	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		F192-5		2.06	38.4	GCA_008326505.1	Vegetable/Fruit/Corn/Plant	99.89	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		CW28		1.98	38.7	GCA_002591805.1	Vegetable/Fruit/Corn/Plant	99.33	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		ATCC_49370	T	1.84	39	GCA_004354555.1	Collection strain	99.89	0.00	NR_041727	99.8
G11	<i>Leuconostoc</i>	<i>citreum</i>		NRIC_1776		1.81	38.9	GCA_008326545.1	Vegetable/Fruit/Corn/Plant	99.89	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		WiKim0096		1.81	39	GCA_016406245.1	Vegetable/Fruit/Corn/Plant	99.70	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		CBA3624		1.89	39	GCA_007954565.1	Vegetable/Fruit/Corn/Plant	99.89	0.56		
G11	<i>Leuconostoc</i>	<i>citreum</i>		EFEL_2700		1.92	39	GCA_002804045.1	Vegetable/Fruit/Corn/Plant	99.33	0.00		

Group	Genus	Species	Subspecies	Strain	Type strain	Size (Mb)	GC%	Assembly	Isolation source	Completeness (%)	Contamination (%)	16S Genbank accession	16S rRNA sequence identity (%)
G11	<i>Leuconostoc</i>	<i>citreum</i>		TR116		1.83	38.8	GCA_004359915.1	Vegetable/Fruit/Corn/Plant	99.89	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		TMW21194		1.74	38.9	GCA_009792915.1	Food	99.89	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		WiKim0101		2.02	38.9	GCA_009707745.1	Vegetable/Fruit/Corn/Plant	99.16	0.19		
G11	<i>Leuconostoc</i>	<i>sp.</i>		LN180020		1.91	39	GCA_014844115.1	Vegetable/Fruit/Corn/Plant	99.89	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		CBA3623		1.97	38.9	GCA_007954705.1	Vegetable/Fruit/Corn/Plant	99.33	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		NRRL_B-1299		1.75	39	GCA_000820985.2	-	99.89	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		TR153		1.9	38.7	GCA_006406175.1	Vegetable/Fruit/Corn/Plant	99.89	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		SG0255		1.87	38.8	GCA_014060965.1	Vegetable/Fruit/Corn/Plant	99.89	0.07		
G11	<i>Leuconostoc</i>	<i>citreum</i>		LBAE_C11		1.97	38.7	GCA_000239915.2	Vegetable/Fruit/Corn/Plant	99.89	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		LBAE_E16		1.8	38.9	GCA_000239935.2	Vegetable/Fruit/Corn/Plant	99.89	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		BD1707		1.76	38.9	GCA_013432155.1	Dairy	99.89	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		NRRL_B-742		1.72	39.2	GCA_000820965.2	-	99.25	0.00		
G11	<i>Leuconostoc</i>	<i>citreum</i>		KM20		1.9	38.9	GCA_000026405.1	Vegetable/Fruit/Corn/Plant	99.89	0.00		
G12	<i>Leuconostoc</i>	<i>palmae</i>		JCM 16944	T	1.39	35.4	GCF_019656075.1	Palm wine	99.33	0.56	NR_042695	99.9
G13	<i>Leuconostoc</i>	<i>litchii</i>		MB17	T	1.87	35.8	GCA_008107645.1	Vegetable/Fruit/Corn/Plant	100.00	0.52	LC259518	99.5
G14	<i>Leuconostoc</i>	<i>carnosum</i>		WC0322		1.85	37	GCA_008690865.1	Meat	99.48	1.05		
G14	<i>Leuconostoc</i>	<i>carnosum</i>		WC0323		1.77	37.2	GCA_008690845.1	Meat	99.48	1.05		
G14	<i>Leuconostoc</i>	<i>carnosum</i>		WC0324		1.77	37.1	GCA_008690825.1	Meat	99.48	1.05		
G14	<i>Leuconostoc</i>	<i>carnosum</i>		WC0328		1.81	37.1	GCA_008689695.1	Meat	98.95	1.05		
G14	<i>Leuconostoc</i>	<i>carnosum</i>		WC0319		1.7	37.1	GCA_008690925.1	Meat	99.48	1.05		
G14	<i>Leuconostoc</i>	<i>carnosum</i>		JB16_JB16		1.77	37.1	GCA_000300135.1	Vegetable/Fruit/Corn/Plant	99.48	1.05		
G14	<i>Leuconostoc</i>	<i>carnosum</i>		DSM_5576	T	1.82	37	GCA_014207505.1	Meat	99.48	1.05	NR_040811	99.9
G14	<i>Leuconostoc</i>	<i>carnosum</i>		WC0318		1.75	37.2	GCA_008690985.1	Meat	99.48	1.05		
G14	<i>Leuconostoc</i>	<i>carnosum</i>		MFPC16A2803		1.81	37	GCA_900322415.1	Meat	99.48	1.05		
G14	<i>Leuconostoc</i>	<i>carnosum</i>		WC0325		1.83	37.2	GCA_008690785.1	Meat	99.48	1.05		
G14	<i>Leuconostoc</i>	<i>carnosum</i>		WC0327		1.77	37.2	GCA_008689755.1	Meat	99.48	1.05		
G14	<i>Leuconostoc</i>	<i>carnosum</i>		WC0326		1.77	37.2	GCA_008689765.1	Meat	99.48	1.05		
G14	<i>Leuconostoc</i>	<i>carnosum</i>		WC0321		1.8	37.1	GCA_008690875.1	Meat	99.48	1.05		
G14	<i>Leuconostoc</i>	<i>carnosum</i>		MFPA29A1405		1.67	37.3	GCA_900322425.1	Meat	99.48	1.05		

Group	Genus	Species	Subspecies	Strain	Type strain	Size (Mb)	GC%	Assembly	Isolation source	Completeness (%)	Contamination (%)	16S Genbank accession	16S rRNA sequence identity (%)
G14	<i>Leuconostoc</i>	<i>carnosum</i>		CBA3620		1.7	37.3	GCA_007954525.1	Vegetable/Fruit/Corn/Plant	99.48	1.05		
G14	<i>Leuconostoc</i>	<i>carnosum</i>		WC0320		1.81	37.1	GCA_008690965.1	Meat	99.48	1.05		
G14	<i>Leuconostoc</i>	<i>carnosum</i>		WC0329		1.65	37.2	GCA_008689675.1	Meat	99.48	1.05		
G15	<i>Leuconostoc</i>	<i>miyukkimchii</i>		JCM 17445	T	1.91	36.3	GCF_019656055.1	Brown algae kimchi	99.44	0.97	NR_109072	99.9
G16	<i>Leuconostoc</i>	<i>inhae</i>		KSL4-2		2.09	36.6	GCA_900016165.1	Food	99.33	0.11		
G16	<i>Leuconostoc</i>	<i>inhae</i>		C120c		1.99	36.5	GCA_900009505.1	Vegetable/Fruit/Corn/Plant	99.44	0.00		
G16	<i>Leuconostoc</i>	<i>gelidum</i>	<i>gasicomitatum</i>	CBA3613		1.81	36.9	GCA_013403485.1	-	100.00	0.11		
G16	<i>Leuconostoc</i>	<i>gelidum</i>	<i>gasicomitatum</i>	LMG_18811	T	1.95	36.7	GCA_000196855.1	Meat	99.44	0.00	NR_074997	100
G16	<i>Leuconostoc</i>	<i>gelidum</i>	<i>gasicomitatum</i>	MFPA44A1401		1.89	36.8	GCA_900218135.1	Meat	100.00	0.00		
G16	<i>Leuconostoc</i>	<i>inhae</i>		KCTC_3774	T	2.3	36.4	GCA_000166735.2	Vegetable/Fruit/Corn/Plant	95.81	6.07	NR_025204	99.8
G16	<i>Leuconostoc</i>	<i>gelidum</i>	<i>gasicomitatum</i>	TMW_2.1619		1.92	36.9	GCA_009296125.1	Meat	99.64	0.56		
G16	<i>Leuconostoc</i>	<i>gelidum</i>	<i>gasicomitatum</i>	PB1e		2.09	36.5	GCA_900016175.1	Vegetable/Fruit/Corn/Plant	99.44	0.00		
G16	<i>Leuconostoc</i>	<i>gelidum</i>	<i>gasicomitatum</i>	LEKG1		2.05	36.9	GCA_001536305.1	Vegetable/Fruit/Corn/Plant	100.00	0.11		
G16	<i>Leuconostoc</i>	<i>inhae</i>		PB1a		2.08	36.6	GCA_900016185.1	Vegetable/Fruit/Corn/Plant	99.44	0.56		
G16	<i>Leuconostoc</i>	<i>inhae</i>		PL111		2.06	36.5	GCA_900016205.1	Vegetable/Fruit/Corn/Plant	99.44	0.00		
G16	<i>Leuconostoc</i>	<i>gelidum</i>	<i>gasicomitatum</i>	C122c		2.01	36.6	GCA_900016145.1	Vegetable/Fruit/Corn/Plant	98.88	0.19		
G17	<i>Leuconostoc</i>	<i>gelidum</i>	<i>gelidum</i>	TMW_2.1618		1.82	36.9	GCA_006770305.1	Meat	99.44	0.19		
G17	<i>Leuconostoc</i>	<i>gelidum</i>	<i>gelidum</i>	KCTC_3527	T	1.96	36.6	GCA_000166715.2	Vegetable/Fruit/Corn/Plant	99.44	0.19	AF175402	99.8
G17	<i>Leuconostoc</i>	<i>gelidum</i>		JB7		1.89	36.7	GCA_000298875.1	Vegetable/Fruit/Corn/Plant	99.44	0.19		
G18	<i>Leuconostoc</i>	<i>fallax</i>		ATCC_700006	T	1.66	37.7	GCA_004354645.1	Vegetable/Fruit/Corn/Plant	99.30	0.52	AF360738	100
G18	<i>Leuconostoc</i>	<i>fallax</i>		KCTC_3537		1.64	37.5	GCA_000165675.2	Vegetable/Fruit/Corn/Plant	95.67	0.52		

5.4. RESULTS

5.4.1. Species reclassification in the genus *Leuconostoc*

All the genome sequences available for the species belonging to the genus *Leuconostoc*, including some type strains, were included in the analysis. Based on CheckM analysis, most of the deposited sequences presented low or negligible contamination ($\leq 5\%$), except for six genomes. Five of them were discarded, while the sequence of *L. inahae* KCTC 3774 (contamination = 6.1%) was retained, being a type strain. As a whole, 216 genomes were analyzed. They were ascribed to 16 correctly named species (17 *L. carnosum*, 29 *L. citreum*, 3 *L. falckenbergense*, 2 *L. fallax*, 10 *L. gelidum*, 1 *L. holzapfelii*, 5 *L. inhae*, 2 *L. kimchii*, 19 *L. lactis*, 1 *L. litchii*, 1 *L. miyukkimchii*, 83 *L. mesenteroides*, 1 *L. palmae*, 34 *L. pseudomesenteroides*, 1 *L. rapi*, and 6 *L. suionicum*), to the not-recognized species “*L. garlicum*” (1 strain), and broadly assigned to the genus *Leuconostoc* (5 strains) (Table 10).

Pairwise ANI values were calculated for the whole data set. Considering 95% of ANI as the less stringent cut-off for species designation as suggested by Richter and Rosselló-Móra (2009), the 216 strains ascribed to 16 species were redistributed in 18 groups, each encompassing strains with pairwise ANI $> 96\%$ (Figure 5). Major revisions to the current classification were suggested by ANI analysis and are herein presented.

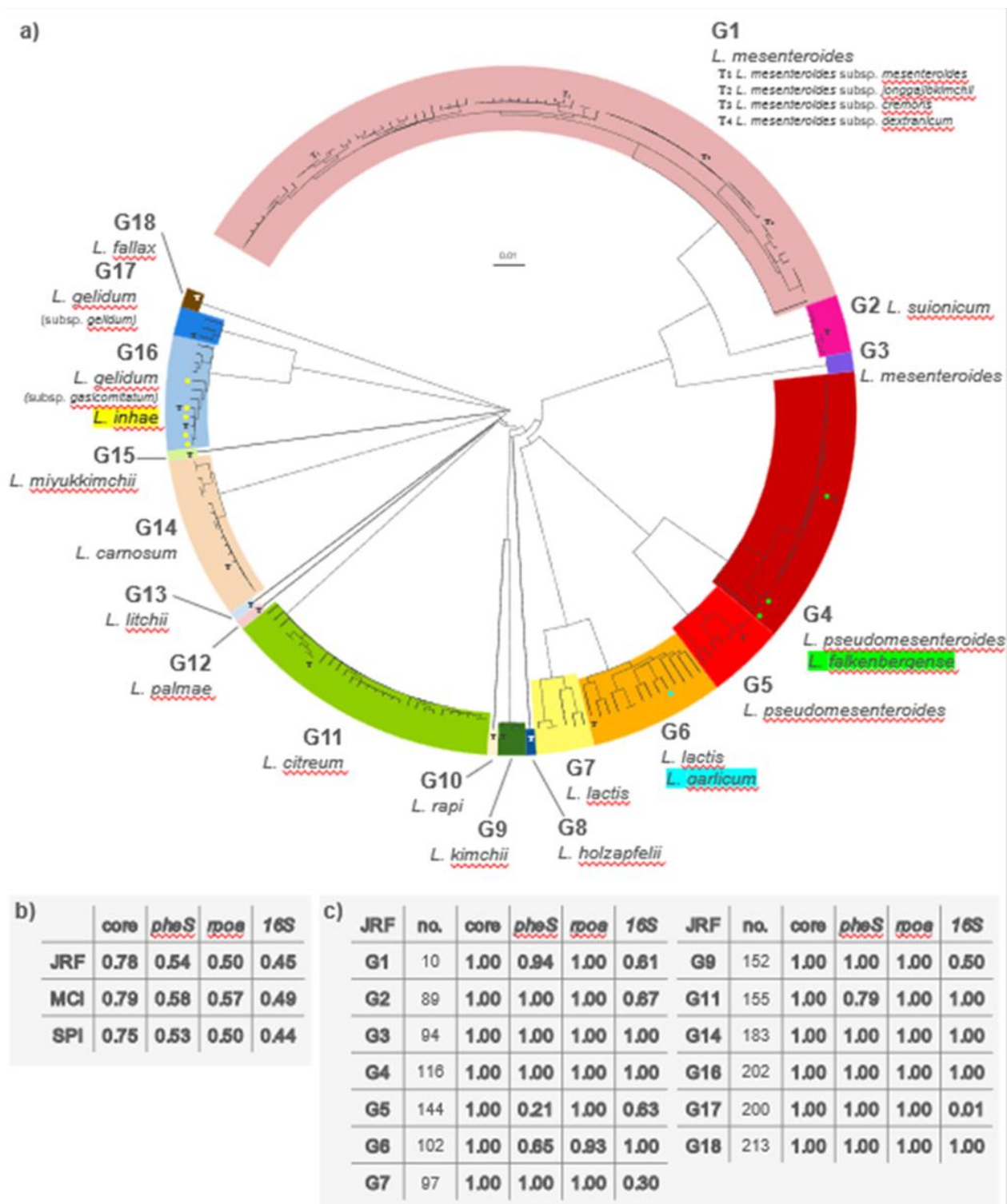


Figure 5. Phylogenomic and Phylogenetic analysis of *Leuconostoc* genus. a) UPGMA unrooted tree based on ANI distances matrix, reporting the 18 proposed clades with values > 96% between encompassed strains; T, type strain. b) Comparison of the ANI tree with those constructed from the alignment of 16S rRNA gene, *pheS*, *rpoA*, or the core genome. Normalized similarity score obtained with the generalized' Robinson-Foulds metrics Jaccard–Robinson–Foulds (JRF), Mutual Clustering Information (MCI), and Shared Phylogenetic Information (SPI) are reported. c) JRF

scores of the 15 groups identified in the ANI tree compared with those constructed from the alignment of 16S rRNA gene, pheS, rpoA, or the core genome.

Most of the *L. mesenteroides* (80) clustered in G1 resulting in the largest species within the genus. All the strains belonging to the subspecies *L. mesenteroides subsp. cremoris* (including the type-strain ATCC 19254), *L. mesenteroides subsp. dextranicum* (including DSM 20484T), *L. mesenteroides subsp. mesenteroides* (including ATCC 8293T), *L. mesenteroides subsp. joggajibkimchii* (including DRC1506T), and *L. mesenteroides subsp. sake* (including NBRC 102481T) were encompassed in G1. Albeit the presence of the subspecies, the strains were highly similar, with a mean ANI within the group of 99.1%.

Another strain of *L. mesenteroides* and a *L. mesenteroides subsp. mesenteroides* clustered in a diverse clade, G3, closely related to G1 and G2, the latter grouping the *L. suionicum* strains.

The strains attributed to *L. pseidomesenteroides* were redistributed in the closely related groups G4 and G5. The mean value of pairwise ANI between strains from the two distinct groups was 91.8%, suggesting the presence of two distinct species. G4 held 26 *L. paramesenteroides* with an ANI mean value of 99.4%, among them 3 strains currently ascribed to *L. falkenbergense*, including the type strain *L. falkenbergense* LMG 10779T, and an additional *Leuconostoc* sp. strain were comprised. The type strain *L. pseudomesenteroides* NCDO 768T, 7 other *L. paramesenteroides*, and a *Leuconostoc* sp. were gathered together in G5, with a mean ANI value of 98.3%.

The strains belonging to the species *L. lactis* were split into 2 phylogenetically correlated groups, G6 and G7. G6 encompassed the type strains of *L. lactis* (strain JCM 6123T), the putative “*L. garlicum*” KFRI01 (species currently not recognized), *L. citreum* 1300_LCIT, and other 12 *L. lactis*. G7 included 6 *L. lactis* strains, among which *L. lactis* KCTC 3773, the type strain of the species *L. argentinum*, a taxon not recognized so far. Pairwise ANI between G6 and G7 was always < 94.5%, while it was always > 96.9% within the 2 groups.

The strains of the two subspecies of *L. gelidum* (*L. gelidum subsp. gasicomitatum* and *L. gelidum subsp. gelidum*) clustered in two separated but closely related groups, corresponding to G16 and G17, the former also including *L. inhae* (Figure 5). G16 comprised all the *L. gelidum subsp. gasicomitatum* (7) and all the *L. inhae* (5), while G17 comprised 2 *L. gelidum subspecies gelidum*, among which the type strain, and another *L. gelidum* strain.

5.4.2. Phylogenetic relationships

Based on 16S rRNA, *pheS*, and *rpoA* gene sequences alignment, the phylogenetic trees of the whole set of 216 *Leuconostoc* strains were constructed using the maximum likelihood method. To evaluate the diverse approaches to study the evolutionary relationships between closely related genomes, the ANI tree (Figure 5.1) was then compared to the three trees obtained analyzing the single conserved genes (Figure 5.2, 5.3, and 5.4) and with the tree obtained aligning the core genomes (Figure 5.5). Three metrics were used to measure the differences in topology between the trees.

Tree scale: 0.1

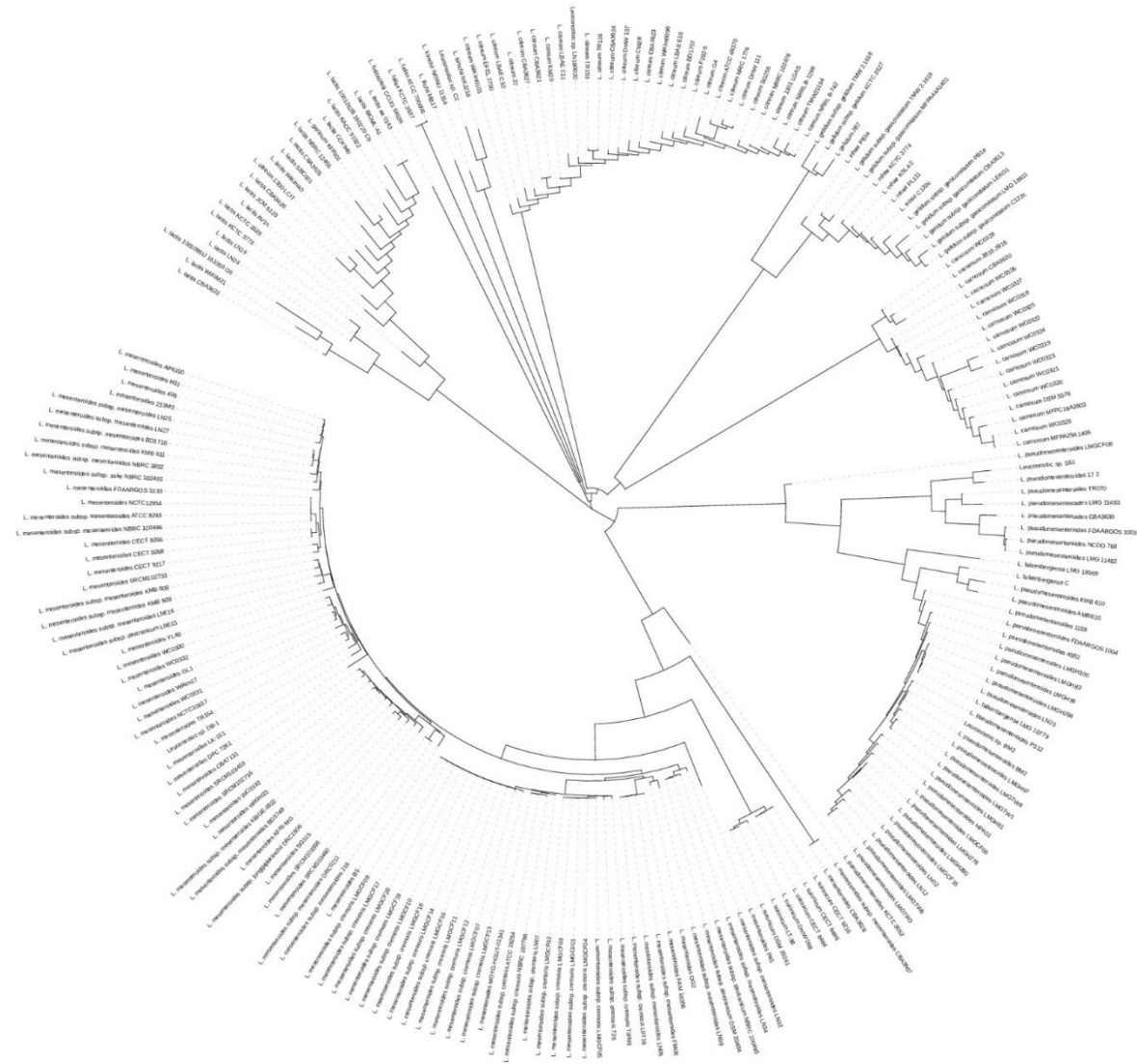


Figure 5.1 Neighbor-joining unrooted tree based on ANI distances matrix.

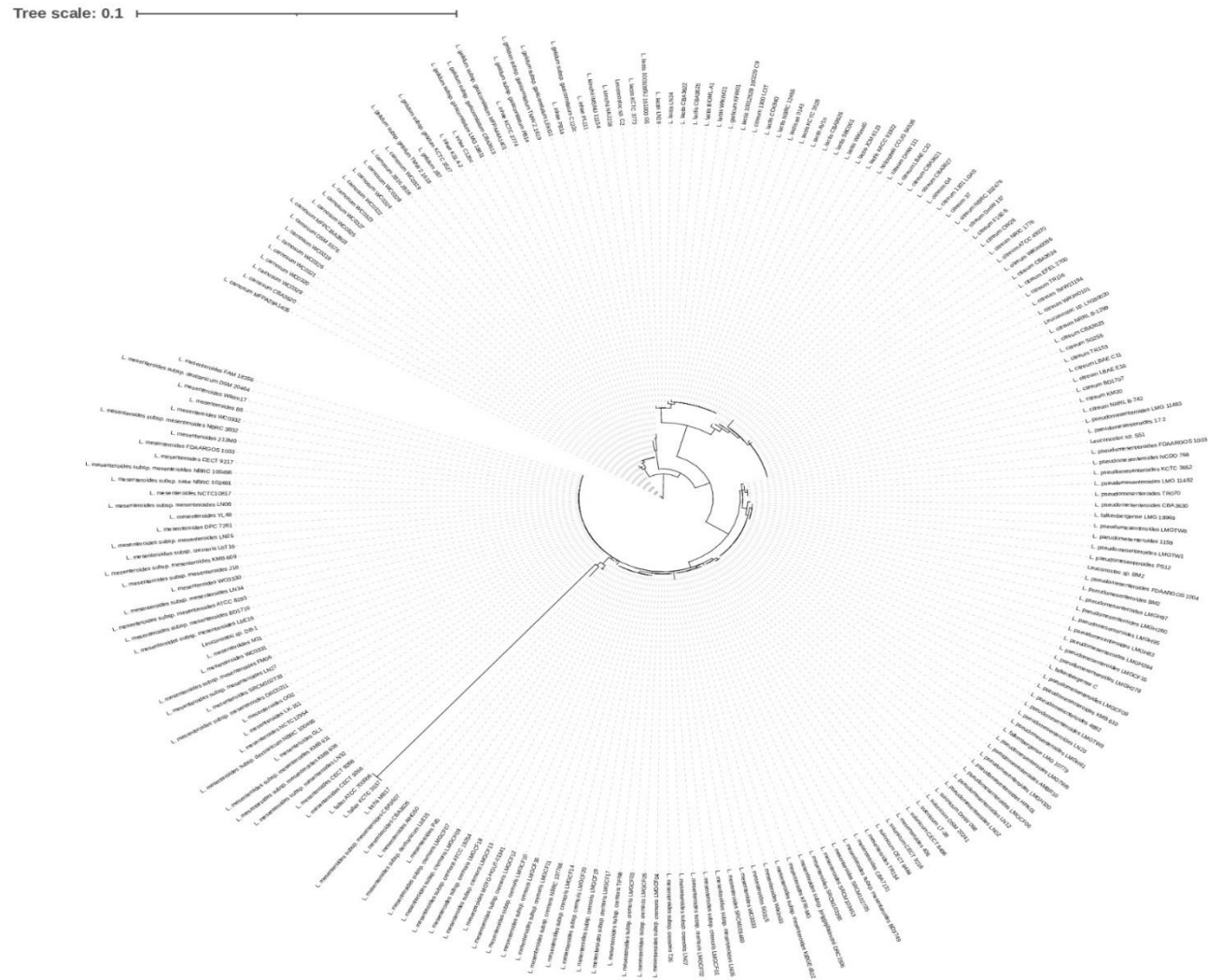


Figure 5.2 Phylogenetic trees constructed with the maximum likelihood method based on the alignments of 16S rRNA gene

ANI tree exhibited the most similar topology with the core genome tree, suggesting that single-gene trees are unable to capture the complete diversity among species belonging to the same genus and generally provide a weak phylogenetic insight that does not always reflect the true evolutionary history of a genus. Among the single genes, the *pheS* tree was the most similar to the ANI one, followed by trees based on the gene *rpoA* and the 16S rRNA gene.

According to JRF metrics, the splits that define the proposed groups in the ANI tree were consistent with the corresponding brackets in the core genome and *rpoA* trees, most of them presenting a JRF score of 1.00. Furthermore, for G3, G4, G14, G16, and G18, the ANI trees were entirely consistent also with those obtained by 16S rRNA and *pheS* genes (JRF = 1.00). On the other hand, the groups G1, G2, G5, G6, G7, G9, G11, and G17 did not perfectly match corresponding splits in *pheS* and particularly in 16S rRNA gene trees (JRF ranging between 0.01 – 0.66).

5.5. DISCUSSION

Emended organization of the genus *Leuconostoc*

ANI results indicated that some of the current *Leuconostoc* species were paraphyletic, suggesting that the taxonomy of this genus needed some re-organization. The proposed revisions of the current taxonomy are reported in Table 11 and the assignment of each analyzed strain is indicated in Table 12.

Table 11. List of the 18 *Leuconostoc* groups identified based on ANI > 96%. Current classification, the proposed name of the species, and the corresponding type strains are reported. “—” indicates an unmodified classification.

Group	Current taxonomy	Proposed taxonomy	Type strain
G1	<i>L. mesenteroides</i>	—	
G2	<i>L. suionicum</i>	—	
G3	<i>L. mesenteroides</i>	<i>L. unimore</i> sp. nov.	CBA3628
G4	<i>L. falkenbergense</i> and <i>L. pseudomesenteroides</i>	<i>L. falkenbergense</i>	LMG 10779
G5	<i>L. pseudomesenteroides</i>	—	
G6	<i>L. lactis</i> and “ <i>L. garlicum</i> ”	<i>L. lactis</i>	JCM 6123
G7	<i>L. lactis</i>	<i>L. argentinum</i>	KCTC 3773
G8	<i>L. holzapfelii</i>	—	
G9	<i>L. kimchi</i>	—	
G10	<i>L. rapi</i>	—	
G11	<i>L. citreum</i>	—	
G12	<i>L. palmae</i>	—	
G13	<i>L. litchi</i>	—	
G14	<i>L. carnosum</i>	—	
G15	<i>L. miyukkimchii</i>	—	
G16	<i>L. inhae</i> and <i>L. gelidum</i> subsp. <i>gasicomitatum</i>	<i>L. inhae</i>	KCTC 3774
G17	<i>L. gelidum</i> subsp. <i>Gelidum</i>	<i>L. gelidum</i>	KCTC 3527
G18	<i>L. fallax</i>	—	

Table 12. List of the reassigned strains

Assembly	Old name	Reclassification
GCA_002092295.1	<i>L. pseudomesenteroides</i> LMGTW8	<i>L. falkenbergense</i> LMGTW8
GCA_000686465.1	<i>L. pseudomesenteroides</i> 1159	<i>L. falkenbergense</i> 1159
GCA_000686505.1	<i>L. pseudomesenteroides</i> PS12	<i>L. falkenbergense</i> PS12
GCA_002092235.1	<i>L. pseudomesenteroides</i> LMGTW1	<i>L. falkenbergense</i> LMGTW1
GCA_002095565.1	<i>Leuconostoc</i> sp. BM2	<i>L. falkenbergense</i> BM2
GCA_016127035.1	<i>L. pseudomesenteroides</i> FDAARGOS_1004	<i>L. falkenbergense</i> FDAARGOS_1004
GCA_002072585.1	<i>L. pseudomesenteroides</i> LMGH97	<i>L. falkenbergense</i> LMGH97
GCA_002092535.1	<i>L. pseudomesenteroides</i> BM2	<i>L. falkenbergense</i> BM2
GCA_002072555.1	<i>L. pseudomesenteroides</i> LMGH280	<i>L. falkenbergense</i> LMGH280
GCA_002072565.1	<i>L. pseudomesenteroides</i> LMGH95	<i>L. falkenbergense</i> LMGH95
GCA_002072475.1	<i>L. pseudomesenteroides</i> LMGH83	<i>L. falkenbergense</i> LMGH83
GCA_002072575.1	<i>L. pseudomesenteroides</i> LMGH284	<i>L. falkenbergense</i> LMGH284
GCA_002092375.1	<i>L. pseudomesenteroides</i> LMGCF15	<i>L. falkenbergense</i> LMGCF15
GCA_002072495.1	<i>L. pseudomesenteroides</i> LMGH278	<i>L. falkenbergense</i> LMGH278
GCA_003346375.1	<i>L. pseudomesenteroides</i> KMB 610	<i>L. falkenbergense</i> KMB 610
GCA_000297375.1	<i>L. pseudomesenteroides</i> 4882	<i>L. falkenbergense</i> 4882
GCA_002092355.1	<i>L. pseudomesenteroides</i> LMGTW3	<i>L. falkenbergense</i> LMGTW3
GCA_002072515.1	<i>L. pseudomesenteroides</i> LMGH61	<i>L. falkenbergense</i> LMGH61
GCA_002092645.1	<i>L. pseudomesenteroides</i> LN23	<i>L. falkenbergense</i> LN23
GCA_002092255.1	<i>L. pseudomesenteroides</i> LMGTW6	<i>L. falkenbergense</i> LMGTW6
GCA_901830415.1	<i>L. pseudomesenteroides</i> AMBR10	<i>L. falkenbergense</i> AMBR10
GCA_002072505.1	<i>L. pseudomesenteroides</i> LMGH100	<i>L. falkenbergense</i> LMGH100
GCA_002092075.1	<i>L. pseudomesenteroides</i> HPK01	<i>L. falkenbergense</i> HPK01
GCA_002092035.1	<i>L. pseudomesenteroides</i> LMGCF06	<i>L. falkenbergense</i> LMGCF06
GCA_002092555.1	<i>L. pseudomesenteroides</i> LN02	<i>L. falkenbergense</i> LN02
GCA_002092635.1	<i>L. pseudomesenteroides</i> LN12	<i>L. falkenbergense</i> LN12
GCA_002092165.1	<i>L. pseudomesenteroides</i> LMGCF08	<i>L. falkenbergense</i> LMGCF08
GCA_000179875.1	<i>L. lactis</i> (<i>argentinum</i>) KCTC 3773	<i>L. argentinum</i> KCTC 3773
GCA_015553465.1	<i>L. lactis</i> 1001095IJ_161003_G05	<i>L. argentinum</i> 1001095IJ_161003_G05
GCA_002092695.1	<i>L. lactis</i> LN24	<i>L. argentinum</i> LN24
GCA_002092595.1	<i>L. lactis</i> LN19	<i>L. argentinum</i> LN19
GCA_007954625.1	<i>L. lactis</i> CBA3622	<i>L. argentinum</i> CBA3622
GCA_001411775.1	<i>L. lactis</i> WIKIM21	<i>L. argentinum</i> WIKIM21
GCA_900016165.1	<i>L. inhae</i> KSL4-2	<i>L. inhae</i> KSL4-2
GCA_900009505.1	<i>L. inhae</i> C120c	<i>L. inhae</i> C120c
GCA_013403485.1	<i>L. gelidum</i> subsp. <i>gasicomitatum</i> CBA3613	<i>L. inhae</i> CBA3613
GCA_000196855.1	<i>L. gelidum</i> subsp. <i>gasicomitatum</i> LMG_18811	<i>L. inhae</i> LMG_18811
GCA_900218135.1	<i>L. gelidum</i> subsp. <i>gasicomitatum</i> MFPA44A1401	<i>L. inhae</i> MFPA44A1401
GCA_000166735.2	<i>L. inhae</i> KCTC_3774	<i>L. inhae</i> KCTC_3774

GCA_009296125.1	<i>L. gelidum</i> subsp. <i>gasicomitatum</i> TMW_2.1619	<i>L. inhae</i> TMW_2.1619
GCA_900016175.1	<i>L. gelidum</i> subsp. <i>gasicomitatum</i> PB1e	<i>L. inhae</i> PB1e
GCA_001536305.1	<i>L. gelidum</i> subsp. <i>gasicomitatum</i> LEKG1	<i>L. inhae</i> LEKG1
GCA_900016185.1	<i>L. inhae</i> PB1a	<i>L. inhae</i> PB1a
GCA_900016205.1	<i>L. inhae</i> PL111	<i>L. inhae</i> PL111
GCA_900016145.1	<i>L. gelidum</i> subsp. <i>gasicomitatum</i> C122c	<i>L. inhae</i> C122c
GCA_006770305.1	<i>L. gelidum</i> subsp. <i>gelidum</i> TMW_2.1618	<i>L. gelidum</i> TMW_2.1618
GCA_000166715.2	<i>L. gelidum</i> subsp. <i>gelidum</i> KCTC_3527	<i>L. gelidum</i> KCTC_3527
GCA_000298875.1	<i>L. gelidum</i> JB7	<i>L. gelidum</i> JB7
GCA_001062635.1	<i>L. citreum</i> 1300_LCIT	<i>L. lactis</i> 1300_LCIT
GCA_001998805.1	“ <i>L. garlicum</i> ” KFRI01	<i>L. lactis</i> KFRI01

To resolve paraphyly of the strains currently attributed to *L. mesenteroides*, according to the results of ANI and phylogenomic trees, the strains currently ascribed to this species need to be split into two diverse taxon. According to the International Code of Nomenclature of Prokaryotes, the two strains belonging to G3 should form a new species that would require characterization and formal definition. With the designation of G3 as a separate species, G1 would maintain the present name *L. mesenteroides* since it encompasses most of the strains of *L. mesenteroides*, including the subspecies *L. mesenteroides* subsp. *cremoris*, *L. mesenteroides* subsp. *dextranicum*, *L. mesenteroides* subsp. *jonggajibkimkii*, and *L. mesenteroides* subsp. *mesenteroides*. Likewise, the reported data indicate that the strains currently attributed to the species *L. lactis* should be split into two separate species, corresponding to the groups G6 and G7. Group G6 corresponds to “true” *L. lactis*, since it encompasses the type strain of the species *L. lactis* JCM 6123T, strain KFRI01, improperly named “*L. garlicum*”, a name species that is not recognized, and *L. citreum* 1300_LCIT. On the other side, group G7 should be renamed *Leuconostoc argentinum*, as it harbors *L. lactis* KCTC 3773, which was previously identified as the type strain of *L. argentinum* (Dicks et al., 1993; Vancanneyt et al., 2006). Thus, we suggest transferring all the *L. lactis* within G7 into *L. argentinum*, with *L. lactis* KCTC 3773 renamed into *L. argentinum* KCTC 3773T as the corresponding type strain.

The two subspecies *L. gelidum* subsp. *gasicomitatum* and *L. gelidum* subsp. *gelidum* should be referred to as two diverse species, respectively corresponding to G16 and G17. Since *L. inhae* is included in G16, with the type strain *L. inhae* KCTC 3774T, we suggest naming this group *L. inhae* and referring the species G17 to as *L. gelidum* due to the presence of the type strain *L. gelidum* KCTC 3527T.

Also, the strains currently recognized as *L. pseudomesenteoides* should be split into two species, G4 and G5. The former incorporates strains assigned to *L. falkbergense*, including the type strain *L. falkenbergense* LMG 10779T, then we suggest keeping the name *L. falkenbergense*. For G5, we propose maintaining *L. pseudomesenteoides*, the original name of the species, including the type strain *L. pseudomesenteoides* FDAARGOS_1003T (DSM20193).

6. Project 4

Effect of rearing temperature on growth and microbiota composition of *Hermetia illucens* and stability over time of insect meal

6.1. INTRODUCTION

According to the United Nations Food and Agriculture Organization (FAO) estimates, the world's population was 7.2 billion in 2014. Of these, 2 billion people joined in the period 1990-2014. Estimates of this continued growth indicate that the human population will reach 9 billion by 2050 (FAOSTAT, 2015). Meeting the corresponding food requirements will require an increase in crops and livestock farming, which, in turn, will lead to a further increase in agricultural production for the feed industry.

The increase in demand has been matched by the economic and environmental crisis of recent decades, so it will be increasingly essential to study methods of rearing and food products capable of dealing with the various problems. Phenomena such as water and soil pollution caused by industrial production, agriculture and intensive farming, deforestation caused by excessive grazing production, and rising temperatures cause of the increasing greenhouse gases have required special attention and a different approach than in the past.

Feeding insects, or ingredients obtained from insects, can have many advantages: their rearing involves a limited emission of greenhouse gases, requires less space for growth, and has a shallow water requirement since it does not require intensive management (Oonincx et al., 2012). In addition, insects have also revealed a high nutritional value (Finke, 2002), being rich in protein, fat, vitamins, and minerals (Rumpold and Schluter, 2013).

According to the FAO (FAO, 2013), there are already 1900 species of insects in the world that humans eat. Globally, the most consumed insects are beetles (31%), butterfly caterpillars (18%), bees, wasps and ants (14%), grasshoppers, locusts, and crickets (13%). Moreover, there are many countries in which entomophagy is developed: in Mexico, the eggs of the giant ants *Liometopum* are considered the "caviar of the insects" and are cooked fried in butter or boiled; in various countries of Central Africa, the bedbugs after being dipped in lukewarm water to remove the typical smell, are boiled and dried in the sun to be consumed as a snack; in South Africa, instead, are commonly consumed the caterpillars of the butterflies (*Imbrasia belina*) called mopane caterpillars, which, after drying, are processed in different ways (Glew et al., 1999). More than 300 species are commonly included in the diet in Southeast Asia. In Cambodia, for example, tarantulas are fried and served as street food. Other insects used as food are the palm weevil at the larval stage, cicadas, termites, water bugs, and grasshoppers.

Insects for feeding in the zootechnical field occur in various areas such as aquaculture and poultry rearing, as they are already present in the usual diet and pig farming (Ramos-Elordy, 2009). However, this growing need to introduce insects into human and animal nutrition has raised the question of the potential biological dangers that could be encountered. In particular, the microbiota (composed of bacteria, viruses, and fungi) is potentially dangerous, both that intrinsically associated with insects derived from their lifestyle and the one introduced during rearing and processing.

The microbiota present in the gut, essential for their metabolism, functioning, and survival, is conditioned by the lifestyle of insects both in nature and farms. Although during rearing for feed purposes, a step provides for a period of fasting to allow insects to empty the intestine, the fulfilled remains in the growing medium and may cause contamination. The microbiota of insects is composed of different genera of bacteria: *Staphylococcus*, *Streptococcus*, *Bacillus*, *Proteus*, *Pseudomonas*, *Escherichia*, *Micrococcus*, *Lactobacillus*, and *Acinetobacter* (Agabou and Alloui, 2010; Amadi et al., 2005; Braide et al., 2011; Giaccone, 2005). It is, therefore, a question of whether these microbes may be pathogenic to humans and animals and, if so, whether they may be transmitted via feed and food containing insects or their derivatives. As the hosts are phylogenetically different, pathogenic intestinal insect bacteria are considered harmless to animals and humans (FAO, 2013). Therefore, the possible bacterial hazards come mainly from the microbiota, linked to rearing conditions (substrate and feed), handling, processing, and storage (ANSES, 2015). A study carried out by Klunder et al. (2012) showed how the microbial gut composition varies depending on the substrate and entomological species considered. Further

research has been carried out by the Belgian Federal Food Chain Safety Agency (FASFC, 2014), focusing on the microbiological status of insects reared explicitly for feed.

Insects to act as vehicles of zootechnical pathogens, such as *E. coli*, *Salmonella* and *Campylobacter* in farm animals (Blazar et al., 2011). Among the insects investigated are cockroaches, beetles, and flies. In addition, pathogens have been detected in substrates used for entomological rearing. Although the active replication of these bacteria does not occur in the intestinal tract of insects, it is widely demonstrated in farmed animals such as poultry and pigs, where bacteria like *Salmonella* have their ecological niche. The role of arthropods as vehicles of this pathogen has been amply demonstrated in several studies (Agabou and Alloui, 2010; Davies and Breslin, 2003; Goodwin and Waltman, 1996; Holt et al., 2007). Insects such as flies can easily acquire and spread *Salmonella* (Holt et al., 2007; Leffer et al., 2010; Roche et al., 2009), including serotypes relevant to public health like *Salmonella enteridis* (Leffer et al., 2010).

Campylobacter has also been isolated from insects in contact with infected poultry, and it has been shown that flies can be vectors of infection for healthy individuals (Strother et al., 2005; Templeton et al., 2006; Wales et al., 2010). However, under experimental conditions, *Campylobacter* can survive only a short period in insects (Hazeleger et al., 2008; Strother et al., 2005; Templeton et al., 2006).

Insects can also be carriers of moulds and yeasts with potential risks for animals and humans. Yeasts and moulds have been found in considerable quantities in fresh, freeze-dried, and frozen insects, particularly in *Tenebrio molitor* and *Locusta migratoria* (FASFC, 2014). The importance of proper processing, handling, drying, and storage has been underlined after unacceptable levels of aflatoxins have been documented in some commercial batches of Phane worms (*Gonimbrasia belina*: *Saturniidae*) (Schabel, 2010). From the same species, dried under laboratory conditions, some fungi (*Aspergillus* spp., *Penicillium* spp.) have been isolated, among which also the mycotoxigenic species such as *Aspergillus niger*, *A. flavus*, *A. parasiticus*, and *A. ochraceus* (Simpanya et al., 2000).

In general, any risk arising from microbes associated with insects produced for food and feed or introduced during agriculture, processing, and storage could be mitigated by hygienic measures throughout the production chain. Insects, at the European level, since 2017 have been considered raw materials to produce animal feed-falling into the category of “terrestrial invertebrates” [Circular Ministry of Health of 05/05/2017]. Their use is at the discretion of each European Member State and only for feeding pet animals.

On the other hand, the animal meal was already considered suitable for the feed area, according to a strict legal framework that establishes categories, production criteria, authorization, hygiene requirements [Reg, EC n. 1069/2009, EU Reg n. 142/2011]. Furthermore, insect meal foods are already on sale in some countries like the Netherlands and Belgium. Therefore, these meals were generally already accepted in the food chain, limited to pets, fur, zoo, and farm animals that were not part of the food chain.

The need to find new food sources, however, has led the European Union to update the directive on "novel food", which from January 2018 allows the introduction of insects on the European market only if they have a proven history of safe consumption in some parts of the world. However, countries in which entomophagy is already present often have a poor health system and collection of epidemiological information. Moreover, the transposition of the legislation and its implementing regulations are not straightforward in terms of timing and methods, so the discussion of practical aspects and concrete perspectives appears premature.

The list of insects accepted since 2017 to produce the animal meal includes Black soldier fly (*Hermetia illucens*), housefly (*Musca domestica*), mealworms (*Tenebrio Molitor*), litter beetle or lesser mealworm (*Alphitobius diaperinus*), House cricket (*Acheta domesticus*), decorated cricket (*Gryllodes sigillatus*) and Silent cricket (*Gryllus assimilis*).

6.1.1. *Hermetia illucens* morphology

Hermetia illucens is also called Black Soldier Fly (BSF) for its peculiar morphology reminds a soldier in uniform with a helmet (Figure 6).



Figure 6. Adult of black soldier fly (<https://naturdata.com>)

It belongs to the order of the Diptera, suborder Brachycera, the family of the Stratiomyidae (Tab. 13). It has reduced hind wings, which act as barbells, and membranous fore wings, typical of the dipterans. The colour of the body appears dark with metallic reflections. The adults can reach a length ranging from 15 to 22 mm (Sheppard et al., 2002) and have a mouthpiece of sucking type.

Table 13. Scientific Classification of *Hermetia illucens*

Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Diptera
Family	Stratiomyidae
Genus	<i>Hermetia</i>
Species	<i>H. illucens</i>

The antennae are composed of three regions: the scape, the pedicel, and the scourge. The first two regions are single segments, while the third region, the longest, is composed of eight flagellomeres (Pezzi et al., 2017).

The adults have evident sexual dimorphism: in the females, the last urites become thinner and elongated, giving shape to an apparatus with telescopic capacity that is extruded in case of mating or oviposition; in the males instead, the last urites give rise to a complex apparatus, integrated with the external genital organs, called hypopygia (Fig. 6.1).

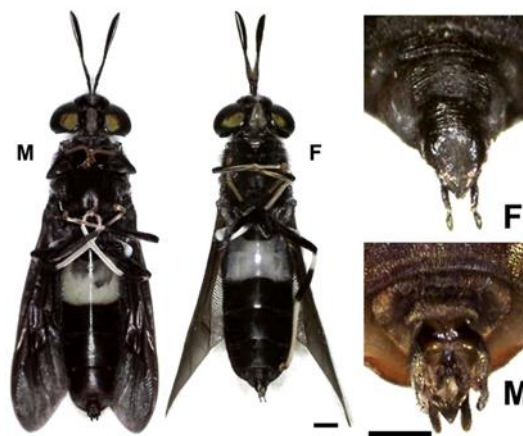


Figure 6.1 Left, ventral view of an adult male and an adult female, right magnification of the respective genitals (Ooonix, 2016)

The morphology of the pupae of *H. illucens* is reported in figure 6.2. During development, the cuticle gradually becomes opaque and sclerotic and turns to a dark brown colour. The body of the pupae is flat with evident cuticle mosaic ornaments. The intra-puparial body shortens when the amount of tissue in the front of the puparium decreases. The posterior region of the puparium is folded by about 45° towards the ventral area. Each segment of the body has many bristles (Lianliang et al., 2016).



Figure 6.2 Pupae of *Hermetia illucens* (<https://entnemdept.ufl.edu>)

The larvae of Black Soldier Fly, before to be adults, exceed six stages like each other in morphology but different in size and weight (Figure 6.3). They have a whitish, opaque colour, with a slight protruding head equipped with a tearing mouthpiece apparatus, with two buccal hooks and a cephalopharyngeal skeleton (Kim et al., 2010). Mature larvae can reach 27mm in length and 6mm in width (Hall and Gerhardt, 2002).



Figure 6.3 Larvae at 6^o stage (www.majalahsains.com)

The eggs of *H. illucens* (Fig. 6.4) are oval, pale yellow, or creamy white and have a size of about 1mm (NCIMPI, 1998).



Figure 6.4 *Hermetia illucens* eggs laid in heaps (zenyrgarden.com)

6.1.2. Biology of *Hermetia illucens*

H. illucens has a saprophagous diet that can feed on a wide variety of organic material, including pig manure, poultry or cattle, kitchen waste, and agricultural waste, but also waste in an advanced state of decomposition or generally difficult to treat as the materials rich in cellulose. The water contained in the substrates must not be less than 70% (Yu et al., 2014), but in the case of rearing, it is necessary to avoid a stagnation of water, which could prevent the access of the larvae to food.

The optimal growth temperatures of the larvae are between 27 and 30 °C, in substrates with 60-90% humidity (Sheppard et al., 2002; Tomberlin et al., 2009), like the habitat of the temperate and warm tropical regions where it is more diffused. Temperature differences, even of only three degrees, determine variations in the survival of the larvae and the duration of the biological cycle and the weight of the same females (Tomberlin et al., 2009).

The larvae of *H. illucens* take about 30 days to complete the development and face six larval stages, of which the last is that of prepupae. In the latter stage, the insect migrates from the alimentary substratum to a drier and more protected place, where it pupates in the larval cuticle. The adult flickers after about 14 days, and in short, it can reproduce. Although the adult's life is influenced by nutrition, it can vary from 9 days, in case of lack of food, to 74 days when water and sugar are available (Nakamura et al., 2016).

Mating (Fig. 6.5) usually occurs in the presence of light and preferably in a dry place (Booth and Sheppard, 1984). The females can lay from the second day after the flickering and oviposition

in small crevices close to a possible feeding source. Adult females can lay up to 1000 eggs at a time, hatching within 102-105 hours after, at 24 °C. (Booth and Sheppard 1984).



Figure 6.5 *Hermetia illucens* mating (singapore.biodiversity.online)

The ability to grow in hostile environments, such as fertilizers and compost, and to make such habitats unsuitable for fungi and bacteria, many studies have been carried out on the predisposition of *H. illucens* to inhibit microbial growth. It was highlighted how they could produce AMP and other antimicrobial substances, including anti-MRSA (Soon-Ik Park et al., 2014). In the past, it has been shown how an extract of the larvae obtained with methanol can counteract the growth of Gram-negative bacteria, such as *Klebsiella pneumoniae*, *Nesseria gonorrhoeae*, and *Shigella sonnei*. Besides, the larvae of *H. illucens* have been tested with positive results for reducing *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Enteridis in poultry manure.

6.1.3. Circular economy and role of *Hermetia illucens*

Hermetia illucens is a valuable source in the field of the circular economy thanks to the ability to feed on every organics waste and/or leftover, and allocate it to a new use. Moreover, after having reached the prepupae stage, it has the peculiarity to self-harvesting, that is, they instinctively leave the substrate and move to a high and clean place, not requiring specifically dedicated systems (Hoc et al., 2019). Also, the adults are not invasive for the man, do not sting, and do not transmit pathogens (Čičková et al., 2015; Sheppard et al., 2002).

BSF is the most tolerant species, able to nourish itself with innumerable varieties of organic substrate materials, besides presenting great advantages such as the low environmental impact of rearing, in terms of small quantities of water and small growth areas required.

H. illucens has already been used with satisfactory results in the management of waste on a small-scale using soil consisting of manure (Sheppard, 1983; Yu et al., 2009), food waste (Green and Popa, 2012), cereals used in distillation (Webster et al., 2016), faecal slurries (Lalander et al., 2013; Banks et al., 2014), animal offal, food leftovers and more (Nguyen et al., 2015). The process allowed the waste material to be exploited and close the nutrient cycle, limiting the emission of potential pollutants and the disposal costs.

The *H. illucens* rearing can be undertaken in developing countries that do not present advanced technologies and cannot afford high capital investments to produce food or feed for other farm animals. On the other hand, it is also possible to set up automated plants to obtain high-quality, safe, and enormous quantities of products (Van Huis et al., 2013).

Compared to other species, BSF larvae have a higher conversion ratio of substrates, consisting of 42% crude protein and 29% fat (Oonincx et al., 2015). Furthermore, thanks to the lipids accumulated in the juvenile stages for the survival of the adult stage, the larvae get a good response as a nutritional source and be converted into biodiesel (Li et al., 2011; Lalander et al., 2015). In addition, dried larvae left after extraction of the fat part can also be used as protein feed (Li et al., 2011).

6.1.4. *Hermetia illucens* applications in non-food waste recovery

The BSF shows multiple applications for recovery or promoting recycling of every organic waste. It can reduce the initial mass of pig manure by 56% and concentrate most of the elements and nutrients by 40-50% (Pivato et al., 2015), offering benefits in terms of hygiene, the reduction of parasitic insects, and lower nutrient pollution from runoff (Pivato et al., 2015; Mallin and Cahoon, 2003). Rice straw and restaurant solid waste are suitable substrates to raise *H. illucens* and obtain, a lipid fraction convertible into an alternative renewable fuel, with most of the properties of this energy source responding to the requirements of the EN 14214 (Zheng et al., 2012).

From 10 tons of food waste can be obtained 300 kg of dried larvae, and 3,346 kg of compost (Solomon et al., 2017). Furthermore, oil derived from BSF larvae has a high concentration of medium chain saturated fatty acids (67% of total fatty acids) and a low concentration of polyunsaturated fatty acids (13% of total fatty acids) making food waste an ideal substrate to produce high-quality biodiesel.

Manure, especially poultry manure, treated with *H. illucens* can be used as a good quality compost improver. It has been reported if zeolite is added to manure, it is possible to reduce up to 50% the contribution of chemical fertilizers in crops (Bertolini et al., 2020). In the project Valoribio project, coordinated by UNIMORE proteins were extracted from the prepupae stage of *H. illucens* to obtain biodegradable plastics. These bioplastics have an essential advantage, i.e. avoiding the use of plants for feeding, promoting the release of nitrogen during biodegradation (www.valoribio.eu).

6.1.5. Applications of *Hermetia illucens* for feed recovering organic waste and food by-products

BSF larvae are a valid substitute for traditional animal feed. In experiments with meat quail (*Coturnix japonica*), no difference was found between the control diet and BSF larval meal in terms of production performance, meat weight, and yield (Cullere et al., 2016). Furthermore, the meal larvae did not affect the sensory, and gustatory aspects of meat, oxidative state, cholesterol composition, improved amino acid content, and increased the levels of less desirable saturated and monounsaturated fatty acids. Similar effects were detected by integrating BSF larvae in the diet of chickens (*Gallus gallus domesticus*), highlighting that defatted BSF reduced the negative impact of the fatty acid profile, thus making this feed a promising protein source for poultry feeding (Schiavone et al., 2017).

These insects are also greatly appreciated by chickens, seeking such a protein source among the food offered, consisting mainly of soy meal (Ruhnke et al., 2017). The rearing of these insects directly on the manure of the birds would allow their immediate use in foodstuffs, at the same time allowing the exploitation and recycling of waste (Allegretti 2017, 2018).

A comparative study of eggs produced by hens fed only with soldier fly meal rather than soy meal has shown that this substitution is also possible with good results. Eggs from BSF-fed hens had a higher proportion of yolk than the soy diet and a higher level of tocopherol, lutein, beta carotene, total carotenoids and 11% lower cholesterol (Secci et al., 2018).

Good results have also been obtained in the use of BSF larvae in fish farming, with the possibility of getting protein-rich biomass at lower costs. Larvae can contribute significantly to sustainable aquaculture (Kroeckel et al., 2012; Saeley et al., 2011; Aniebo et al., 2009), and as a partial or total alternative in meals (Diener et al., 2009; Magalhaes et al., 2017; Bondari et al., 1981) also for aquatic invertebrates such as shrimps (Cummins et al., 2017).

6.2. AIM OF THE PROJECT

In recent years there has been a growing interest in new protein sources for human consumption and food industry. The availability of proteins of alternative animal origin allows the development of innovative food products for a demanding group of consumers with specific ethical and health requirements. On the other hand, the production of protein sources, exploiting industrial waste in the context of circular economies, would reduce the environmental impact of the food industry.

The use of insects as a source of proteins, particularly *Hermetia illucens*, is part of this framework as a concrete possibility of transforming vegetable by-products of the food industry into protein-rich ingredients with high nutritional properties. Rearing *H. illucens* on different waste streams in a biorefinery approach requires attention to chemical and microbiological safety issues. The European Food Safety Authority reported the lack of data regarding microbiology, virology, parasitology, and toxicology of insects reared, addressing the potential hazards of non-processed insects compared with other non-processed sources of protein animal origin (EFSA, 2015). Hygiene issues of edible insects can originate from the substrate and the microbial community of the insect gut and may be affected by processing steps linking farming and consumption.

Recent works reported the microbiota composition of BSF larvae and prepupae (De Smet et al., 2018; Osimani et al., 2018; Wynants et al., 2018; Terova et al., 2019). However, little attention has been paid to microbial dynamics associated with extrinsic parameters, such as the rearing temperature. From a bioeconomy perspective, the biotransformation of wastes toward new valuable compounds should be a low-energy process that can be exploited in different districts with good tolerance to temperature changes, minimizing the requirement of heating or cooling.

This part of the work was supported by the Flies4value project, aimed to offer an economic, efficient, competitive, and low-impact system to enhance the waste of regional agri-food industries by exploiting the effectiveness of *Hermetia illucens* larvae, bioconverting insects, for the production of feed for laying hens and other substances with high added value for the food and agriculture industries.

From this standpoint, BSF larvae were reared at different temperatures to determine the effect of this parameter on the growth rate and the biomass yield of prepupae. Since temperature may be a primary driver of microbial population composition, the microbiota of prepupae reared at 20, 27, and 33 °C was characterized. Furthermore, the larvae were fed a basal substrate to avoid biases due to the feed's intrinsic different microbial communities (Jeon et al., 2011; Zheng et al., 2013; Bruno

et al., 2019). The microbial load has also been estimated by using selective media to research mesophilic bacteria, Enterobacteriaceae, lactobacilli, yeasts, and molds.

With the perspective to monitor from diverse points of view the potential risks of these new foods or feeds, our attention has been focused on the contamination of molds because of the potential production of mycotoxins. During rearing, moulds have been isolated from the substrates and identified by ITS gene sequencing to assess the potential risk. These latter experiments evaluated the potential use of meal BSF larvae before entering as a diet of laying hens, where the poultry farmers mainly use soy as a protein source. Therefore, the continuous increase in the prices of this raw material and the consumer aversion to GMO products, like most of the imported soybeans, pushes towards the search for alternative protein sources. The microbiota of this larval biomass (i.e. Enterobacteria, *Salmonella*, *Clostridium perfringens*, and total mesophilic aerobes bacteria) has been analyzed to ensure safe animal feeding.

6.3. MATERIALS AND METHODS

6.3.1. Effect of rearing temperature on growth and microbiota composition of *H. illucens*

6.3.1.1. Rearing of *H. illucens*

BSF larvae from a colony preserved at the Applied Entomology Laboratory, BIOGEST-SITEIA (Reggio Emilia, Italy), established initially with prepupae purchased from CIMI srl (Cuneo, Italy), were reared. The vegetable substrate (S) to rear BSF larvae was composed of 25% zootechnical use cornmeal (mill waste), 15% wheat bran, 10% alfalfa meal, and 50% water (Hogsette 1992). The second and third larval stages were obtained from eggs hatched in S and incubated in climatic chambers under the controlled conditions of 27 ± 0.5 °C, 65% humidity, and 16:8 light / dark cycles. One hundred small larvae (L) were collected and reared in glass containers (20 × 12 × 8 cm) containing 400 g of substrate S within climatic chambers at 20 ± 0.5 , 27 ± 0.5 , or 33 ± 0.5 °C. Two independent experiments, starting with lots of L, were performed, where diverse rearing temperatures (20, 27, and 33 °C) were assayed in triplicate. Test checks were carried out every three days, starting from day seven until the end of the development period: the experiments were considered concluded when a minimum of 90% of the initial larvae became prepupae (PP). The time point presenting the highest PP appearance in a single control was registered as the peak

of PP. At the end of the experiment, insects were collected from the frass (F). PP and the remaining larvae (L_{final}) were counted and weighed to determine total biomass weight, the mean growth rate of each insect, and larvae mortality, according to the following:

$$\text{Total biomass (g)} = \text{total PP weight} + L_{final} \text{ weight (1)}$$

$$\begin{aligned} \text{Mean growth rate (g/d)} = \\ (\text{mean PP weight} + \text{mean } L_{final} \text{ weight} - \text{mean L weight}) / \text{development period (d) (2)} \end{aligned}$$

$$\text{Larvae mortality \%} = 100 \times (N L - N L_{final} - . PP) (3)$$

Standard hygienic conditions were applied throughout all the rearing phases without using surface disinfection of larvae, substrate sterilization, or aseptic incubation. Samples of L, S, PP20 °C, PP27 °C, PP33 °C, F20 °C, F27 °C, and F33 °C from each experiment were frozen and maintained at –80 °C until analyzed.

6.3.1.2. Culture dependent microbiological analyses

Five grams of S, PP, or F samples were suspended (10% w/v) in buffered peptone water (Tryptone 1g/L, Sodium chloride 9 g/L) and homogenized for 2 minutes by Ultra Turrax (T25 Ika, Staufen, Germany). Proper dilutions were spread on selective media provided by BD Difco (Franklin Lake, NJ, USA). On Plate Count Agar (PCA, Peptone 5 g/L, Yeast extract 2.5 g/L, Glucose 1 g/L, Agar 18 g/L), total mesophilic aerobes were counted after incubation at 30 °C for 72 h. Aerobic sporulating bacteria were cultured on PCA plates supplemented with 2 g/L of starch, incubated at 37 °C for 48 h, with samples heated at 80 °C for 10 min to inactivate vegetative bacteria. To detect lactic acid bacteria, plates of Lactobacilli MRS agar (Proteose peptone No 3 10 g/L, Beef extract 10 g/L, Yeast extract 5 g/L, Dextrose 20 g/L, Polysorbate 80 1 g/L, Ammonium citrate 2 g/L, Sodium acetate 5 g/L, Magnesium sulfate 0.1 g/L, Manganese sulfate 0.05 g/L, Dipotassium phosphate 2 g/L, Agar 18 g/L) were incubated at 30 °C for 72 h under microaerophilic conditions (GasPack EZ, BD Difco). Enterobacteriaceae were enumerated on Violet Red Bile Agar (VRBGA; Gelatin peptone 7 g/L, Yeast extract 3 g/L, Sodium chloride 5 g/L, Bile salts 1.5 g/L, Glucose monohydrate 10 g/L, Neutral red 0.03 g/L, Crystal violet 0.002 g/L and Agar 15 g/L) plates incubated at 37 °C for 24 h. Yeasts and molds were counted on plates of Dichloran Rose Bengal

Chloramphenicol Agar (DRBCA; Protease peptone No.3 5 g/L, D-hypsim 10 g/L, Monopotassic phosphate 1 g/L, Magnesium sulfate 0.5 g/L, Dicloran 2 mg/L, Bengal rose 25 mg/L, Chloramphenicol 0.1 g/L, Agar 18 g/L, pH corrected to 5.6) after incubation at 25 °C for 5 days. To detect and enumerate the food pathogens *Bacillus cereus*, *Campylobacter* spp.; *Clostridium perfringens*, *Listeria monocytogenes*, *Listeria* spp.; *Salmonella* spp.; *Staphylococcus aureus*, and coagulase-positive staphylococci, standard methodologies were used (ISO 7932:2004; ISO 7937:2004; ISO/TS 6579-2:2012; ISO 10272-2:2017; ISO 11290-2:2017; ISO 6579-1:2017; ISO 6888-1:1999/AMD 2:2018). The protocols provided by ISO consisted of seeding *B. cereus* on *Bacillus cereus* Agar base (MYP, Meat extract 1 g/L, Peptone 10 g/L, D-mannitol 10 g/L, Sodium chloride 10 g/L, Phenol red 0.025 g/L, Agar 12 g/L) supplemented with Polimixin B sulfate 10⁶ I.U. and 10 mL Egg yolk emulsion 20%. Plates were incubated at 37 °C for 24 h. *Campylobacter* spp. were spread on *Campylobacter* blood-free medium base (Bolton) (mCCDA, Beef extract 10 g/L, Peptone 10 g/L, Tryptone 3 g/L, Sodium chloride 5 g/L, Charcoal 4 g/L, Sodium desoxychoate 1 g/L, Sodium pyruvate 0.25 g/L, Ferrous sulfate 0.25 g/L, Agar 15 g/L), and added one vial of Bolton CCDA antimicrobial supplement reconstituted with 5 mL of sterile distilled water. The plates were incubated in microaerophilic conditions at 41.5 °C for 48 h. *Clostridium perfringens* was enumerated on TSCA plates (Eezymatic digest of casein 15 g/L, Soy peptone 5 g/L, Yeast extract 5 g/L, Sodium metabisulphite anhydrous 1 g/L, Ferric ammonium citrate 1 g/L, and Agar 15 g/L) supplemented with D-Cycloserine 4 g/L, the plates were incubated anaerobically at 37 °C for 20 h. *Staphylococcus aureus*, and coagulase-positive staphylococci were enumerated onto Baird-Parker agar plates (BPA, Tryptone 10 g/L, Beef extract 5 g/L, Yeast extract 1 g/L, Sodium pyruvate 10 g/L, Glycine 12 g/L, Lithium chloride 5 g/L, Agar 15 g/L) supplemented with 50 mL of egg yolk tellurite emulsion 20%. According to the ISO, for the detection of *Listeria monocytogenes* or *Listeria* spp., 5 g of sample was suspended 10% (w/v) in a primary selective enrichment half Fraser broth (HFB, Enzymatic digest of animal tissues 5 g/L, Enzymatic digest of casein 5 g/L, Meat extract 5 g/L, Yeast extract 5 g/L, Sodium chloride 20 g/L, Disodium hydrogen phosphate dihydrate 12 g/L, Potassium dihydrogen phosphate 1.35 g/L, Aesculin 1 g/L, Lithium chloride 3 g/L), supplemented with the sodium salt of nalidixic acid 0.1 g dissolved in 10 mL of Sodium hydroxide 0.05 mol/L, Acriflavine hydrochloride 0.25 g dissolved in 100 mL of distilled water and Ammonium iron (iii) citrate 5 g dissolved in 100 mL of distilled water. The samples were incubated at 30 °C for 25 h. Then, tubes of secondary selective enrichment Fraser broth (FS) same composition of HFB, with an amount Sodium salt of nalidixic acid 0.02 g dissolved in 5 mL of Sodium hydroxide (0.05 mol/L), were inoculated with 0.1 mL of primary selective enrichment and incubated at 37 °C for 24 h. After the two enrichment *Listeria monocytogenes* and *Listeria* spp.

were encountered on Agar *Listeria* according to Ottaviani and Agosti (ALOA, Peptone 18 g/L, Tryptone 6 g/L, Yeast extract 10 g/L, Sodium pyruvate 2 g/L, Glucose 2 g/L, Magnesium glycerophosphate 1 g/L, Magnesium sulfate 0.5 g/L, Sodium chloride 5 g/L, Lithium chloride 10 g/L, Disodium hydrogen phosphate anhydrous 2.5 g/L, 5-bromo-4-chloro-3-indolyl- β -D-glucopyranoside 0.05 g/L, Agar 13.5 g/L), supplemented with Nalidixic acid sodium salt 0.02 g dissolved in 5 mL of Sodium hydroxide, Polymyxin B sulfate 76700 I.U. dissolved in 5 mL distilled water, Cycloheximide dissolved in 1:1 ethanol and distilled water, and Soy lecithin 2 g/L content from 9% to 15% phosphatidylinositol non-fractionated dissolved in 50 mL distilled water. The plates were incubated at 37 °C for 48 h. For the presence/absence of *Salmonella*, 0.1 mL of initial suspension in buffer peptone water incubated at 37 °C for 18 h was used to inoculate tubes of 10 mL Rappaport Vassiliadis Soy enrichment (RVS, Soy peptone 4.5 g/L, Sodium chloride 7.2 g/L, Monopotassium phosphate monobasic 1.26 g/L, Dipotassium phosphate 0.18 g/L, Magnesium chloride 13.4 g/L, Malachite green oxalate 0.036 g/L) and a second aliquot of 1 mL culture used to inoculate tubes of Muller Kauffmann tetrathionate broth (MKTTn, Enzymatic meat hydrolysate 4.3 g/L, Enzymatic casein hydrolysate 8.6 g/L, Sodium chloride 2.6 g/L, Calcium carbonate 38.7 g/L, Sodium thiosulfate anhydrous 30.3 g/L, Bile salts 4.78 g/L, Brilliant green 9.6 mg/L) supplemented with Novobiocin sodium salt 0.04 g dissolved in 5 mL of distilled water and iodine solution constituted by Iodine 20 g, and Potassium iodide dissolved in 100 mL of distilled water. Tubes RVS were incubated at 41.5 °C for 24 h, and MKTTn at 37 °C for 24 h. Cultures obtained from RVS and MKTTn were spread onto Xylose Lysine Deoxycholate agar plates (XLDA, Yeast extract 3 g/L, Sodium chloride 5 g/L, Xylose 3.75 g/L, Lactose 7.5 g/L, Sucrose 7.5 g/L, L-Lysine 5 g/L, Sodium thiosulfate 6.8 g/L, Iron (iii) ammonium citrate 0.8 g/L, Phenol red 0.08 g/L, Sodium deoxycholate 1 g/L, Agar 15 g/L), and incubated at 37 °C for 48 h.

Triplicate samples from the two rearing batches were analyzed (n = 6). The observed microbial contamination was compared with recommended thresholds of international authorities (EFSA, 2015; NVWA 2014 and 2019).

6.3.2. Rearing of *H. illucens* for feeding laying hens

In the context of the Flies4value project, growth of larvae was performed on a set of vegetable wastes, to produce larvae on agro-industrial by-products to feed laying hens. The rearing was performed inside plastic boxes with a 30 x 40 cm surface, using four larvae per cm² as larval density. Each larva was fed with 1 g of moist substrate consisting of 40% ground tomato, 30% bran,

and 30% ground green beans. Whey was added in a ratio of 1: 2 (v/w) of the vegetable by-products to obtain the complete substrate (S). The first larval stages (small larvae) were added in S and incubated in climatic chambers under the controlled conditions of 27 ± 1 °C, 60-70 % humidity. Then, the larvae in S were fed and reached maturity in 7-8 days. Finally, the larvae were killed and stored at -20 °C before obtaining the meal.

6.3.2.1. Fungal contaminants of vegetable wastes for larval diet

During the rearing experiments on vegetable by-products, growth of molds generally occurred. In order to investigate the potential impact on aflatoxin production and contamination of larvae with these toxic metabolites, fungi were isolated from food waste ingredients and contaminated frass. Morphologically different colonies were collected and taxonomically identified sequencing the Internal Transcribed Spacer region (ITS). Food by-products (Table 14) were provided to the larvae in two different forms, coarse and ground.

Table 14. Matrices used to feed H. illucens

Kind of substrate	N° samples	Havarest data
Coarse tomato	2	27-07-2020
Ground tomato	1	27-07-2020
Coarse pineapple	1	31-07-2020
Ground pineapple	1	27-07-2020
Coarse orange	1	27-07-2020
Ground orange	1	27-07-2020
Control 1 (the Gainesville diet)*	1	27-07-2020
Coarse Cranberry bean	0	
Ground Cranberry bean	1	15-07-2020
Coarse Cannellini bean	2	15-07-2020
Ground Cannellini bean	2	15-07-2020
Coarse green beans	1	15-07-2020
Ground green beans	1	15-07-2020
Coarsa chickpea	1	15-07-2020
Ground chickpea	1	15-07-2020
Chaff	1	15-07-2020
Control 2 (the Gainesville diet)*	1	15-07-2020
Carrot	1	15-07-2020
Carrot + chaff	1	15-07-2020

*The Gainesville diet contains corn, bran, and alfalfa and is commonly used for rearing larvae.

From the above matrices, fungi have been isolated using the following culture media: Potato dextrose agar (PDA; Potato starch 4 g/L, Dlysis 20g/L, Agar 18 g/L, pH corrected to 5.1), Malt extract agar (MEA; Malt extract 50 g/L, ZnSO₄ - 7H₂O 0.01 g/L, CuSO₄ - 5H₂O, Agar 18 g/L, pH corrected to 5.4), and DRBCA obtained from BD (Difco, Sparks, USA). The plates were incubated at 25 °C for 5-7 days. The colonies presenting diverse morphologies were isolated and purified. DNA was extracted according to the protocol of Möller and Bahnweg (1992). The sequence encoding the ITS sequence was amplified by PCR using 100 µM of the following mixture: Dream Taq Green buffer (Thermo Fisher Scientific, Waltham, MA), 0.25 mM dNTPs mix, 0.2 µM ITS1 primer (5'-TCC GTA GGT GAA CCT TGCGG-3'), 0.2 µM ITS4 primer (5'-TCC TCC GCT TAT

TGA TAT GC-3'), 5 U Taq polymerase (DreamTaq, Thermo Fisher Scientific), and 10 µL DNA template. The thermocycler was set as follow: 95 °C for 5 min; 35 cycles at 94 °C for 45 sec, 50 °C for 45 sec, 72 °C for 1 min; 72 °C for 7 min. The PCR products were separated on agarose gel (TAE 1%) for 90 min at 80 V, adding Gene Ruler DNA Ladder Mix (100-10.000 bp) as molecular marker. ITS amplicons were cut, purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany), normalized at 10 ng/µL, and sent to the service to be sequenced (Eurofins Genomics, Ebersberg, Germany).

6.3.2.2. Larval meal production and stabilization

Larvae of *H. illucens* were processed for meal production to feed laying hens Larvae were defrosted, blanched for 30 sec, then dried in the oven at 55 °C for 24 h, then ground to obtain the meal. The larval meal was stored at 4 °C and analyzed within 24 h (A, B, and D), or processed to obtain a stabilized product (C, E, F, and G):

- A. Larval meal untreated (lot 1);
- B. Larval meal untreated (lot 2);
- C. Larval meal autoclaved at 121 °C for 15 min (lot 2);
- D. Larval meal untreated (lot 3);
- E. Larval meal heated at 110 °C for 30 min (lot 3);
- F. Larval meal heated at 110 °C for 30 min and autoclaved at 121 °C for 15 min (lot 3);
- G. Larval meal heated at 110 °C for 30 min and autoclaved at 121 °C for 30 min (lot 3).

The microbiological stability of samples G was assessed. The larval meal was analyzed after:

- G1. one month at room temperature;
- G2. one month at room temperature in modified atmosphere (N₂);
- G3. one month at 38 °C and 90 % of humidity.

A further time point after 2 weeks since preparation was analyzed for G3 meal. Total mesophilic bacteria, Enterobacteriaceae, and *Clostridium perfringens* were enumerated in PCA, VRBGA, and TSCA plates, respectively. in The presence/absence of *Salmonella* sp. in 25 g of sample was determined following the Commission Regulation (EU) No 142/2011.

6.3.3. Statistical analysis

Plate counts and growth parameters are presented as means $\pm$ SD. ANOVA followed by Tukey's post hoc test was carried out with SPSS Statistics software (v. 21, IBM, Armonk, NY, USA). Differences were considered statistically significant for $p < 0.05$. Spearman's rank correlation was utilized to estimate the correlation with temperature.

6.4. RESULTS

6.4.1. Growth performance of *Hermetia illucens* at different temperatures

The effect of temperature on the growth of *H. illucens* was evaluated at 20, 27, and 33 °C measuring growth rate, larvae biomass production and larvae mortality (Table 15). The longer development period (40 d; $p < 0.05$), was observed at 20 °C, while it was similar, approximately 24 d, at 27 and 33 °C ($p > 0.05$). Rearing at 20 °C resulted in the lowest total biomass yield and mean growth rate (15.9 g and 4.1 mg/d; $p < 0.05$). No significant differences were observed between 27 °C and 33 °C (approximately 21 g and 10 mg/d). The highest larval mortality (14.5%) was registered at 33 °C ($p < 0.05$), whereas it was similar at 20 and 27 °C (3.8 and 5.5%, respectively). The temperature also significantly affected the time point at which the peak of PP was observed. The higher the temperature, the earlier came the peak (30.0, 19.7, and 16.0 d at 20, 27, and 33 °C, respectively).

Table 15. Growth parameters of *H. illucens* at the three tested temperatures. Within each line, values marked with different letters indicate significant differences (ANOVA followed by Tukey's post hoc test, $p < 0.05$).

Growth parameter	20 °C	27 °C	33 °C
Development period (d)	40.0 ± 0 ^a	24 ± 1.1 ^b	23.67 ± 1.0 ^b
Total biomass (g)	15.9 ± 2.3 ^b	21.3 ± 3.8 ^a	21.0 ± 1.3 ^a
Mean growth rate (mg/d)	4.1 ± 0.6 ^b	9.4 ± 1.5 ^a	10.4 ± 0.7 ^a
Larvae mortality (%)	3.8 ± 2.0 ^b	5.5 ± 5.3 ^b	14.5 ± 7.4 ^a
Peak of PP	30.0 ± 1.5 ^a	19.7 ± 2.6 ^b	16.0 ± 1.6 ^c

6.4.1.1. Culture dependent microbiological analysis

The viable count of total mesophilic aerobes in PP positively correlated with temperature ($p < 0.05$), being the lowest at 20 °C (7.4 Log cfu/g) and approximately one magnitude higher at both 27 and 33 °C (Table 16). Aerobic spore-forming bacteria were less abundant than total aerobes at all the tested temperatures ($p < 0.05$). Lactic acid bacteria and enterobacteriaceae lay in the range of 6.2–6.9 and 3.7–5.2 Log cfu / g, respectively, without significant differences associated with temperature. A low load of cultivable yeasts and moulds was observed, between 0.7 and 1.2 Log cfu/g, without significantly influence of temperature. *Bacillus cereus*, *Campylobacter* spp.; *Clostridium perfringens*, coagulase-positive staphylococci, and listeriaceae were detected in PP reared at all the tested temperatures. The load of *Campylobacter* and listeriaceae was positively

correlated to temperature: when the temperature increased from 20 °C to 33 °C, *Campylobacter* passed from 3.2 to 4.7 Log cfu/g, and listeriaceae from 4.8 to 5.8 Log cfu/g. Coagulase-positive staphylococci, *B. cereus*, and *C. perfringens* laid in the ranges of 3.7–4.2, 2.3–3.2, and 0.8–1.6 Log cfu/g, respectively, and were not affected by the temperature. *Salmonella* spp. was found in 7 out of 18 samples. Among the positive samples, only one, PP reared at 20 °C, allowed the recovery of colonies by direct plating at a concentration of 1.1 Log cfu/g.

The microbiological analysis of the growth substrate revealed a mean viable charge of 4.5 Log cfu/g total mesophilic aerobes, 4.9 Log cfu/g aerobic sporulating bacteria, 2.9 Log cfu/g lactic acid bacteria, and 3.4 Log cfu/g fungi. In contrast, enterobacteriaceae, *B. cereus*, *Campylobacter* spp. *C. perfringens*, coagulase-positive staphylococci, and *Salmonella* spp. were not detected.

Table 16. Cultivable microorganisms observed in the substrate (S) and prepupae biomass (PP) grown at 20, 27 and 33 °C. Counts are expressed as Log cfu/g. For *Salmonella* spp. the number of positive samples out of six is reported.

Viable Counts	PP				Contamination Limits ¹
	S	20 °C	27 °C	33 °C	
Total mesophilic aerobes	4.5 ± 0.2	7.4 ± 0.9 ^b	8.5 ± 0.4 ^a	8.7 ± 0.3 ^a	< 5.7
Aerobic spore-forming bacteria	4.9 ± 0.8	6.8 ± 1.3 ^a	7.9 ± 0.5 ^a	7.9 ± 0.2 ^a	n.s
Lactic acid bacteria	2.9 ± 0.7	6.6 ± 0.4 ^a	6.9 ± 0.7 ^a	6.2 ± 0.5 ^a	n.s
Enterobacteriaceae	-	3.7 ± 0.3 ^b	5.2 ± 0.6 ^a	4.8 ± 1.3 ^{ab}	< 3.0
Yeast and moulds	3.4 ± 0.3	0.7 ± 0.9 ^a	1.2 ± 1.0 ^a	0.9 ± 0.8 ^a	n.s.
<i>Bacillus cereus</i>	-	2.3 ± 0.9 ^a	3.2 ± 0.7 ^a	2.3 ± 0.7 ^a	< 5.0
<i>Campylobacter</i> spp.	-	3.2 ± 0.3 ^c	4.2 ± 0.4 ^b	4.7 ± 0.2 ^a	absent in 25g
<i>Clostridium perfringens</i>	-	0.8 ± 0.7 ^a	1.6 ± 1.3 ^a	1.0 ± 0.8 ^a	< 5.0
Coagulase-positive staphylococci	-	3.9 ± 0.7 ^a	4.2 ± 0.7 ^a	3.7 ± 0.9 ^a	< 5.0
Listeriaceae	2.6 ± 0.4	4.8 ± 0.4 ^b	5.5 ± 0.3 ^a	5.8 ± 0.4 ^a	< 2.0
<i>Salmonella</i> spp.	0/6	2/6	1/6	4/6	absent in 25g

Values are means ± SD (n = 6). - indicates < 2 Log cfu/g in the 6 replicates. For PP, within each line, values marked with different letters indicate significant differences (ANOVA followed by Turkey's post-hoc test, p < 0.05). ¹ Reference values according to NVVWA and EFSA (EFSA, 2015; NVWA, 2014 and 2019). n.s., limit not specified.

6.4.2. Isolation of fungi from vegetable by-products

During the experiment of *H. illucens* rearing on vegetable by-products, the matrix where larvae were developing was subjected to an abundant growth of moulds. In order to assess a potential risk due to the fungal production of aflatoxins, filamentous fungi were isolated and taxonomically characterized. Approximately 22 different fungal colonies were selected from RBCA and PDA plates, starting from the samples reported in Table 17. The ITS sequences of the purified fungi were obtained and blasted on the NCBI platform to define the species. As a whole, the isolated biotypes belonged to 5 species: *Rhizopus oryzae*, *Aspergillus oryzae*, *Trichosporon asahii*, *Geotrichum silvicola*, and *Mucor circinelloides*.

Aspergillus oryzae was found in coarse and ground orange, coarse cannellini, coarse green beans, and chaff. Instead, *Rhizopus oryzae* was abundant in carrot + chaff and coarse chickpeas. Furthermore, *Trichosporon asahii* and *Geotrichum silvicola* fungi were isolated only in-ground cannellini. Finally, *Mucor circinelloides* was found in-ground chickpeas.

Table 17. Isolation of Fungi from food waste matrices utilized as substrates for *H. illucens*

	Rhizopus oryzae	Aspergillus oryzae	Mucor circinelloides	Trichosporon asahii	Geotrichum silvicola
Coarse tomato	X	X			
Ground tomato	X	X			
Coarse pineapple	X	X			
Ground pineapple	X	X			
Coarse orange		X			
Ground orange	X	X			
Control 1	X	X			
Control 2	X	X			
Chaff		X			
Ground Cranberry bean					
Coarse Cranberry bean		X			
Ground Cannellini bean				X	X
Coarse green beans		X			
Ground green beans	X	X			
Coarsa chickpea	X				
Ground chickpea			X		
Carrot+chaff	X				

6.4.3. Larval meal of *H. illucens* stabilization tests

Different substrates composed by food by-products were compared for *H. illucens* growth on the basis of larval yield. Larvae for feeding laying hens were reared at pilot scale with the best performing vegetable-based substrate (data not shown), composed as following: 40% ground tomato, 30% bran, and 30% ground green beans. The larvae were processed according to the protocol above mentioned (6.3.2.2), then subjected to diverse stabilization treatments.

Viable count obtained on PCA plates of three batch of larvae, analysed before stabilization treatments, indicated a charge of total mesophilic aerobes (TMA) ranging between 7.4 and 8.7 Log cfu/g. In the same samples, enterobacteriaceae ranged between 6.3 and 8.5 Log cfu/g. *Clostridium perfringens* was detected in TSC plates at concentration between 3.6 and 5.7 Log cfu/g. Presence of

Salmonella spp. in 25 g of sample was detected only in lot 3 where, according to the most probable number analysis, reached 3 Log cfu/g.

The same meal samples underwent different stabilization procedures. Treatment with dry heat at 110 °C for 30' was not effective in the reduction of TMA, enterobacteria, and *C. perfringens* ($p>0.05$; Table 18). Conversely, with autoclave protocol a significant reduction ($p<0.01$) of 2.5 log of TMA and 1.2 log of *C. perfringens* was observed as well as a reduction under the limit of detection of enterobacteriaceae (< 2 Log cfu/g; $p<0.01$). Combining dry heat and autoclave procedures resulted in the depletion of both *C. perfringens* and enterobacteriaceae but TMA charges remained up to 7.2 Log cfu/g regardless of the treatment time in the autoclave. All the stabilization procedures applied to lot 3, the sole originally contaminated with *Salmonella* spp., were effective for the complete inactivation of this microorganism.

Table 18. Microbial contamination in larval meal before and after the stabilization procedures

	Untreated	Treated at 110 °C x 30'	Treated in autoclave at 121 °C x 15'	Treated at 110 °C x 30' + 121 °C x 15' in autoclave	Treated at 110 °C x 30' + 121 °C in autoclave x 30'
PCA					
Lot 1	8.8± 0.1 ^a		4.9 ± 0.1 ^b		
Lot 2	7.4± 0.2 ^a				
Lot 3	8.7 ± 0.0 ^a	8.7 ± 0.1 ^a		7.2 ± 0.1 ^b	7.1 ± 0.1 ^b
TSCA					
Lot 1	4.9± 0.1 ^a		2.1 ± 0.3 ^b		
Lot 2	3.3± 0.4 ^a				
Lot 3	5.7 ± 0.0 ^a	2.8 ± 0.1 ^b		-	-
VRBGA					
Lot 1	8.3± 0.1 ^a				
Lot 2	6.3± 0.1 ^a		-		
Lot 3	8.5 ± 0.1 ^a	8.3 ± 0.0 ^a		-	-

Values are means ± SD (n = 3). - indicates < 2 Log cfu/g in the 3 replicates. Values marked with different letters indicate significant differences (Paired t-test followed by Anderson-Darling Normality test, p < 0.05).

6.4.3.1. Microbiological analysis of stabilized larval meal

After stabilization, the microbial load of larval meal was monitored for one month keeping samples at room temperature (sample RT), at room temperature under N₂ atmosphere (sample N2), and at 38 °C and 90% of humidity (sample T38).

At the starting point of the test (T0), TMA were 7.1 ± 0.1 Log cfu/g whereas enterobacteria, *Salmonella* spp., and *C. perfringens* were absent or under the detection limit. Enterobacteriaceae and *Salmonella* remained under the limit of detection in all samples regardless the storage conditions. On the other hand, TMA reached 9.4 ± 0.0 Log cfu/g after one month at room temperature and in samples N2 and T38 the enumeration was not possible, likely for the presence of motile bacteria with high microbial load (>10⁹cfu/g), that impaired the detection of colonies even at high dilutions. At the end of the test, *C. perfringens* was present in all samples reaching up to 4.9 Log cfu/g (Table 19). At room temperature, the storage under N₂ atmosphere significantly affect (p<0.05) the *C. perfringens* load that was lower in presence of oxygen compared to the N₂ atmosphere (3.1 and 4.9 Log cfu/g, respectively).

Table 19. Viable count of *Clostridium perfringens* during the stability test of larval meal

<i>Clostridium perfringens</i> (Log cfu/g)	
T0	-
RT	3.1 ± 0.9 ^b
N2	4.9 ± 0.5 ^c
T38	4.1 ± 0.4 ^{bc}

Values are means ± SD (n = 3). -indicates < 2 Log cfu/g in the 3 replicates. Values marked with different letters are indicative of significant differences.

6.5. DISCUSSION

In recent years, interest in insects as a relevant future source of sustainable raw materials for animal feed has grown in many countries worldwide. Insects are able to meet the dietary needs of animals in terms of nutritional composition, amino acid profile and, as part of the natural diet of different animal species (Belghit et al., 2019). Furthermore, the mass production of insects has a low environmental impact and reduces CO₂ emissions (Zarantoniello et al., 2019). Insect rearing requires a small land area to produce one kg of protein (Truzzi et al., 2020) and determines a lower competition between feed and food (Belghit et al., 2019). The use of insects in the bioconversion of waste is an innovative approach and a remarkable example of a sustainable circular economy (Bruni et al., 2018). Many insects have been tested as animal feeds, and among the most encouraging species we find the black soldier fly *Hermetia illucens* (Schiavone et al., 2017 and 2018).

The impact of temperature on growth of *H. illucens* is a key point, in the perspective to build production plants all over in the world, and hopefully in developing countries. The production of prepupae of *H. illucens* at three different temperatures (20, 27, and 33 °C) was performed on a standard vegetal substrate, to compare the growth yields. Although with differences in the development rate, the larvae efficiently grew across the range of temperatures, confirming the robustness of *H. illucens*. In the tropics, the growth of *H. illucens* can occur yearlong, while it is restricted to a few generations per year in warm temperate regions (Tomberlin et al., 2001), generally requiring temperatures above 26 °C (Park et al., 2010). In agreement with other studies, we demonstrated that higher temperatures enhanced the larval development and greater final biomass yield, as well as higher mortality (Glicorescu et al., 2018; Tomberlin et al., 2009), suggesting that the best rearing temperature is 27 °C. The flexible rearing temperature enlarges the possibility to built plants allover in the world, with minimal investments in energy-consuming devices for cooling or heating.

The impact of temperatures on microbiota composition has been studied. The rearing temperature slightly affected the microbial load of prepupae: only total mesophilic aerobes and enterobacteriaceae were significantly lower at 20 °C than at higher temperatures. All the assessed pathogens *Bacillus cereus*, *Campylobacter* spp.; *Clostridium perfringens*, *Listeria monocytogenes*, *Listeria* spp.; *Salmonella* spp.; *Staphylococcus aureus*, and coagulase-positive staphylococci were detected in the whole set of prepupae, and the counts of *Campylobacter* and listeriaceae increased with the increasing temperature.

The substrate was hostile to all the tested pathogens except listeriaceae. However, the larvae were not disinfected and were not reared under aseptic conditions, according to hygienic conditions that may be achieved in a production facility, where the substrate (e.g., a not sterilized vegetal waste) is expected to be the primary source of microbial contamination. Therefore, it is likely that enterobacteriaceae, *B. cereus*, *Campylobacter* spp., *C. perfringens*, coagulase-positive staphylococci, and *Salmonella* spp., laying below the limit of detection in the fed substrate, were environmental contaminants or were introduced with small larvae.

In any case, hygienic conditions must be kept under control throughout the multiple manufacturing processing stages because the rearing of prepupae favoured the blooming of the pathogens. Therefore, significant attention must be paid to microbiological optimization through the processes aimed at stabilizing the insects, such as powdering, heating, drying, UV treating, high-energy microwaving, pasteurizing, and acidifying, which are expected to contain the pathogens charge.

In the context of the Flies4values (www.flies4value.it), the design of an integrated plant for mass production of *H. illucens* larvae, suppression, and functional stabilization is in progress, in order to prepare feed for poultry. The feed obtained from lycopene rich tomato wastes is expected to provide natural pigments to obtain eggs with intensely coloured yolk, as required by the market, thus reducing the use of synthetic pigments, expensive and harmful to the health of hens. A main goal of the project has been to choose the best meal to feed larvae of *H. illucens*, exploiting vegetable by-products. Several feeds for *H. illucens* have been assembled and tested (Table 2). However, a major drawback of these rearing experiments was the abundant growth of moulds over the larval growth process. Fungi were isolated and characterized, in order to evaluate a potential risk in terms of mycotoxin contamination.

The detection of fungi occurring in the substrate during the rearing of larvae was carried out to verify whether they could produce mycotoxins or other low molecular weight secondary

metabolites that could compromise food and feed safety, with risk for humans and animals if consumed or inhaled (Bennett and Klich, 2003).

In the frass moulds belonging to the following taxa have been isolated: *Rhizopus oryzae*, *Aspergillus oryzae*, *Geotrichum silvicola*, *Mucor circinelloides* and *Trichosporon asahii*. It is not entirely clear whether the genera *Rhizopus* and *Mucor* produce mycotoxins. However, in some studies, *Rhizopus* and *Mucor* are generally reported as degraders of aflatoxin B1, zearalenone, ochratoxin A (Varga et al., 2004; Hackbart et al., 2014). The species *Mucor circinelloides* presents low cytotoxicity due to the production of 3-nitropropionic acid (Hollmann et al. 2008). In contrast, aflatoxin B1, zearalenone and ochratoxin A can be produced mainly by *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* (Krska et al., 2008; Ten Bosch et al., 2017; Greco et al., 2015; Ismaiel and Papenbrock, 2015).

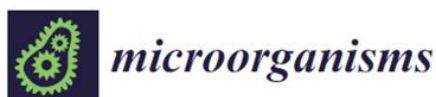
The species *Aspergillus oryzae* is not able to produce aflatoxins but produces other mycotoxins such as cyclopiazonic acid, β -Nitropropionic acid (Frisvad et al., 2018). However, *A. oryzae* is generally considered a safe fungus used to reduce fermented foods (Kim et al., 2014). The role of *Trichosporon asahii* as a mycotoxin-producing species is unknown. Still, this species is considered an opportunistic pathogen for humans, particularly affecting immunodeficiency patients or immunocompromised with blood diseases (Ruan et al., 2009). No data are available regarding the production of mycotoxins by *Geotrichum silvicola*. In the perspective to grow *H. illucens* on these vegetable by-products, it is relevant to take into account that to completely inactivate microorganisms and toxins, a thermal treatment is required (Grabowski and Klein, 2016).

In the present study, a mass rearing of *H. illucens* larvae was performed to obtain livestock feed, particularly laying hens. According to Regulation (EU) No 142/2011, in processed animal protein *C. perfringens* has to be absent in one gram of product, enterobacteria might be less than 10^1 cfu/g in five samples tested, and *Salmonella* spp. might be absent in 25 g of product in five samples tested.

Preliminary microbiological tests were performed to evaluate the product safety. The viable count of total mesophilic aerobes, *C. perfringens*, and *Salmonella* spp. in these samples was very high, and not consistent with regulatory limits. According to Klunder et al. (2012), a heating step is required to reduce the bacterial endospores. Therefore, the larval meal samples underwent different types of heat treatments to make the product suitable as animal feed. The treatment with dry heat at 110 °C for 30 min followed by wet heat at 121 °C for 30 min, abated the microbial load of total mesophilic aerobes and pathogenic bacteria (*Salmonella* spp., *C. perfringens*), albeit not sustainable in terms of energetic requirements. Furthermore, the product is not stable and over the time, within

a time lapse of one month, the microbial load increased and did not longer fulfill the regulatory requirements for feed use. As a whole, the containment of microbial contamination in a stabilized products resulted very challenging., and it can represent a major drawback to the exploitation of these larvae to produce a sustainable, cheap, and safe feed for poultry.

Part of these results are presented in the paper:



Article

Effect of Rearing Temperature on Growth and Microbiota Composition of *Hermetia illucens*

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7. Project 5

Inflorescences of seven *Cannabis sativa* varieties through a culture-dependent approach

7.1. INTRODUCTION

Cannabis sativa L., commonly known as hemp, is an annual herbaceous plant with a branched hold native to Southeast Asia already from Holocene, spontaneously sprouting along the area that extends from the lower Danube to northern China. Its cultivation is widespread all over the world starting from East Asia passing through Europe up to spread in the Americas (Long et al., 2017; Pisanti and Bifulco, 2018).

In China, back in 2007 b.C., hemp was a fundamental plant for the population for the ease of being able to obtain tissue fibres from the floema of the stem. Later, between 70 and 80 b.C., also in China, hemp was recognized as a raw material for the manufacture of paper. In Europe, on the other hand, it was introduced by the Scythians between the 4th and early 3rd-millennium b.C. and, later, by the Thracians who weaved it to produce clothes. Also, it was custom for these peoples to throw "hemp seeds" on the stones rolled to the fire and then immerse themselves in their fumes by throwing screams of joy (Sorenson and Johannesse, 2009). The first documented case on the use of Cannabis for sacramental and religious purposes is much older and dates back over 120 thousand years, as evidenced by the discovery of seeds, resin, and ash at a Palaeolithic site in the Hindu Kush mountainous area of Pakistan where leaves, buds, and resin, were burned breathing smoke to enjoy psychotropic properties and approach the deity (Clarke and Merlin, 2013; Bonini et al., 2018).

Nowadays this represents the oldest evidence of intentional use of substances for psychotropic purposes by man. In Europe, Cannabis probably reaches around 5000 years ago and is used based

on recipes derived from tradition and folk medicine (Bonini et al., 2018). Since the mid-19th century, more than 100 articles on the medical use of hemp as a powerful narcotic, analgesic, sleeping pill, for the treatment of seizures, hysteria, madness, mental depression, dysmenorrhea, and menorrhagia have been published in Europe and the USA (Reynolds, 1890).

At the beginning of the twentieth century, the use of *Cannabis* is increasingly widespread, so much so that it is included in the official pharmacopeia and is available in European and American pharmacies. Nevertheless, in 1937, it was placed on the drug list in the United States, as a law was passed prohibiting the cultivation of any type of hemp, including for industrial purposes. As a result, its use as a drug also suffers a sharp collapse in Europe, resulting in a reduction in scientific publications dedicated to the subject in the following decades. Medical interest in *Cannabis* awakened in early 1970, following the discovery of Δ^9 -THC (Gaoni and Mechoulam, 1964), its main psychoactive component. In 1988 Devane et al. (1988) described the presence in the human body of a membrane receptor capable of being activated by Δ^9 -THC, opening up important new perspectives for the therapeutic use of *C. sativa* L. For decades now, pharmaceutical companies have been extracting molecules from hemp or producing synthetic cannabinoids, to these are added plant products based on its inflorescences including Bedrocan, Bedrobinol, Bediol, for the treatment of numerous pathologies (cancer, Parkinson's disease, anxiety, stress, epilepsy, fibromyalgia, pain therapies, and many others), or Sativex, a natural extract, authorized for muscle spasms in people with multiple sclerosis (Rekand, 2014).

7.1.1. Therapeutic applications of medical cannabis

Despite the millennial use of the plant and the identification of Δ^9 -THC in 1964, it is necessary to wait until the nineties to understand the mechanism of action of cannabinoids. The way in which these compounds produce their effects was determined by the discovery of cannabinoid-specific receptors called CB1 (cannabinoid receptor type 1) and CB2 (cannabinoid receptor type 2) (Gaoni and Machoulam, 1964; Pacher et al., 2006).

Although CB2 typically show peripheral distribution (spleen, thymus, tonsils, and immune system cells, such as B lymphocytes and NK) and are not responsible for the psychomimetic effects, typical of CB1, its localization is also at the level of the CNS (microglia and brain stem). CB1s are present in vascular tissue, immune system, heart, and testicles (Gong et al., 2006; Onaivi et al., 2006; Van Sickle et al., 2005). Numerous cannabinoid compounds interact with CB receptors. One of the most important cannabinoids is the non-psychoactive compound cannabidiol (CBD). It

is not clear how CBD exert its activity but, since several studies have shown that it decreases the psychotropic activity of THC, it has been hypothesized that it may act as a reverse agonist (Pertwee et al., 2010), also assuming a protective role (Iseger and Bossong, 2015). Cannabinoids have various activities justified by their interaction with cannabinoid receptors: analgesic, myo-relaxing, anti-immunogenic, anti-inflammatory, anti-tumor, from appetite stimulation to mood enhancement (Grotenhermen, 2005).

Scientific research in the therapeutic field of Cannabis has seen a significant strengthening over the years, especially in the international context, regarding its effects on the state of health of patients undergoing treatment. The result of the combination of all substances contained in *Cannabis* is called the "entourage effect" and has been studied in recent years (Blasco-Benito et al., 2018; Cogan, 2020; Finlay et al., 2020). This mechanism describes the process by which cannabinoids work together synergistically and affect the body like the action of its endocannabinoid system. Researchers claim that extracts containing a wide spectrum of substances act better as therapeutic agents, rather than individual cannabinoid extracts (Liu, 2013). One study, for example, shows that a Cannabis extract with 20% THC showed a more marked activity as an antispastic than pure THC (Williamson, 2001).

Below are proposed in summary, the most consistent evidence on the use of cannabinoids and their derivatives.

Antioxidant activity

Reactive oxygen species are highly reactive molecules capable of causing damage to living organisms: hydrogen peroxide (H_2O_2), hypochlorous acid (HClO) and free radicals such as hydroxyl radical ($OH\bullet$) and superoxide anion (O_2^-) can cause damage to DNA, proteins, lipids, etc. CBD performs a neuroprotective antioxidant action, showing a more powerful action of ascorbate and α -tocopherol (Hampson et al., 1998). The peptides generated by the digestion of hydrolyzed hemp seeds, subsequently purified and separated, into eight peptide fractions with HPLC in reverse phase, exhibit a high ability to neutralize radical oxygen species and excellent scavenger properties towards 2,2-diphenyl-1-picrylhydrazyl, superoxide anion, and hydroxyl radical (Girgih et al., 2013).

Anti-tumor activity

Recent studies have reviewed the possibility of using cannabinoids as real anti-cancer drugs. According to the study published in *Anticancer Research* (Scott et al., 2013), "cannabinoids can interfere with the development of cancer cells by slowing their development." There are at least six non-psychoactive cannabinoids that have been shown to inhibit the growth of leukemic cells at all stages of the cell cycle, including cannabidiol (CBD), cannabigerol (CBG), and cannabigerovarin (CBGV). Interestingly, the team observed even greater effects when several cannabinoids were administered together (Scott et al., 2014). Cannabinoids have also shown the ability to reduce tumor growth such as glioma (Solinas et al., 2013), breast cancer (Behrend, 2013), prostate cancer (Orellana-Serradell et al., 2015), colon (Borrelli et al., 2014), and skin cancer (Perez-Gómez et al., 2013; Velasco et al., 2016).

Anti-inflammatory activity

In contrast to Δ^9 -THC, CBD binds much looser to CB1 and CB2 receptors, and has a strong anti-inflammatory activity compared to Δ^9 -THC, with high therapeutic potential (Burstein, 2015). A CBD-derivative, HU-444, has been studied, which cannot be converted by cyclic to Δ^9 -THC, has anti-inflammatory activity (reduces the presence of reactive oxygen intermediates and inhibits the production of TNF- α by macrophages) (Haj et al., 2015). Cannabinoid (CBN), cannabigerol (CBG), cannabichromene (CBC) and cannabidiolic acid (CBDA) are also more powerful prostaglandin inhibitors than Δ^9 -THC (Burstein et al., 1973). The anti-inflammatory activity of hemp terpenoids has long been known eugenol, carvacrol, and p-vinyl phenol overtake cannabinoids in the inhibition of prostaglandins (Burstein et al., 1975). The β -myrcene, the most abundant monoterpene in hemp, has anti-inflammatory activity. The same applies to β -caryophyllene, the most common sesquiterpene in the plant, which also has cytoprotective (on the gastric mucosa) and anti-malarial (McPartland and Russo, 2001).

Immuno-suppressive activity

Cannabinoids present in *Cannabis* mediate their physiological effects by activating the CB1 and CB2 (De Petrocellis et al., 2011). Cannabinoids are powerful immuno-suppressant and anti-inflammatory agents (Di Marzo and Piscitelli, 2015). The mechanism by which they mediate immuno-suppression is only now being understood and can be characterized in four steps:

apoptosis, inhibition of cell proliferation, suppression of cytokine and chemokine production, and induction of regulating T cells.

Muscle relaxation

Tremors are born from an involuntary cycle of contractions and muscle relaxations: they are a disabling symptom very common in various motor-associated pathologies, such as Parkinson's, multiple sclerosis, Huntington's disease, and forms of ataxia (Wagner and Ulrich-Merzenich, 2009). An improvement in symptoms has been observed in hemp smokers (Wright et al., 2013) and given the high density of cannabinoid receptors in the brain area responsible for motor functions, many researchers have wondered whether cannabinoid-associated compounds could be used in therapy to relieve tremors associated with central nervous system disorders (Arjmand et al., 2015).

Anti-epileptic activity

Using rat models, researchers found that cannabidivarin (CBDV) and CBD could be effective not only for seizures but also in treating specifically related genes (Amada et al., 2013). In a recent study, 90% of children with drug-resistant epilepsy, who were given cannabis with a high CBD value (CBD: THC ratio 20:1), experienced a significant decrease in the frequency and intensity of seizures (Tzadok et al., 2016).

Anxiolytic role

In some people Cannabis leads to freeing itself from anxieties, generating confidence and a good mood, in others, it can provoke reactions of the opposite type, from mild worries to states of negativity and agitation. Many of the receptors to which cannabinoids bind are concentrated in the amygdala, the part of the brain that manages emotions and especially our reactions to fear and stress (Katona and Patel, 2014). When THC acts on this part of the brain it modifies its communications and can do so positively or negatively. The effects vary both from person to person, and in different situations and moods.

Sometimes THC can overexcite neural pathways by bringing anxiety-producing states, while others can positively affect, producing well-being (Ramikie et al., 2014). More recently, studies confirm that low dosages of THC reduce anxiety, high dosages increase it (Mechoulam and Parker, 2013). It remains to be remembered that the most efficient cannabinoid for anxiety and associated pathologies, such as post-traumatic disorder syndrome or obsessive-compulsive disorder, is CBD

(Bergamaschi et al., 2011; Crippa et al., 2011). Terpenes also leave a lot of hope, an example of which is β -caryophyllene, a CB2 receptor agonist that produces multiple behavioural changes relevant to anxiety and depression in mice (Bahi et al., 2014).

7.1.2. Uses and purposes of industrial hemp

Hemp, which responded positively to environmental and agronomic needs, could form the basis for a new economy. From an agronomic point of view, it is the typical "reeling" crop, since it is more competitive than most weeds, thanks to its rate of growth and the ability to select light radiation. It is considered "multipurpose" already for the traditional division of production between stem, from which fibre and hemp wood are obtained, and seed, from which oil and flour can be obtained.

In recent years, the use and sale of inflows have been increasing, until the boom of the so-called "cannabis light", which can become a new resource for Italian hemp growers, especially following the entry into force in January 2017 of Ddl n.242, in which inflows are not contemplated from a regulatory point of view but can nevertheless be classified and sold for technical use.

The components of the plant can also be used for various uses: in addition to the traditional ones, more and more new and innovative destinations are expanding for which it is possible to foresee a strong market expansion. To highlight the importance of hemp and the strong interest towards the return of this cultivation, the uses and possible products that can be derived from it will be listed below (Guerriero et al., 2014; 2015; 2016).

Phytoremediation

Its cultivation could prove beneficial in those land withdrawn from production, in weak areas, or for the reclamation of soils contaminated with heavy metals through a process called "phytoremediation". It is considered suitable for this purpose precisely because of its chelating capacity towards contaminants such as arsenic and copper, as well as solvents and pesticides.

Textile

Quality long fibre is intended for the linen type of industry. Hemp fibres are considered more productive because they are more robust (Guerriero et al., 2013) and durable than cotton. It is one of the crops for textile use with a high environmental impact, both because of the high use of pesticides, which are not necessary for the cultivation of hemp and because of the use of water resources. From hemp, you can obtain resistant yarns that can be used in fabrics intended to produce textile products (Cassano et al., 2013).

Paper

The use of hemp in paper production could solve most of the environmental problems: many primary forests in the world have been destroyed to produce paper, at considerable environmental costs. Today, 93% of the paper comes from wood, and only 29% is recycled. The manufacture is used both the fibrous part, which produces high-quality paper, thin and resistant, and the woody part (canapulo) (Warrior et al., 2013), for cardboard and newspaper paper. Hemp paper has characteristics such as strength, extensibility, opacity, and resistance to both tears and lacerations, as well as moisture. Also, unlike tree paper, it can be recycled seven times, always maintaining the same thickness.

Food

Hemp can also be grown for food use. You can obtain the seeds, rich in proteins with high biological value, and an oil-rich in unsaturated fats and ideal to prevent diseases of the cardiovascular system.

Industrial

The oil has exceptional properties: it is possible to produce soaps, waxes, cosmetics, biodegradable detergents. Also the paints made with this raw material, are of higher quality than those produced with petroleum derivatives and are not polluting. About the production of plastics, hemp can represent a concrete alternative to oil thanks to the cellulose contained in the plant, which through a polymerization process, allows obtaining degradable plastic materials (Marsh, 2003) that can be used in the production of packaging and materials to be used with an insulating function.

Energy saving

Hemp, because of its high plant yield, could be a source of biomass for use as an alternative to coal and other fossil fuels. Some companies claim that hemp pellets are the fuel that, thanks to hemp ethanol, burns cleaner. This could provide several energy solutions: the use of this biofuel, unlike fossil hydrocarbons, does not lead to an increase in CO₂ in the atmosphere since, the emission of carbon dioxide during combustion, would be offset by the amount of CO₂ that hemp itself can absorb during its growth.

Bioarchitecture

As for the green building, using some parts of the plant (such as canapulo, short fibres, and other waste materials) felts, mattresses, insulating panels, mortars, and bricks can be produced with sound-absorbing and insulating properties and compared to plastic and glass wool, have greater impact resistance, greater lightness and less susceptibility to deformation under the heat effect. The combination of hemp and lime is also the perfect mix to produce bricks for use in eco-sustainable construction through the combination of canapulo with a binder based on hydraulic lime and water.

7.1.3. Elements of botany

Cannabis is a genus of annual dicotyledon angiosperm plants, belonging to the family *Cannabiaceae* (of which hops are also part, *Humulus lupulus*, L.). *C. sativa* L. is an annual herbaceous plant, the height varies between 1.5 - 2 m, but in cultivated subspecies, it can reach up to 5 meters. It has a long taproot and a fuse, erect or branched, with resinous, angled, sometimes hollow growths, especially above the first pair of leaves. The leaves are pickled and have stipules; each is webbed, consisting of 5-13 elongated lanceolate leaflets, at the serrated margin, with sharp tips. The leaves at the base of the plant are opposite, in the apical part instead tend to grow alternate, simple, or trifurcated, especially after the non-tenth knot of the plant, or sexual maturation occurred. As flowering progresses, the number of leaflets per leaf decreases, up to a single small leaflet that appears before each pair of flowers (Clarke, 1981). Although currently numerous monoecious varieties have been selected, originally the plant was dioicous, and unisexual flowers grow on individuals of different sexes. The male flowers (stamiferous) are gathered in axillary racemes, and each has 5 tepals fused at the base and 5 stamens (Figure 7).



Figure 7. Male and female inflorescence (pevgrow.com)

Female flowers (pistils) are gathered in groups of 2-6 at the base of bracts forming glomerular ears. They have an urceolate calyx surrounding the uniovulate ovarian. The female flower, with ripening, is progressively covered with glandular trichinosis, which represents the main sites of bioaccumulation of the active ingredients, cannabinoids, and terpenes, which give the plant a strong smell (Hillig and Mahlberg, 2004).

Male plants usually die soon after releasing their pollen, while female plants die only after completing seed production, a few months after flowering. Besides, male plants show a faster

increase in height and a faster decrease in leaf size; the leaflets accompanying the male flowers are much smaller in size than the leaflets accompanying the female flowers.

Although it is not possible to distinguish the sex of the plant before flowering, the most recent and modern genetic applications, combined with molecular markers associated with the male sex, allow its early discrimination (Mandolin et al., 1999; Törjek et al., 2002).

Biosynthesis and bioaccumulation of cannabinoids are located in specialized epidermal glands, distributed throughout the aerial surface of the plant. The glands vary in shape, numerical density, and secretory activity, depending on the location site and the phenological phase reached. Cannabinoid production, associated with terpenes, occurs to a greater extent in female blooms than in males, and the female dioicous plant is the main object of therapeutic Cannabis cultivation.

The fruit is bivalve, smooth, indehiscent, ovoid, or almost spherical achenium. The pericarp has a corneal consistency, the colour is not uniform, but is speckled, thanks to a lattice of vascular bundles located under the epidermis. Brown predominates, sometimes olive or tending to reddish, but there is no lack of lighter, whitish, and greenish fruits. Each achenium contains only one seed of varying sizes (the weight range of 1000 seeds vary from 3 to 60 g). The embryo has two cotyledons rich in reserve substances, a poorly developed root, and a stem. The plant germinates in spring and blooms in late summer when daylight hours begin to decrease (short-day plant) (Bocsa and Karus, 1998).

Most of the world's Cannabis cultivation still takes place outdoors and generally, but not necessarily, from seed, because growing Cannabis from seeds means that half of the crop could be unwanted male plants. In intensive greenhouse cultivation, this is generally avoided, through cloning and indoor production, which go hand in hand, often using hydroponic techniques, i.e., cultivation in nutritious solutions rather than in soil.

Industrial cannabis is grown and produced to obtain its seeds and fibres. It includes several varieties of *Cannabis sativa*, specific for agricultural and industrial uses, and is characterized by a low THC content and a high CBD content. In most European countries, the maximum legal limit for THC content in fibres is 0.2%. The collection of fibres occurs at the end of the flowering of female plants and before the formation of seeds.

The life cycle of *C. sativa* has a duration of about 120-150 days and is completed with flowering (Figure 7.1). The flowering cycle can last anywhere between 4 and 12 weeks, depending on variety and environmental conditions. A good sign of maturation is the colour of the stigma, a

structure similar to a fluff. As soon as the flower ripens, these typically shrivel and turn brown. When about 75% of the stigma is brown, the plants are ready for harvesting.

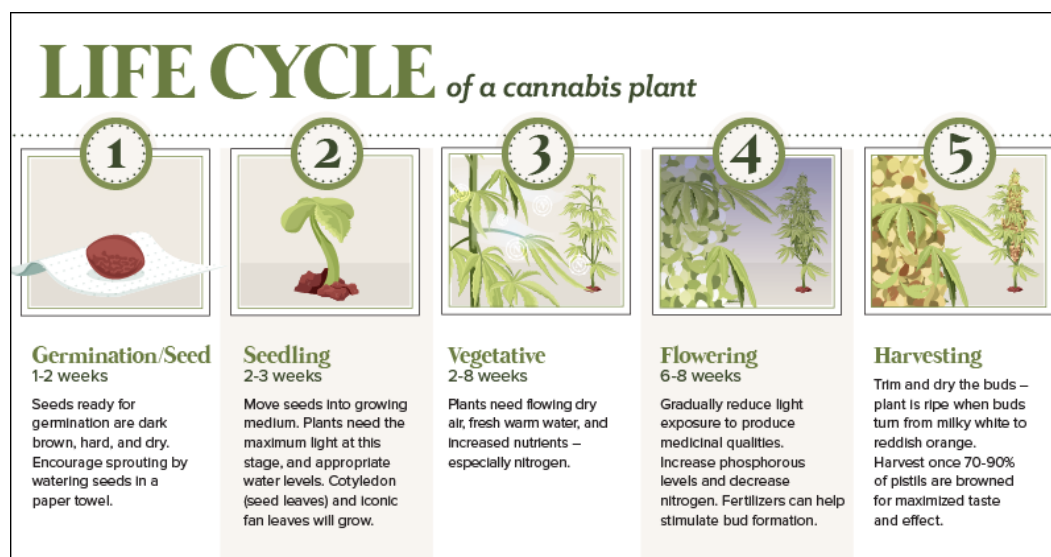


Figure 7.1 Life cycle (www.visualcapitalist.com)

7.1.4. Chemical composition

Cannabis sativa contains a significant variety of active ingredients. To date, at least 554 different compounds have been identified (Aizpurua-Olaizola et al., 2016), among which 112 are phytocannabinoids, some of which are found exclusively in *Cannabis* (Pertwee, 2014; Ahmed et al., 2015), while terpenes, with about 140 members, make up the most abundant class (ElSohly and Slade, 2005).

Cannabinoids or phytocannabinoids have been defined as a group of typical compounds with 21 carbon atoms (Gaoni and Mechoulam, 1964). They are terpenoids, their chemical structure can be described as that of a terpene combined with an alkyl substitution resorcinol, or as that of a benzopyran ring system. They are stateless, they are poorly soluble in water and they are highly soluble in fats. Cannabinoids are more abundantly present in fresh plant material in the form of their carboxylic acid; drying, aging, and heating convert them into neutral forms. The two most abundant cannabinoids are Δ^9 -THC, also called "dronabinol", the main psychoactive constituent of *Cannabis*

and CBD (Pertwee, 2006). The content of THC can vary significantly depending on the origin and type of preparation of the plant.

7.2. AIM OF THE PROJECT

In recent years, we have seen a growing interest in hemp-based products and their contained active ingredients. Particularly attractive for the markets are the inflorescences from which it is possible to extract a wide range of bioactive molecules and aromas with potential applications in the pharmaceutical, cosmeceutical, and food sectors. The parts of the plant that are historically used are the seeds, intended to produce oil and flour, and the fibers, which are obtained from the stalk and used in the production of biomaterials (paper, textiles). Another possible use of hemp is the valorization of the lignocellulosic biomass for the production of renewable energy.

The new law on the cultivation of industrial hemp entered into force in 2017 in Italy, while regulating different aspects of the production chain, does not mention the inflows, therefore it does not explicitly prohibit its marketing, citing a thriving business.

This project takes place from a wider study on *Cannabis sativa* (UNIHEMP project) aimed to create a platform for the exploitation of waste from the cultivation of hemp, for a long time a neglected plant. The activities focused on the estimation and characterization of the microbial load contaminating the inflorescence of *C. sativa*. This apical part of the plant presents a low THC content <0.2% but, is rich in extremely interesting secondary metabolites, in particular other cannabinoids, useful for pharmaceutical and cosmeceutical applications. The inflorescences of 7 varieties of *C. sativa* were purchased from 2 Italian locations (Acireale and Rovigo), the samples were harvested for three years from 2019 to 2021, dried and stored at room temperature. The samples were analyzed to determine the viable microbial load of total aerobic bacteria, enterobacteria, *Salmonella*, *Escherichia coli*, yeasts, and molds.

7.3. MATERIALS AND METHODS

7.3.1. Sampling

The dried inflorescences of seven cultivars of *Cannabis sativa* L. (C.S. Carmagnola, Eletta campana, Carmaleonte, Codimono Bernabeo, and Fibranova) were provided by the CRA departments of Acireale and Bologna, partners of the UNIHEMP project.

The harvest in the open field took place in the three years 2019, 2020, and 2021. The *Cannabis sativa* varieties from CREA of Acireale (CT, Italy) were collected in Libertia and Pietrapescie. The Bologna ones were collected in the Rovigo area (RO, Italy). As soon as they reached maturity, generally in June or July, the inflorescences were harvested by manually cutting the apical portion of the plant and were carefully selected by cleaning them from residues of the stem and larger leaves.

Every year, hemp samples of the 7 varieties were analyzed from both Acireale and Rovigo, in order to identify the cultivable microbiota. As a whole, 42 samples were analyzed according to the guidelines of the European Pharmacopoeia 9.8 for the search of pathogens in non-sterile herbaceous matrices.

7.3.2. Drying

Hemp buds of 30 cm, harvested on different dates according to the various varieties' flowering time and were dried for 72 h at 30-35 °C in a greenhouse. The dried material underwent manual shelling to separate stems, inflorescences, seeds, and leaves. Dried inflorescences were ground to obtain a coarse size. Two passages through sieves of 2 mm diameter were used to separate the seeds.

7.3.3. Culture media

According to the guidelines of the European Pharmacopoeia 9.8, microbiological analyses were carried out using the following culture media: Casein Soya Bean Digest Agar (CSBDA), Casein Soya Bean Digest Broth (CSBDB), Sabouraud Dextrose Agar (SDA), Enterobacteria

Enrichment Broth Mossel (EBM), Violet Red Bile Glucose Agar (VRBGA), MacConkey Broth, MacConkey Agar, Rappaport Vassiliadis Salmonella Enrichment Broth (RVSEB), Xylose, Lysine, Deoxycholate Agar, (XLDA). SDA, EBM, VRBGA, and MacConkey agar were obtained from BD (Difco, Sparks, USA), MacConkey broth from Oxoid (Thermo-Fischer Scientific, Waltham, USA), XLDA from Biolife (Milan, Italy). Unless otherwise indicated, the media were prepared with reagents obtained by Sigma Aldrich (Darmstadt, Germany) and BD (Difco, Sparks, USA). The media were sterilized by autoclaving at 121 °C for 15 minutes.

The composition of the media has been reported for 1 L. SDA, EBM, VRBGA, XLDA, McConkey (broth and agar) were ready to use:

- CSBDA: pancreatic hydrolyzate of casein (BD) 15 g, papic hydrolyzate of soy (BD) 5 g, NaCl 5 g, agar 15 g, pH corrected to 7.3;
- CSBDB: pancreatic casein hydrolyzate (BD) 17 g, papic soy hydrolyzate (BD) 3 g, NaCl 5 g, K₂HPO₄ 2.5 g, glucose monohydrate 2.5 g, pH corrected to 7.3;
- SDA + antibiotics: glucose 40 g, a mixture of pectic hydrolyzate of animal tissues and pancreatic hydrolyzate of casein (1: 1) 10 g, agar 15 g, corrected to pH 5.6. Chloramphenicol 20 µg / mL and ampicillin 100 µg / mL were added to the medium, once autoclaved and cooled to 56 °C;
- EBM: pancreatic gelatin hydrolyzate 10 g, glucose monohydrate 5 g, dehydrated bovine bile 20 g, KH₂PO₄ 2 g, Na₂HPO₄ 2 H₂O 8 g, Brilliant green 15 mg, pH corrected to 7.2;
- VRBGA: yeast extract 3 g, pancreatic gelatin hydrolyzate 7 g, bile salts 1.5 g, NaCl 5 g, glucose monohydrate 10 g, agar 15 g, neutral red 30 mg, crystal violet 2 mg, pH corrected to 7.4;
- MacConkey broth: pancreatic gelatin hydrolyzate 20 g, lactose monohydrate 10 g, dehydrated ox bile 5 g, bromocresol violet 10 mg, pH corrected to 7.3;
- MacConkey agar: pancreatic hydrolyzed gelatin 17 g, meat peptones and casein 3 g, lactose monohydrate 10 g, NaCl 5 g, bile salts 1.5 g, agar 13.5 g, neutral red 30mg, crystal violet 1mg, pH corrected to 7.1;
- RVSEB: soy peptone 4.5 g (BD), MgCl₂ · 6H₂O 29 g, NaCl 8 g, K₂HPO₄ 0.4 g, KH₂PO₄ 0.6 g, malachite green 0.036 g, pH corrected to 5.2;

- XLDA: xylose 3.5 g, L-lysine 5 g, lactose monohydrate 7.5 g, sucrose 7.5 g, NaCl 5 g, yeast extract 3 g, phenol red 80 mg, agar 13.5 g, sodium deoxycholate 2.5 g, sodium thiosulfate 6.8 g, ferric ammonium citrate 0.8 g, pH corrected to 7.4.

7.3.4. Sample preparation and microbiological analysis

The analyses were set up on 42 hemp samples belonging to 7 varieties, collected in two different areas (Acireale and Rovigo) for 3 subsequent years. For each variety, two replicates belonging to the same lot were analyzed.

Each sample was weighed, diluted 1:10 in CSBDB, and homogenized using a Lab blender stomacher (VWR PBI, Milan, Italy) for 4 minutes to obtain the initial suspension. According to European Pharmacopoeia 9.8, once the samples have been homogenized, they were placed at room temperature for 2.5-3 h. Then, 1 mL was 1:10 serially diluted in CSBDB. 0.1 mL from each dilution plated on CSBDA and SDA + antibiotic plates. CSBDA was used to enumerate total aerobes, SDA + antibiotics to enumerate yeasts and molds. The CSBDA plates were incubated at 30 °C for 3-5 days; plates of SDA + antibiotics were placed at 25 °C for 5-7 days.

The detection limit of plate counting is a drawback in the search of dangerous pathogens, and enrichment becomes a necessary step for pathogen detection. In this study, Enterobacteriaceae, *E. coli*, and *Salmonella* were searched through enrichment.

Enterobacteriaceae, in particular, Gram-negative bile-tolerant bacteria, were quantified by the most probable number procedure. Volumes of 1 mL, 0.1 mL, and 0.01 mL of the 1:10 CSDBB suspension were inoculated in 10 mL of EBM enrichment cultures. In addition, to determine the presence/absence in 1 gram of sample, 10 mL of inoculum from 1: 10 CSDBB suspension were picked to inoculate flasks containing 190 mL of EBM. After 48 h of static incubation at 30 °C, an aliquot of the enriched cultures in EBM was used to seed the VRBGA plates to evaluate the growth of Gram-negative bile-tolerant bacteria.

The 1:10 suspension in CSBDB was incubated at 30 °C for 18-24 h. At the end of the incubation, 1 mL of suspension was used to inoculate the enrichment cultures of *Escherichia coli* (flasks containing 100 mL of MacConkey broth), and 0.1 mL to inoculate the enrichment cultures of *Salmonella* (tubes containing 10 mL RVSEB). MacConkey broth cultures were statically incubated at 44 °C for 48 h; RVSEB cultures at 30 °C for 24 h. After incubations, to confirm the pathogen presence/absence, 10 µL of enrichment cultures in MacConkey broth and RVSEB were

used to seed MacConkey agar and XLDA plates, respectively. MacConkey agar plates were incubated at 30 °C for 72 h to detect the presence of *E. coli*. XLDA plates were incubated at 30 °C for 24-48 h to highlight the presence of *Salmonella*.

7.3.5. Statistical analysis

The data obtained of microbiological counts on selective media were compared using Maxstat Pro (Maxstat, version 3.6) software. The univariate analysis of variance was followed by Tuckey's post hoc B test (one-way Anova), setting the significance level $p < 0.05$.

7.4. RESULTS

7.4.1. Cultivable bacterial enumeration

Seven varieties of *Cannabis sativa*, from two Mediterranean Italian locations (Acireale and Rovigo) were collected and analysed in the years 2019, 2020, and 2021 to verify microbial contaminants in the inflorescences. The load of total mesophilic aerobes (TMA) were evaluated on CSBDA plates. In the samples collected in Acireale in 2019 TMA ranged between 3.6 and 4.8 Log cfu/g, except for the CS variety, where TMA were under the detection limit (< 3 Log cfu/g) (Figure 7.11A). Among the contaminated varieties, Eletta campana (4.4 ± 0.5 Log cfu/g) showed significantly higher contamination than Codimono (3.6 ± 0.0 Log cfu/g; $p < 0.05$), whereas no significant differences were detected among the other ones ($p > 0.05$). The corresponding samples in 2020 presented higher charges, with mesophilic bacteria ranging from 3.7 to 7.8 Log cfu/g (Fig. 7.11B). Eletta campana and Fibranova were less contaminated than the other varieties ($p < 0.001$), with a mean load of 3.8 ± 0.1 Log cfu/g and 5.7 ± 0.0 Log cfu/g, respectively. CS and Bernabeo presented the highest load (7.9 ± 0.5 and 7.8 ± 0.0 Log cfu/g, respectively; $p < 0.01$) In 2021, the corresponding samples presented charges of TMA ranging from 4.9 to 7.0 Log cfu/g, and no significant differences were found between varieties ($p > 0.05$) (Fig.7.11C).

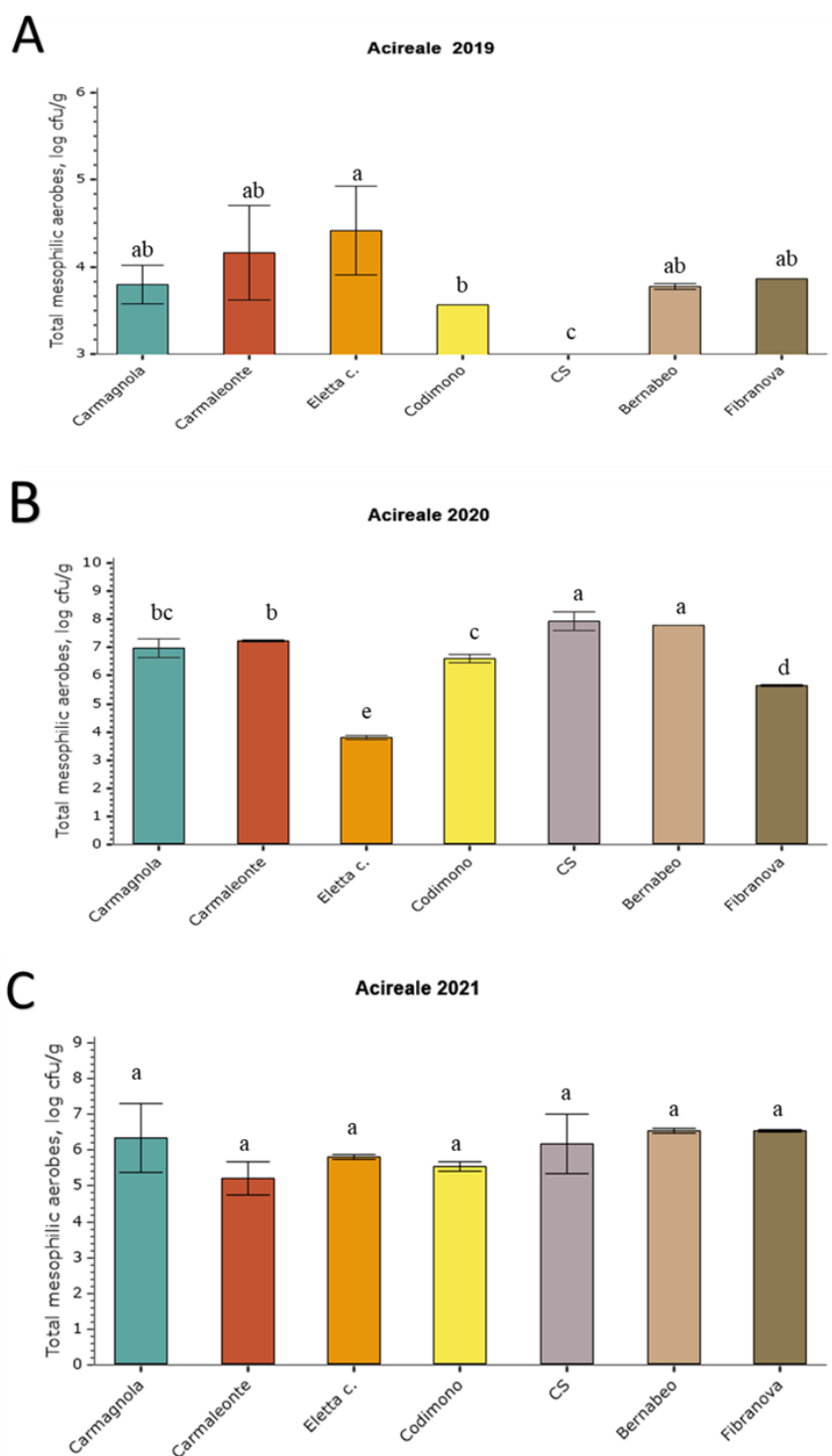


Figure 7.11 Total mesophilic aerobes bacteria, counted on CSBDA plates, in the hemp varieties grown in Acireale, for three years, (A) 2019, (B) 2020, (C) 2021. The reported values are means $\pm$ SD, $n = 2$. In each panel means with the same letter are not significantly different ($P > 0.05$, one-way ANOVA with Tukey post-hoc test).

C. sativa samples of the same varieties cultivated in Rovigo were also analyzed. In the 2019 harvest, the TMA concentration ranged between 5.6 and 7.3 Log cfu/g (Figure 7.12A). The varieties Carmagnola, Carmaleonte, Eletta campana, and Codimono showed significantly higher values than CS, Bernabeo, and Fibranova (on average 6.9 and 5.8 Log cfu/g, respectively).

The hemp varieties collected in 2020 showed a range of TMA between 6.7 and 9.1 Log cfu/g (fig. 7.12B), with Carmagnola and Bernabeo presenting the highest and the lowest levels of contamination, respectively. The corresponding samples in 2021 showed lower charges of TMA ranging from 5.8 to 6.8 Log cfu/g (fig 7.12C), with Codimono at the highest levels of contamination (6.8 ± 0.1 Log cfu/g; $p < 0.05$).

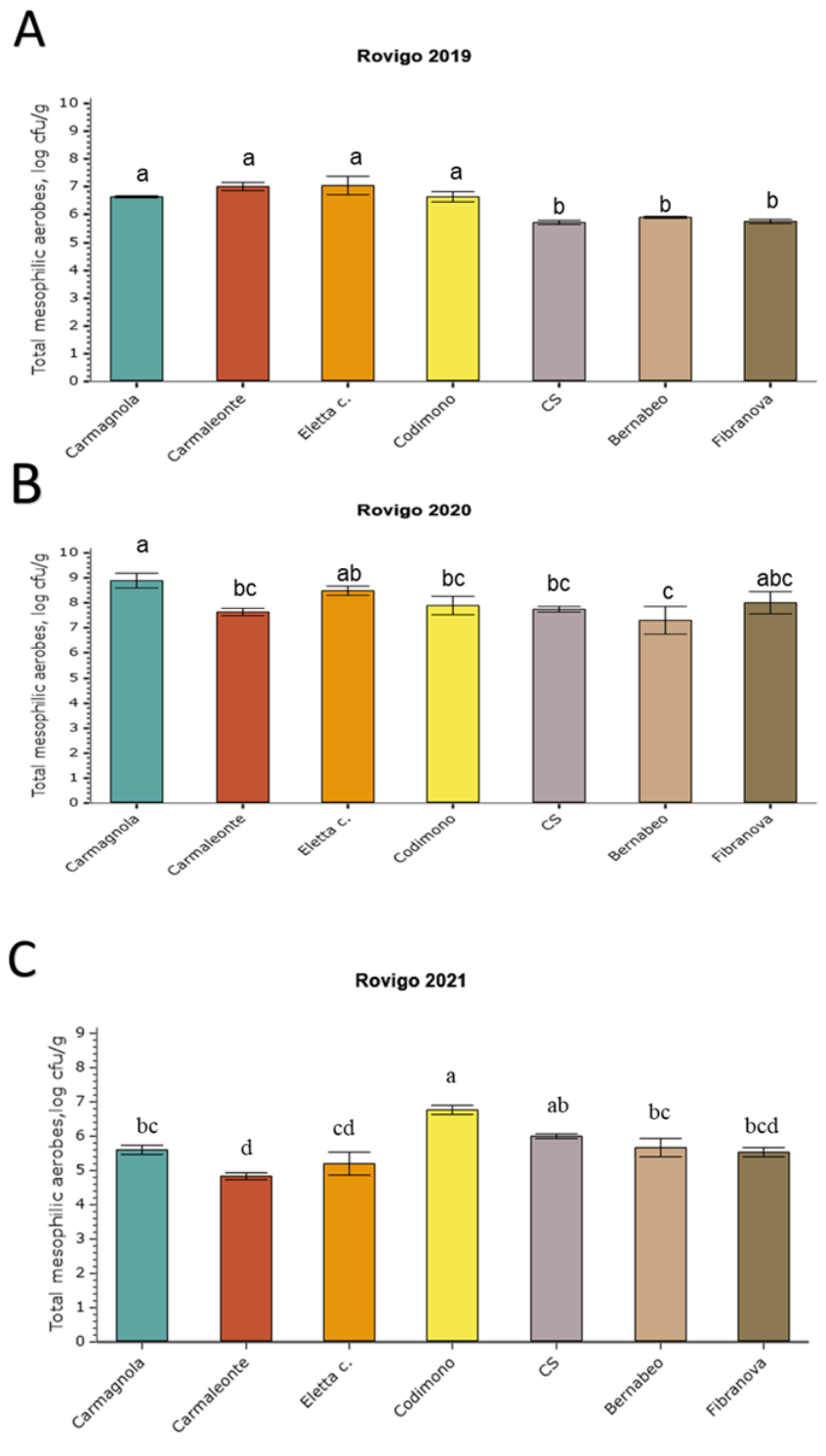


Figure 5.12 Total mesophilic aerobes bacteria, counted on CSBDA plates, in the hemp varieties grown in Rovigo, for three years, (A) 2019, (B) 2020, (C) 2021. The reported values are means $\pm$ SD, $n = 2$. In each panel means with the same letter are not significantly different ($P > 0.05$, one-way ANOVA with Tukey post-hoc test).

Comparing the single variety in the three years of studies from Acireale (Fig. 7.12a), the load of TMA of Carmagnola and Carmaleonte were significantly higher in 2020 than 2019 ($p < 0.05$) while no significant differences of contamination were found in 2021 compared to 2019 and 2020 ($p > 0.05$). The CS variety was more contaminated in 2020 and 2021 than 2019 ($p < 0.01$), no significant differences were observed between 2020 and 2021. Bernabeo, Codimono and Fibranova presented the lowest contamination in 2019 and the highest level of contamination in 2020 ($p < 0.05$). Eletta campana showed higher levels of contamination in 2021 than 2020 ($p < 0.05$), while 2019 did not have significant differences of contamination compared to 2020 and 2021 ($p > 0.05$).

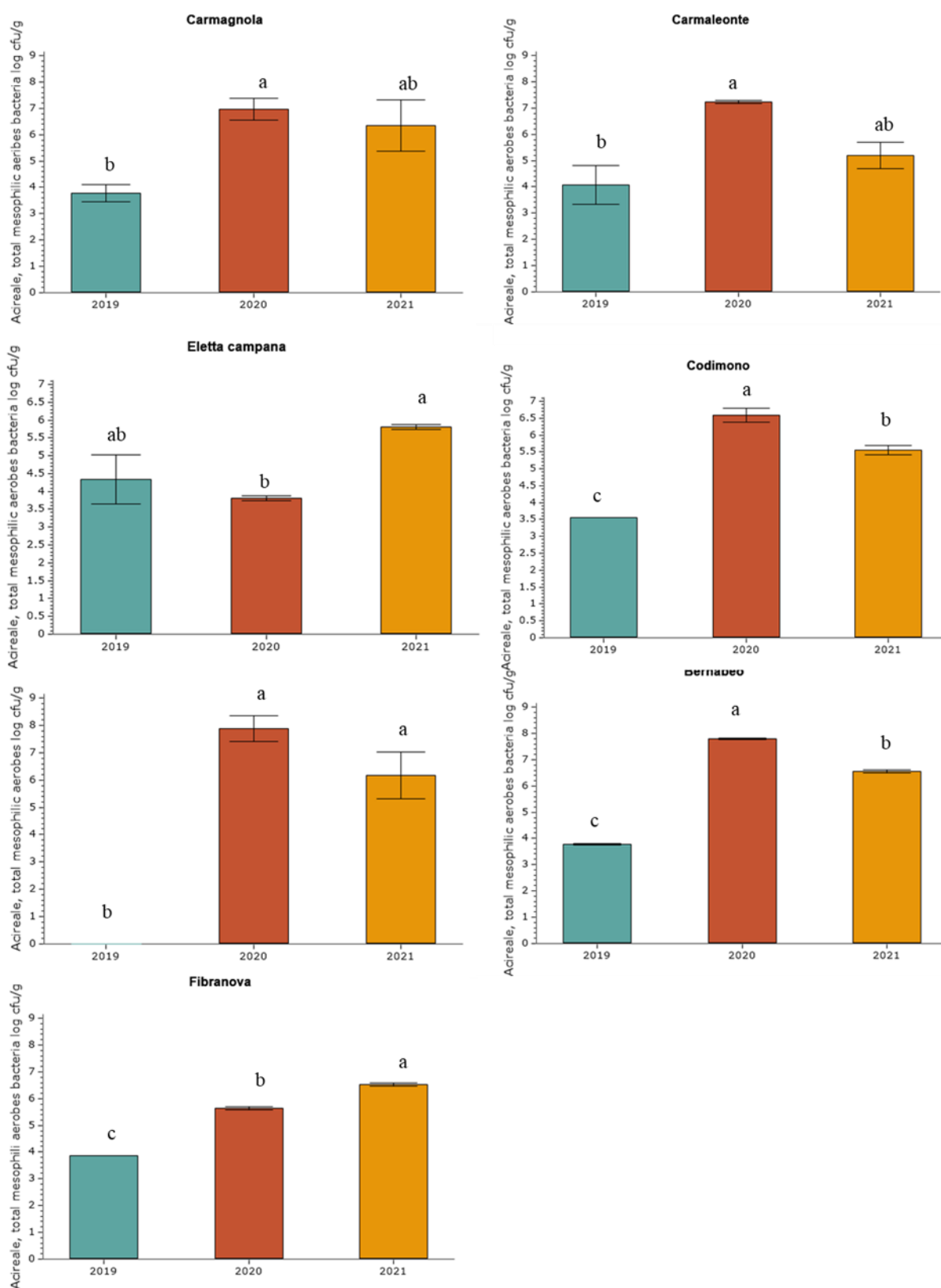


Figure 7.12a Total mesophilic aerobes bacteria, counted on CSBDA plates, in the hemp varieties grown in Acireale, compared in the three years. The reported values are means $\pm$ SD, $n = 2$. In each panel means with the same letter are not significantly different ($P > 0.05$, one-way ANOVA with Tukey post-hoc test).

On the other site, comparing each hemp variety cultivated in Rovigo in the three years (Fig. 7.12b), Carmagnola showed higher level of contamination in 2020 than 2019 and 2021 ($p < 0.05$), no significant differences of contamination were observed between 2019 and 2021 ($p > 0.05$). Carmaleonte was less contaminated in 2021 than 2019 and 2020 ($p < 0.01$), no significant differences were observed between 2019 and 2020 ($p > 0.05$). Eletta campana, was highly contaminated in 2020 than 2021 ($p < 0.01$), and lower levels of contamination were detected in 2021 than 2019 ($p < 0.05$). In Codimono and Bernabeo no significant differences were observed in the three years ($p > 0.05$). The contamination of CS, and Fibranova were higher in 2020 than 2019 and 2021 ($p < 0.01$), while no significant differences were found between 2019 and 2021 ($p > 0.05$).

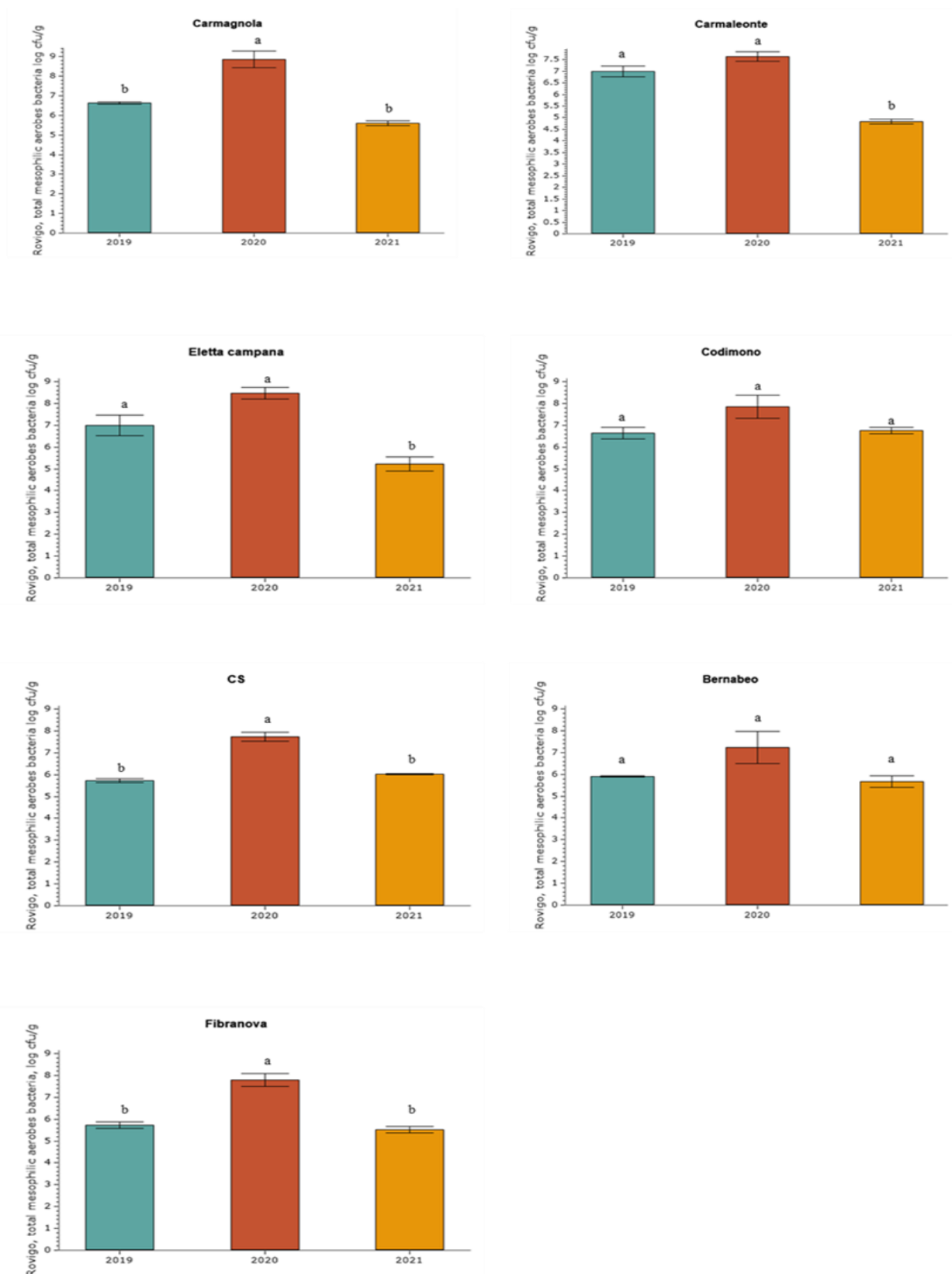


Figure 7.12b Total mesophilic aerobes bacteria, counted on CSBDA plates, in the hemp varieties grown in Rovigo, compared in the three years. The reported values are means $\pm$ SD, $n = 2$. In each panel means with the same letter are not significantly different ($P > 0.05$, one-way ANOVA with Tukey post-hoc test).

7.4.2. Yeast and moulds

The total load of yeasts and moulds (Y&M) was evaluated by enumeration on SDA plates supplemented with antibiotics. In 2019, the concentration of Y&M in the seven hemp varieties from Acireale was in the range from 2.3 and 2.9 Log cfu/g, without significant differences (Figure 7.13A; $p > 0.05$), with the exception of Carmagnola and Fibranova, where fungi were not detected ($< 10^2$ cfu/g).

In samples harvested in 2020, the load of Y&M was generally higher than in 2019, ranging between 1.4 and 4.2 Log cfu/g (Fig 7.13B). Codimono (1.4 Log cfu/g) was significantly less contaminated than Carmagnola, Carmaleonte, Bernabeo, and Fibranova varieties, and presented a similar charge of Eletta campana and CS ($p > 0.05$). The corresponding samples harvested in 2021 showed a contamination of Y&M ranging from 2.7 to 3.4 cfu/g, and no significant differences were found between varieties ($p > 0.05$) (Fig. 7.13C).

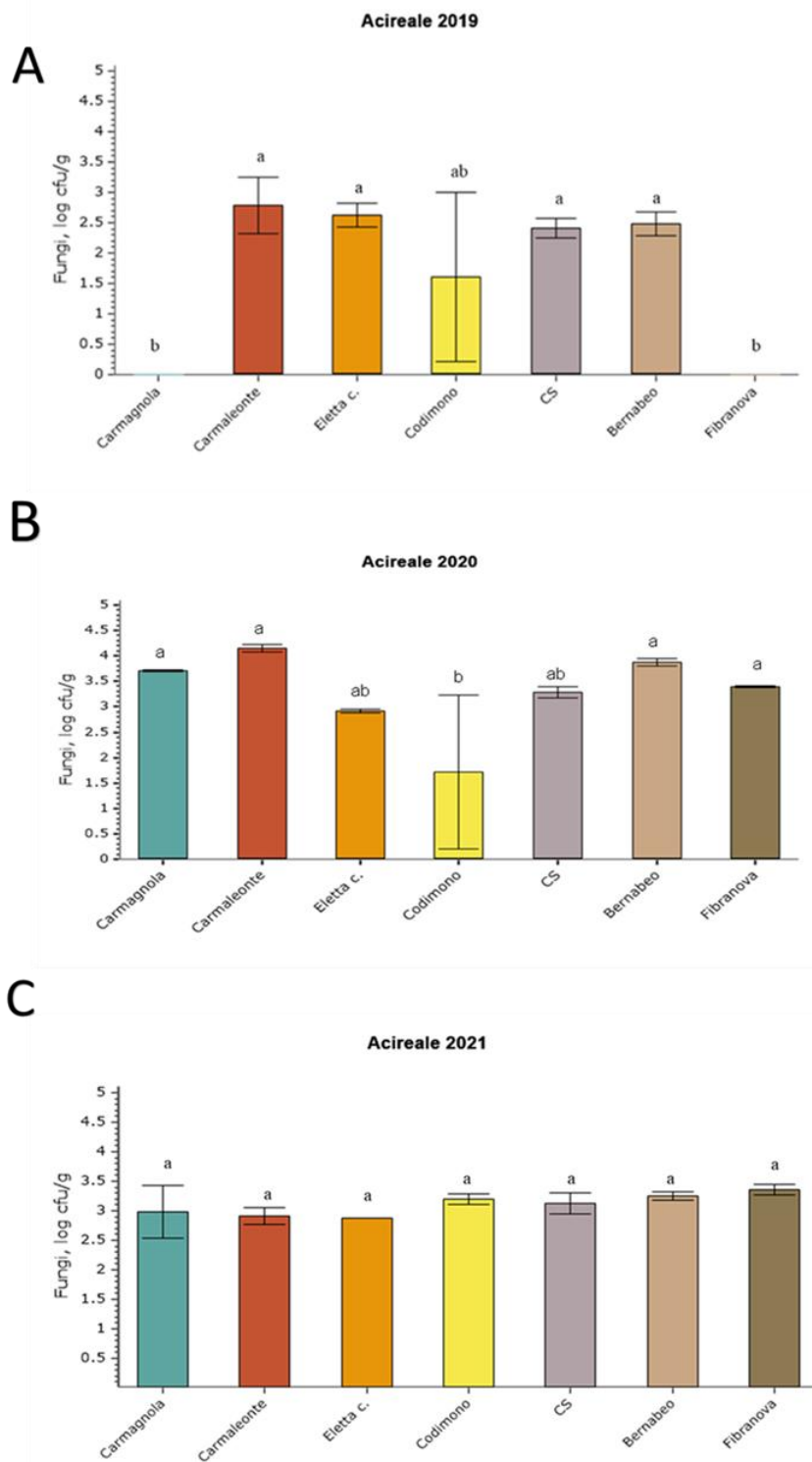


Figure 7.13 Yeasts and moulds, counted on SDA + antibiotics plates, in the hemp varieties grown in Acireale, for three years, (A) 2019, (B) 2020, (C) 2021. The reported values are means $\pm$ SD, $n = 2$. In each panel means with the same letter are not significantly different ($P > 0.05$, one-way ANOVA with Tukey post-hoc test).

The hemp varieties harvested in Rovigo in 2019 showed a concentration of yeasts and moulds ranging between $1.5 \cdot 10^3$ and $3 \cdot 10^6$ cfu/g (Figure 7.14A). The charge of Eletta campana (5.5 ± 1.4 Log cfu/g) was significantly higher than Codimono, CS, and Fibranova (3.8 ± 0.3 Log cfu/g; 3.4 ± 0.1 Log cfu/g; and 3.4 ± 0.1 Log cfu/g, respectively; $p < 0.05$).

On the other side, varieties harvested in Rovigo in 2020 presented a load of yeasts and moulds between $2.8 \cdot 10^3$ and $1 \cdot 10^6$ cfu/g (fig 7.14B). Bernabeo and Fibranova (mean of 5.9 ± 0.1 Log cfu/g and 5.6 ± 0.1 Log cfu/g) were significantly more contaminated than Carmagnola, Carmaleonte, Eletta campana, Codimono and CS (4.4 ± 0.4 Log cfu/g; 4.9 ± 0.3 Log cfu/g; 3.5 ± 0.0 Log cfu/g; 4.2 ± 0.1 Log cfu/g; and 4.4 ± 0.0 Log cfu/g, respectively; $p < 0.01$). In contrast, load of Eletta campana was lower than other varieties ($p < 0.001$).

The corresponding samples in 2021 showed lower contaminations of yeasts and moulds ranging from $1 \cdot 10^4$ to $4.1 \cdot 10^5$ cfu/g (Fig. 7.14C), with Carmagnola, Codimono and Fibranova presented the highest levels of contamination (5.2 ± 0.5 Log cfu/g; 5.2 ± 0.0 Log cfu/g; 5.3 ± 0.5 Log cfu/g, respectively; $p < 0.05$). In contrast, Eletta campana presented the lowest levels of contamination (4.0 ± 0.0 Log cfu/g; $p < 0.05$).

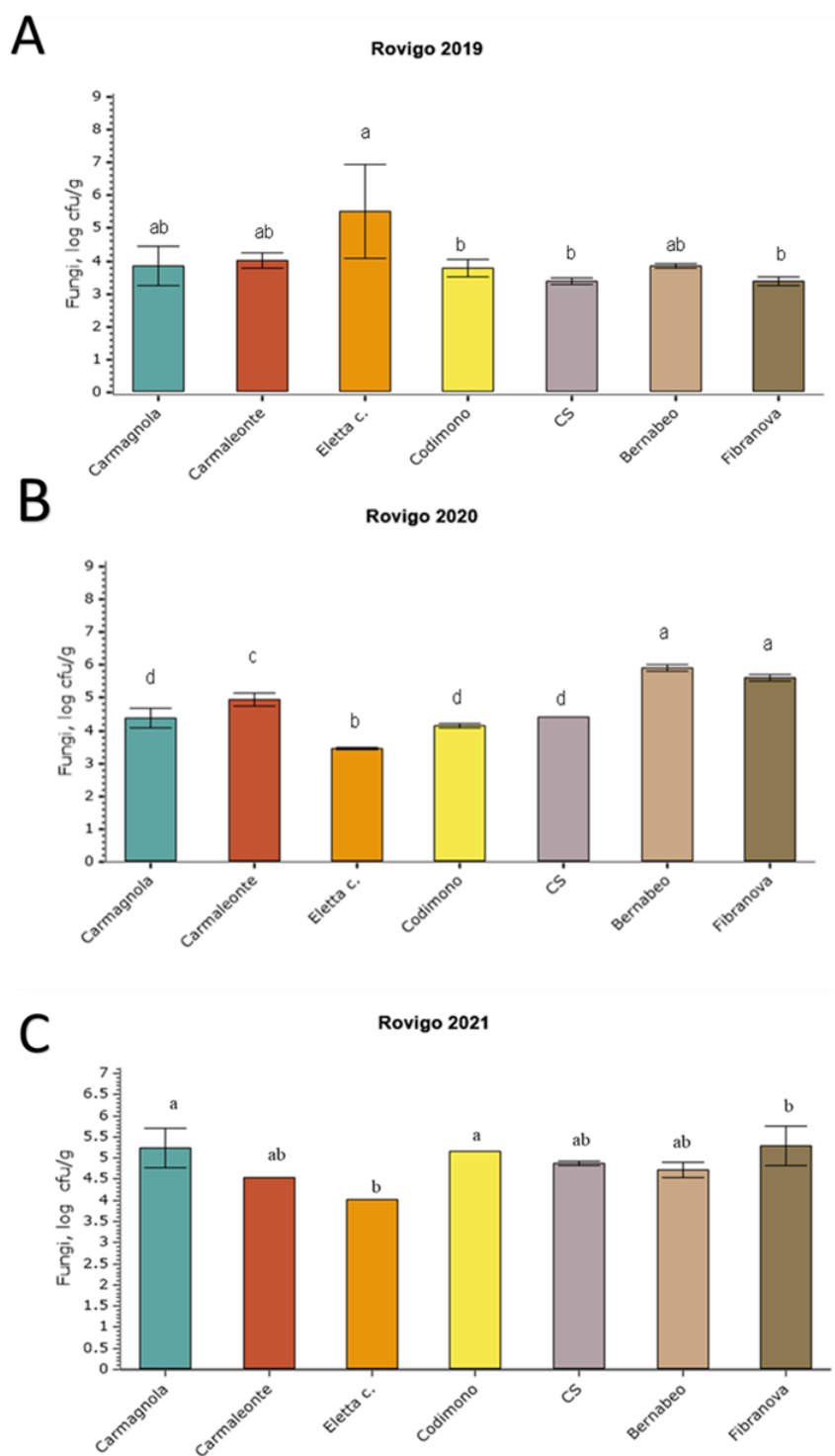


Figure 7.14 Yeasts and moulds, counted on SDA + antibiotics plates, in the hemp varieties grown in Rovigo, for three years, (A) 2019, (B) 2020, (C) 2021. The reported values are means $\pm$ SD, $n = 2$. In each panel means with the same letter are not significantly different ($P > 0.05$, one-way ANOVA with Tukey post-hoc test).

Compering each variety from Acireale collected in the three years of the study (Fig. 7.14a), the charge of Y&M in Carmagnola, Carmaleonte, Eletta campana, Codimono, and CS did not present significant differences during the three years. Bernabeo was more contaminated in 2020 than 2019 ($p < 0.05$), no significant differences were observed between 2019 and 2021 and between 2020 and 2021 ($p > 0.05$). Fibranova showed the lowest contamination in 2019 ($p < 0.001$), in contrast no significant differences were detected between 2020 and 2021 ($p > 0.05$).

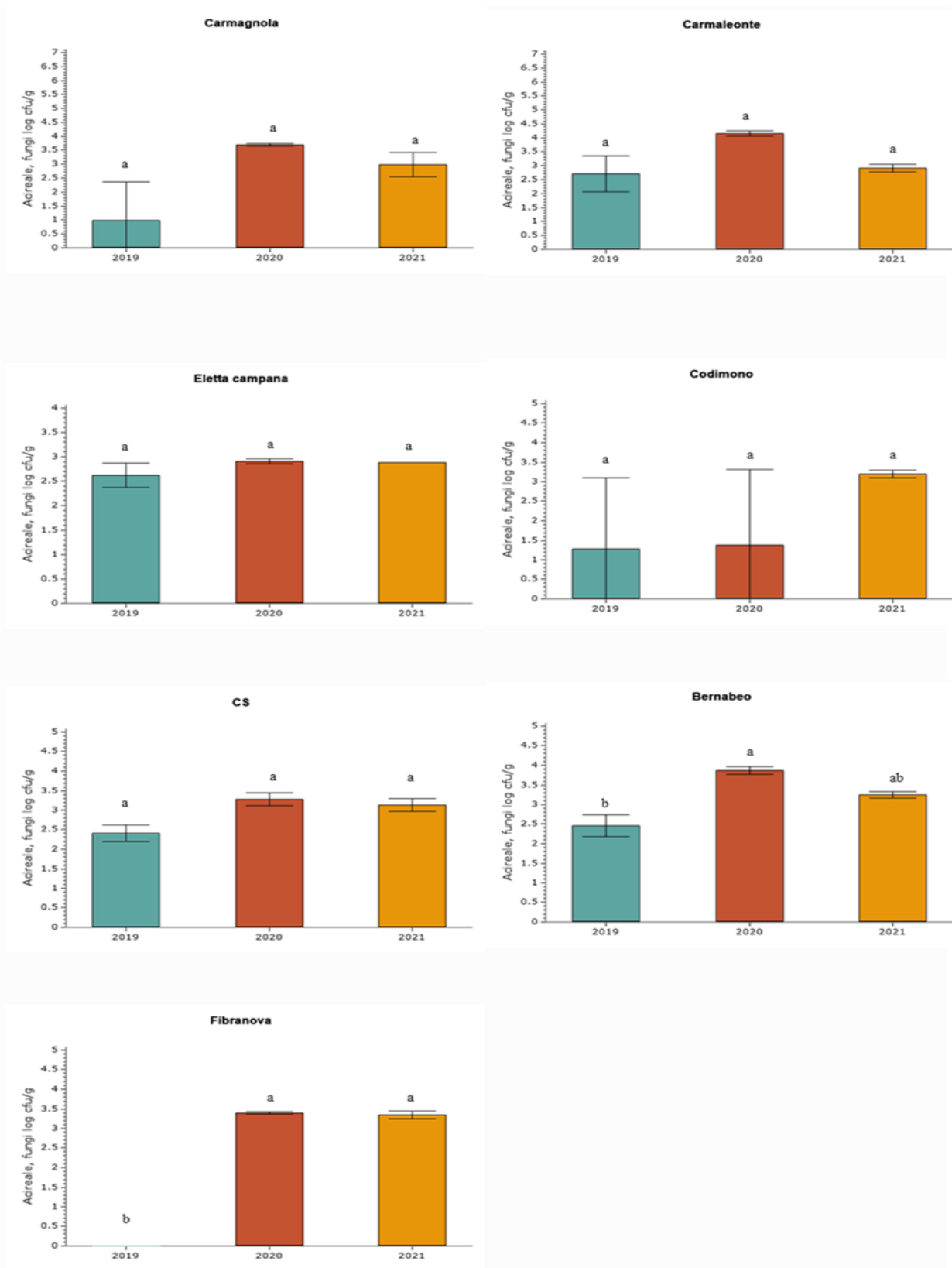


Figure 7.14a Yeasts and moulds, counted on SDA + antibiotics plates, in the hemp varieties grown in Acireale, compared in the three years. The reported values are means $\pm$ SD, $n = 2$. In each panel means with the same letter are not significantly different ($P > 0.05$, one-way ANOVA with Tukey post-hoc test).

Looking at Rovigo site, comparing each variety during the three years (Fig. 7.14b), Carmagnola, Carmaleonte, Eletta campana did not present significant differences among the sampled times ($p > 0.05$). Codimono was highly contaminated in 2021 than 2019 ($p < 0.05$), no significant differences were found between 2019 and 2020 and between 2020 and 2021 ($p > 0.05$). CS showed a Y&M contamination increasing during the time laps of the study ($p < 0.05$). In contrast, Bernabeo showed the highest charge in 2020 ($p < 0.01$) and the lowest charge was detected in 2019 ($p < 0.05$). Finally, Fibranova was less contaminated in 2019 than 2020 and 2021 ($p < 0.05$).

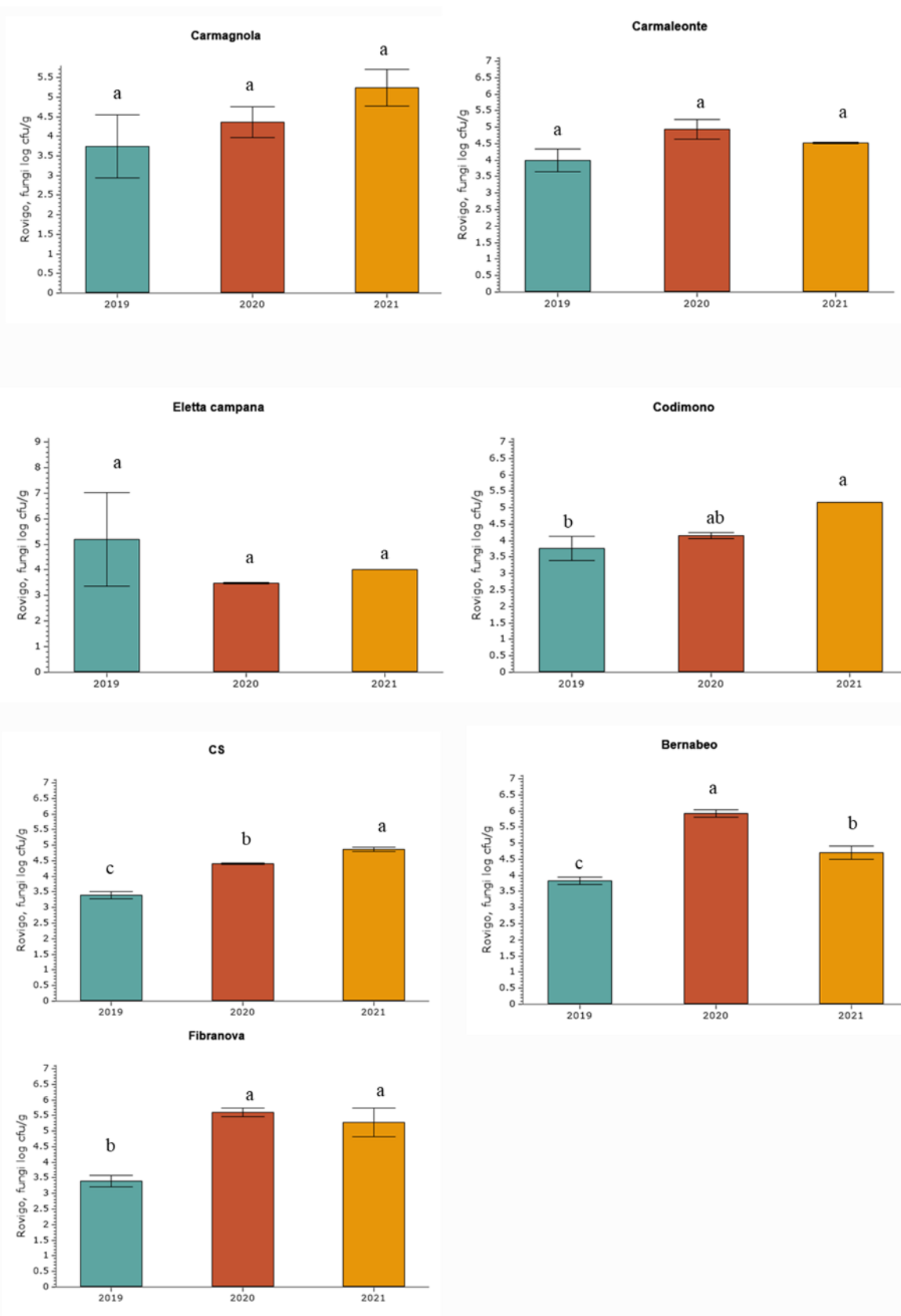


Figure 7.14b Yeasts and moulds, counted on SDA + antibiotics plates, in the hemp varieties grown in Rovigo, compared in the three years. The reported values are means $\pm$ SD, $n = 2$. In each panel means with the same letter are not significantly different ($P > 0.05$, one-way ANOVA with Tukey post-hoc test).

7.4.3. Enterobacteria

Contamination by Enterobacteriaceae was evaluated through enrichment cultures in EBM and estimation of the most probable number. In 2019, all the hemp varieties from the Acireale site (Table 20), showed a concentration of Enterobacteriaceae lower than 100 cfu/g, except for Carmagnola with an enterobacteria load comprised between 100 and 1000 cfu/g. Carmaleonte, Codimono, and Fibranova contained less than 10 cfu/g. In 2020, the concentration observed was always higher than 1000 cfu/g, regardless of the variety. In 2021, the enterobacteria load was comprised between 100 and 1000 cfu/g, except for Codimomo and Fibranova which presented a concentration higher than 1000 cfu/g. In samples from Rovigo site, the Enterobacteriaceae concentration was always $> 10^3$ cfu/g regardless of the hemp variety and the harvest year.

7.4.4. *E. coli* and *Salmonella*

The presence or absence of *E. coli* and *Salmonella* spp. was evaluated in one gram of sample by enrichment in MacConkey and RVSEB broth, respectively. *E. coli* was observed in samples from all the harvest years, in good agreement with the concomitant presence of a high load of total Enterobacteriaceae. In 2019, in samples from both Acireale and Rovigo, only Carmagnola, Carmaleonte, and CS varieties were contaminated with *E. coli*. In 2020, the varieties Bernabeo and CS were positive to *E. coli* from both the two production sites. In 2021, all samples from Rovigo contain *E. coli* as well as the majority of those from Acireale, except for Bernabeo and Eletta campana.

Salmonella spp. was sporadically detected in the hemp inflorescences. The samples collected in 2019 and 2021 from both Acireale and Rovigo were not contaminated at all (Table 20). In 2020, the varieties Carmagnola, Carmaleonte, Eletta campana, and Fibranova from Rovigo showed positive samples as well as samples of Carmaleonte and CS harvested in Acireale.

Table 20. Presence or absence of 7 varieties from Acireale and Rovigo harvested in the three years

	Acireale			Rovigo		
	Enterobacteria	<i>E.coli</i>	<i>Salmonella</i>	Enterobacteria	<i>E.coli</i>	<i>Salmonella</i>
2019						
Carmagnola	<1000 e >100	±	-	>1000	±	-
Carmaleonte	<10	-	-	>1000	-	-
Eletta C.	<100 e >10	±	-	>1000	±	-
Codimono	<10	-	-	>1000	-	-
CS	<100 e >10	+	-	>1000	+	-
Bernabeo	<100 e >10	-	-	>1000	-	-
Fibranova	<10	-	-	>1000	-	-
2020						
Carmagnola	>1000	-	-	>1000	-	±
Carmaleonte	>1000	-	+	>1000	-	±
Eletta C.	>1000	-	-	>1000	-	±
Codimono	>1000	-	-	>1000	-	-
CS	>1000	+	±	>1000	+	-
Bernabeo	>1000	+	-	>1000	+	-
Fibranova	>1000	-	-	>1000	-	±
2021						
Carmagnola	<1000 e >100	±	-	>1000	+	-
Carmaleonte	<1000 e >100	+	-	>1000	+	-
Eletta C.	<1000 e >100	-	-	>1000	+	-
Codimono	>1000	+	-	>1000	+	-
CS	<1000 e >100	+	-	>1000	+	-
Bernabeo	<1000 e >100	-	-	>1000	+	-
Fibranova	>1000	+	-	>1000	+	-

7.5. DISCUSSION

In recent years the cultivation of industrial hemp has been attracting great interest, with more than 25,000 products currently on the market and forecasts of strong growth on the global market. Unlike medical *Cannabis*, whose THC content varies from 1 to 22%, industrial cultivars are devoid of psychotropic properties (THC <0.02%) while representing a highly varied source of multiple phytocannabinoid and terpene bioactive molecules (Schlottenhofer and Yuan, 2017; Bonini et al., 2018). Thanks to the potential use of all the plant fractions in different application fields (Andre et al., 2016), to the modest water needs, and to the strong adaptability even to marginal land, industrial hemp represents a model of multipurpose, eco-compatible, and highly sustainable farming. The recent re-evaluation of the positive effects on human health of other non-psychotropic phytocannabinoids, especially CBDA (most common phytocannabinoid in industrial hemp inflorescences) and CBD (derived from CBDA decarboxylation), led to a reassessment of the interest for inflorescences of industrial cultivars, scarcely used until a few years ago, that recently attracted attention as sources of such molecules and were at the center of an unexpected growing business.

Hemp plants and derived products are subjected to microbial contamination caused by molds or bacteria, usually occurring during growth or subsequent processing and handling. Most molds are relatively harmless and are widespread in the environment. Some can threaten health if inhaled, especially for immunosuppressed individuals, such as some cannabis users for medical purposes (Dryburgh et al., 2018).

To assess the potential risk of Cannabis exploitation, we determined the microbial charge of *Canabis sativa* flower heads, addressed to the production of nutraceutical and cosmeceutical products. Most microorganisms that have been isolated are not pathogens, and are common members of the hemp plant microbiota (Taghinasab and Jabaji, 2020). The microbial endophytes component in industrial hemp cultivars is rich of Pseudomonadaceae (*Cellvibrio* sp.), Oxalobacteraceae, Xanthomonadaceae, Actinomycetales, Sphingobacteriales, *Pantoea* spp., Staphylococcaceae, and Bacillaceae. Recurrent fungi are *Aureobasidium* spp., *Alternaria* spp., *Cochliobolus* spp., and *Cladosporium* spp. (Scott et al., 2018; Montoya et al., 2020; Taghinasab and Jabaji, 2020). These endophytic microorganisms exert a role in defence of the plant against the attack of pathogens.

The concentration of total mesophilic aerobes bacteria (TMA) in hemp for medical use according to the proposed directives in Canada and the Netherlands must be less than 10² cfu/g

(McPartland and McKernan, 2017). In the samples that were analyzed in this study, TMA concentration varied consistently among samples, reaching values up to 10^9 cfu/g in 2020 and 10^7 cfu/g in 2019 and 2021, many orders of magnitude higher than those expected. This suggests the presence of a relevant load of bacteria that was affected by farming place and harvest year and that was not easily predictable.

According to literature, the microorganisms contaminating hemp plants that caused the greatest damage belonged to Enterobacteriaceae. In some cultivars harvested in Massachusetts, *Acinetobacter baumannii*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Ralstonia pickettii*, *Salmonella enterica*, *Stenotrophomonas maltophilia*, and *Clostridium botulinum* were detected (Mckernan et al., 2016). On the other hand, *Klebsiella*, *Streptococcus*, and *Enterobacter* are minor contaminants (Ungerleider et al., 1962).

The level of enterobacteria found in the hemp varieties from Acireale and Rovigo was usually higher than 10^3 cfu/g, except for some cultivars harvested in 2019, regardless of the site of cultivation. Samples have been also investigated for the presence/absence of other pathogens such as *Salmonella* spp. and *E. coli*. *Salmonella* can cause diarrhea, enteritis, as well as fever, and vomiting (Daley et al., 2013), representing a substantial risk for inflorescences utilization. In the analyzed hemp biomass, the presence of *Salmonella* spp. was sporadic, being observed only in a few samples from the 2020 harvest. As reported in the literature, also pathogenic *E. coli* may cause relevant symptoms in hemp consumers (Kim, 2016; Crofts et al., 2018; Valilis et al., 2018). *E. coli* contamination in the harvested samples was more frequent than *Salmonella*, and strictly correlated to the presence of a high load of Enterobacteriaceae. The presence of *E. coli* was reported in samples of the three years. As a whole, the presence of enterobacteria, *E. coli*, and *Salmonella* spp. in the analyzed samples poses a threat to the health of potential inflorescences utilizes.

The literature reported that bacterial contamination could occur in hemp, but it is less relevant than that caused by fungi (Mckernan et al., 2016). Conversely, in the samples analyzed in this study a higher load of bacteria has been detected, compared to fungi and molds. The process of drying after the harvest of hemp biomass represents a critical phase for bacterial containment and requires more attention in future studies to verify the reduction of water activity. Likely, the presence of residual humidity or the conduction of a too slow drying process favored the growth of bacteria. The presence of a predominant fungal population may be characteristic of better-dried hemp samples, in which the growth of xerophilic fungi is not entirely compromised.

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PUBLICATIONS

1. Candeliere, F., Raimondi, S., **Spampinato, G.**, Tay, M. Y. F., Amaretti, A., Schlundt, J., Rossi, M. (2020). Draft Genome Sequences of 12 *Leuconostoc carnosum* Strains Isolated from Cooked Ham Packaged in a Modified Atmosphere and from Fresh Sausages. **MICROBIOLOGY RESOURCE ANNOUNCEMENTS**, 9(2). doi.org/10.1128/MRA.01247-19
2. Raimondi, S., **Spampinato, G.**, Macavei, L. I., Lugli, L., Candeliere, F., Rossi, M., Maistrello, L., Amaretti, A. (2020). Effect of Rearing Temperature on Growth and Microbiota Composition of *Hermetia illucens*. **MICROORGANISMS**, 8(6), 902 doi.org/10.3390/microorganisms8060902
3. Candeliere, F., Raimondi, S., **Spampinato, G.**, Tay, M. Y. F., Amaretti, A., Schlundt, J., Rossi, M. (2021). Comparative genomics of *Leuconostoc carnosum*. **FRONTIERS IN MICROBIOLOGY**. doi.org/10.3389/fmicb.2020.605127
4. Raimondi, S., **Spampinato, G.**, Candeliere, F., Amaretti, A., Brun, P., Castagliuolo, I., Rossi, M. (2021). Phenotypic Traits and Immunomodulatory Properties of *Leuconostoc carnosum* Isolated From Meat Products. **FRONTIERS IN MICROBIOLOGY**. doi.org/10.3389/fmicb.2021.730827
5. **Spampinato, G.**, Candeliere, F., Amaretti, A., Licciardello, F., Rossi, M., Raimondi, S. (2022). Microbiota Survey of Sliced Cooked Ham During the Secondary Shelf Life. Submitted to **FRONTIERS IN MICROBIOLOGY** accepted the 25/01/22. In press.

REPORT OF ACTIVITIES

1st Year

PARTICIPATION TO THE SCHOOL COURSES

Date: 12/11/18; 13/11/2018

Title/subject: Medicinal plants as sources of potential pharmaceuticals products and Cameroonian wild mushrooms from the Bamoun Region: Nutritional and Medicinal Properties

Date: 14/01/19; 15/01/19; 16/01/19; 28/01/19; 29/01/19; 30/01/19.

Title/subject: Scientific English

Date: 29/05/19

Title/subject: The model organism *Saccharomyces cerevisiae* mitochondrial inheritance as case study

Date: 12/06/19; 14/06/19; 17/06/19

Title/subject: Applications of multivariate analysis in the agri-food context

Date: 04/07/19

Title/subject: Management and exploitation of bioresources the fundamental roles of the microbial culture collections

Date: 18/09/19

Title/subject: Microbial biotechnologies for Biorefineries

Date: 02/10/19

Title/subject: Model Plants I

Date: 04/10/19

Title/subject: Animal models

Date: 09/10/19

Title/subject: Model plants II

PARTICIPATION TO SYMPOSIA

Date: 25-26-27 September 2019

Location: Catania

Title: 5TH International Conference on Microbial Diversity 2019

2nd Year

PARTICIPATION TO THE SCHOOL COURSES

Date: 07 08/10/20

Title/subject: Infrared Spectroscopy

PERIODS OF STUDY ABROAD

Where: Teagasc, Cork

How long: 08/03/2020 - 30/08/2020

3rd Year

PARTICIPATION TO THE SCHOOL COURSES

Date: 15-16-17-18/12/20

Title/subject: Scientific writing and publishing

Date: 5/02/21 and 12/02/21

Title/subject: Microbial ecology

PARTICIPATION TO OTHER COURSES

Date 13/10/21

Location: meeting-online

Title/subject: Novel technologies for dairy product innovation