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Lactococcus garvieae and *Morganella morganii*: two bacterial models to study quality and safety of fish products

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Abstract

ABSTRACT

This PhD thesis research project aims to improve the current systems of management and control of food safety, focusing the attention on the study of two contaminating emerging pathogens in fish products: *Lactococcus garvieae*, the etiological agent of a hemorrhagic septicemia in aquaculture sector, and *Morganella morganii*, the most active histamine-producing species.

In the first part of the research the molecular characterization and genetic polymorphism of *L. garvieae* strains coming from different food niches were carried out revealing an early separation of *L. garvieae* population into two independent genetic lineages. Subsequent phylogenetic analyses through a MLST approach confirms the significant genetic heterogeneity in this species, the separation into two principal evolution lineages, and the presence of a third lineage totally separated from the others, that could be the ancestor branch of the main lineages. Genome sequencing of representative selected strains and the comparison with other *L. garvieae* sequences already present in databases, confirmed the subdivision in the three different subgroups. Within each subgroup the nucleotide similarity was 99%, while comparing subgroups the similarity decreased (94-95%). Moreover, this preliminary comparison reveals that the core genome of *L. garvieae* contains, in additions to genes codifying for the main metabolic pathways, genes encoding putative virulence factors, such as hemolysins and adhesin. Genes related to carbohydrate fermentation (such as lactose and sucrose) seemed part of the dispensable genome, as well as the gene cluster related to the capsule formation. Experiments carried out to evaluate the expression of virulence-related genes in representative *L garvieae* strains, indicated an adaptive response to different environmental conditions, specific at the strain-level.

Regarding the second part of the research, we started exploring the bacterial population of fresh tuna samples and evaluated the histamine content. Despite low levels of histamine were detected (<10 ppm), many samples showed high total viable counts after temperature abuse. The 10% of the 141 enteric bacteria isolated from samples were identified by 16S rDNA sequence analysis, as *M. morganii*, the most active histamine-producing species. Subsequently a new quantitative PCR assay for the detection of *M. morganii* in potentially contaminated fish products before histamine accumulates was developed, with a detection limit of 0.563 pg of pure DNA, corresponding to DNA extracted from approximately nine cells. To improve the knowledge at genome level of the histidine decarboxylase of *M. morganii* (*hdc* gene), a region of 7681 bp was sequenced. Results showed the presence of genes encoding two putative histidine/histamine antiporters upstream and downstream the *hdc* gene, followed by an histidyl-tRNA synthetase. Gene expression analysis indicates that in *M. morganii hdc* was inducible, showing the highest expression under acidic conditions, mainly in presence of histidine supplementation. Finally, with the aim to prepare a significant collection of different *M. morganii* strains from different ecological niches, we compared several clinical biotypes, in addition to strains isolated from fish through REP and RAPD PCR analysis. The cluster analysis confirmed the existence of two main branches related to the habitat of isolation, which were separated at a level of similarity of 19%.

RIASSUNT

***Lactococcus garvieae* e *Morganella morganii*: due modelli batterici per studiare la qualità e la sicurezza dei prodotti ittici.**

Con questo progetto di tesi di dottorato si intende contribuire ad implementare gli attuali sistemi di gestione e controllo della salubrità dei prodotti alimentari, focalizzando l'attenzione sullo studio di due potenziali patogeni emergenti dei prodotti ittici: *Lactococcus garvieae*, un batterio che causa setticemia nei pesci d'acquacoltura e *Morganella morganii* la specie maggior produttrice d'istamina.

Nella prima parte della ricerca sono stati effettuati esperimenti di caratterizzazione molecolare e polimorfismo genetico di ceppi di *L. garvieae* provenienti da diverse nicchie alimentari, rivelando una separazione della popolazione di *L. garvieae* in due indipendenti linee evolutive. Successive verifiche filogenetiche tramite analisi MLST hanno confermato la notevole eterogeneità genetica di questa specie, la separazione in due principali linee evolutive, e la presenza di un terzo raggruppamento totalmente separato dagli altri, che potrebbe rappresentare il ramo antenato delle due linee principali. Il sequenziamento dei genomi di ceppi rappresentativi della collezione e il confronto con altre sequenze di *L. garvieae* già presenti in banca dati, hanno confermato la suddivisione in tre differenti sottogruppi. Ciascun sottogruppo mostrava un'omologia nucleotidica del 99%, mentre comparando i raggruppamenti, la similarità era inferiore (94-95%). Questo confronto preliminare indicava anche che il "core genome" di *L. garvieae* contiene, oltre ai geni codificanti per le principali vie metaboliche, anche geni codificanti putativi fattori di virulenza, come emolisine e adesine. I geni relativi alla fermentazione di carboidrati come il lattosio e il saccarosio sembravano invece parte del "dispensable genome", così come il cluster genico relativo alla formazione di una capsula. Esperimenti condotti per valutare l'espressione di geni correlati alla virulenza in ceppi rappresentativi della collezione di *L. garvieae*, hanno evidenziato una risposta adattativa in differenti condizioni ambientali, specifica a livello di ceppo.

Per quanto riguarda la seconda parte della ricerca, si è iniziato ad esplorare la popolazione batterica di campioni di tonno fresco e valutarne il contenuto di istamina. Nonostante i bassi livelli di istamina detectati (<10 ppm), molti campioni mostravano elevate cariche batteriche vitali, dopo un abuso di temperatura. Il 10% dei 141 enterobatteri isolati dai campioni di tonno sono stati identificati, mediante analisi della sequenza del gene 16S rDNA, come *M. morganii*, la specie maggior produttrice d'istamina. Successivamente è stata sviluppata una nuova metodica di PCR quantitativa per la rilevazione di *M. morganii* in prodotti ittici potenzialmente contaminati, avente un limite di rilevamento di 0,563 pg di DNA puro, corrispondente al DNA estratto da circa nove cellule. Per migliorare la conoscenza genetica del gene codificante per l'istidina decarbossilasi (*hdc*) di *M. morganii*, è stata sequenziata una regione di 7681 bp. I risultati mostravano la presenza di due geni codificanti per due putativi istidina / istamina antiporters a monte e a valle del gene *hdc*, seguiti da un istidil-tRNA sintetasi. L'analisi dei livelli di espressione genica ha evidenziato che in *M. morganii* il gene *hdc* è inducibile e mostra la più alta espressione in condizioni di acidità, soprattutto in presenza di istidina. Infine, allo scopo di creare una collezione significativa di ceppi di *M. morganii* provenienti da diverse nicchie ecologiche, sono stati confrontati diversi biotipi clinici, con i ceppi isolati da pesce, attraverso esperimenti di REP e RAPD PCR. Le analisi hanno confermato l'esistenza di due gruppi principali in funzione dell'habitat di isolamento, che mostravano un livello di somiglianza del 19%.

PREFACE

Fish is one of the most important foods for its high nutritional value. In fact, it contains a great source of high-quality protein (a portion of 150 g of fish provides about 50–60 % of the daily protein requirements for an adult), vitamins and healthful polyunsaturated fatty acids.

The demands of a growing population, particularly in poorer countries, now far outstrip the sustainable yield of the seas. At the same time as fishing has become more industrialised (considerably influenced by globalization in food systems and by innovations and improvements in processing, transportation, distribution, marketing and food science and technology), and wild fish stocks increasingly depleted, aquaculture production—fish and shellfish farming—has grown rapidly to address the shortfalls in capture fisheries (Tidwell & Allan, 2001). During the last two decades the general picture of international trade in seafood (fish and shellfish harvested from capture fisheries and aquaculture production) has changed in size and structure. Seafood is traded within a porous and complex international market that is changing at an impressive pace and scale as compared to other commodities (FAO, 2010). Seafood contributes at least 15% of average animal protein consumption to 2.9 billion people (United Nations Department of Economic and Social Affairs/Population Division, 2009). Capture fisheries and aquaculture supplied the world with about 148 million tonnes of fish in 2010. World aquaculture production attained another all-time high in 2010, at 60 million tonnes (FAO, 2012).

Fish it is a very high perishable product with a shelf life limited by microbial growth, due to the presence of native and external bacteria. The producers demand, and, above all the request of the consumers, to have a foodstuff with good quality and safe, is very strong, in relation with the diffusion of new food models that provide the consume of raw fish, typical of Japanese tradition (FAO, 2005). This implies a strong regulatory action combined with a depth control along the entire food chain to ensure the respect of quality and safety parameters of the final product, at local level in the free trade (Ababouch, 2006).

Health hazards in seafood can be roughly classified into two categories: pre-harvest and post-harvest risks. Pre-harvest risks are those originating in the environment (Opara et al., 2007). These include chemicals accumulated in fish tissues in the environment, ''natural'' toxins, and pathogenic microorganisms. Regarding biological components, it is an accepted fact that fish products are in general considered to be safe but they can cause illness when fished from contaminated waters. *Vibrio* spp., *Listeria monocytogenes* and *Clostridium botulinum* can be part of the native microbial population in fish (Huss et al., 2003). Other micro-organisms and viral components, among which *Salmonella* spp., *Shigella* spp., *Escherichia coli*, Norwalk and Calicivirus, may arise from the aquatic contaminated environment (Martinez et al., 2005). Further microbiological risks are related to the stages of transport, processing and distribution of fish, above all when the food is processed under conditions that do not respect hygiene standards, or as a result of insufficient maintenance of the cold chain (De Silva, 2011). Moreover, fish can contain potentially harmful substances, such as biogenic amines, low molecular weight organic bases, formed by microbial decarboxylation of amino acids (Visciano et al., 2012). Biogenic amines are characterized by vasoactive and psychoactive properties that make them able to interact with human metabolism. The most known intoxication is due to histamine, that causes the "scombroid fish poisoning" (Prester, 2011).

The true incidence of diseases transmitted by foods is not known. Seafood is at least as significant a source of food-borne illness as other animal protein products. The source of risk in seafood is, on the whole, however, quite different from other protein commodities and requires a significantly richer regulatory approach to ensure effective risk mitigation.

Despite numerous efforts to improve the quality of the production process of fish, and to develop more suitable methods for assessing product freshness, quality and healthfulness, there are still some limitations, typically microbiological, related to several factors, among which the incidence and

diffusion in fish of new microbial species, potential emerging pathogens, or new biovars of known bacterial species (Mor-Mur & Yuste, 2010). In this context, the traditional food inspection systems are insufficient, because knowledge of the emerging pathogens is still incomplete. To determine the concept of healthy food, it is crucially important expend efforts for a comprehensive study of new emerging pathogens present in food products for which studies related to their dissemination, impact and role are insufficient.

This PhD thesis reports the study of two bacterial species, *Lactococcus garvieae* and *Morganella morganii*, that represent models of contaminating emerging pathogens in fish products (Ferrario, 2010, 2011, 2012).

During the last decades, one of the most remarkable problems in aquaculture is the contamination with a pathogen typical of the fisheries sector, *Lactococcus garvieae*, the etiological agent of an hemorrhagic septicemia, known as Lactococcosis (Vendrell et al., 2006), which causes important economic losses, considering the high rate of mortality in fishes, reaching 70%. Despite numerous studies to reduce such contamination, little is yet known about the origin of contamination, the factors related to pathogenicity and the possible interventions to control the development of this emerging pathogen. As *Lactococcus garvieae* has been associated to human infections and founded in other food sectors, overall in dairy products (Fortina et al., 2003), it could represent a potential emerging pathogen for human. *L. garvieae* could be a model of Gram positive potential dangerous bacteria, whose knowledge deserves to be thoroughly investigated.

In the fishing sector problems are related to find quality and freshness indicators and are more complex because due to type of fish, fishing areas, water contamination and type of fishing. Tuna trades is strongly increased in the last few years (primarily related to its raw consumption) and its healthiness is linked to the limitation of bacterial spoilage, and to the absence of specific pathogenic bacteria that can decarboxylate amino acids producing biogenic amines. In particular, among these compounds histamine, can cause the scombroid syndrome after consumption of spoiled altered fish products (Economou et al., 2007). Among enterobacteria implicated in the tuna fish contamination, *Morganella morganii* could be considered a species model: it is one of the most detected species and one of the main histamine producer bacteria (Kim et al, 2003). Until today, there are not methods that can significantly evaluate food freshness and healthiness compatible with the processing time and product marketing. Moreover, for this pathogen the dynamic of growth in terms of intrinsic and extrinsic parameters is not yet investigated.

Preface

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1. AQUACULTURE SECTOR MODEL: *Lactococcus garvieae*

1. Aquaculture sector model: *Lactococcus garvieae*

1.1. STATE OF THE ART

1.1.1. Problems in the aquaculture sector

With the rapid and ongoing development of aquaculture sector, the need of a better health management and improved capacity to face new health challenges, is required.

During the last three decades, aquaculture has expanded based on movements of live aquatic animals and animal products. New outlooks and directions have accelerated the accidental spread and incursion of diseases into new populations and geographic regions, for example, through movements of hatchery produced stocks, new species for culture, enhancement and development of the ornamental fish trade (Vendrell et al., 2006).

Transmission of pathogenic microorganisms to fish is mainly due to horizontal mechanisms. Among co-habiting fish, direct transmission of pathogens through the water is the principal way, especially when macroscopic and microscopic injuries occur on fish body surfaces, becoming potential portals of entry for bacterial pathogens (Amaro et al., 1995). Several causes of fish infections could be identified. Viral diseases cause serious problems in every aspect of aquaculture, with severe economic losses. Infectious hematopoietic necrosis, infectious pancreatic necrosis, and viral hemorrhagic septicemia are the major viral diseases of trout and salmon (Meyer, 1991).

Bacterial pathogens probably cause more disease problems overall than all other causes combined. Septicemias, cutaneous lesions, and destruction of the shell are among the manifestations of bacterial infections. Gram-negative bacilli are the most frequent cause of bacterial diseases in finfish: furunculosis (*Aeromonas salmonicida*), bacterial hemorrhagic septicemia (*A. hydrophila*), vibriosis (*Vibrio* spp.), enteric redmouth disease (*Yersinia ruckerii*) and others caused by *Pseudomonas* spp., *Flavobacterium* sp. and *Pasteurella* sp. Only a few of Gram-positive affect fish, among which *Lactococcus garvieae*. Since its first occurrence in yellowtail (Kusuda et al., 1976), this species has been considered an important etiological agent of Lactococcosis in various fish species, including yellowtail (*Seriola quinqueradiata*) and rainbow trout (*Onchorrrynchus mykiss*).

1.1.2. Lactococcosis in aquaculture sector

Lactococcosis is an emerging pathology affecting a variety of fish species all over the world, and it is particularly devastating in the freshwater culture of salmonid fish and marine cultured species, especially when water temperature increases over 16 °C in summer months. Pathogenicity and clinical signs of Lactococcosis has been defined as a hyper acute and hemorrhagic septicemia (Bercovier et al., 1997). It has been observed in several experimental tests that incubation period of the disease is very short and the microorganism acts with high virulence: symptoms are visible 2–3 days post-inoculation (Itami et al., 1996).

L. garvieae is the causative agent of this infection: outbreaks affecting rainbow trout have been reported in several countries, such as Australia, South Africa, Japan, Taiwan, England and countries of the Mediterranean area. The losses produced can exceed approximately 50–80% of the total production (Ghittino & Prearo, 1992).

A strain of *L. garvieae*, previously described as *Streptococcus garvieae*, was originally isolated in the United Kingdom from a mastitic udder and was identified as the reference strain (ATCC43921) for this species (Collins et al., 1983). In 1991, it was proposed as a new species, *Enterococcus seriolicida*, in order to bring together a number of Gram-positive isolates recovered from Lactococcosis outbreaks in Japanese yellowtail (*Seriola quinqueradiata*) over the preceding 20 years (Kusuda et al., 1991). The first outbreak of Lactococcosis in rainbow trout from Spanish fish farms occurred in 1988 (Palacios et al., 1993). Initially, the agent was described as an *Enterococcus* sp. until it was definitely identified as *L. garvieae*, which showed biochemical characteristics very similar to *E. seriolicida* (Prieta et al., 1993). In the following years, *L. garvieae* has been isolated from several septicemic processes in fish. Phenotypical and molecular taxonomic studies confirmed the same agent as *E. seriolicida*. This species was reclassified as a junior synonym of *L. garvieae* (Eldar et al., 1996). From this time on, Lactococcosis was progressively spread in several countries and aquatic organisms. It was isolated in Italy (Ghittino & Prearo, 1992), Australia and South Africa (Carson et al., 1993), Taiwan (Chen et al.,

1. Aquaculture sector model: *Lactococcus garvieae*

2001) and in Turkey (Diler et al., 2002).

1.1.3. Who is *Lactococcus garvieae*?

L. garvieae is a facultative anaerobic, non-motile, non-spore forming, Gram positive coccoid, occurring in pairs and short chains. It produces α-hemolysis on blood agar. This species is able to grow in a wide range of temperature (4–45 °C), with optimal growth at 37°C, in presence of NaCl, bile salts, at pH 9.6 and in methylene blue-milk (Vendrell et al., 2006).

Several studies have been carried out to demonstrate the phenotypic heterogeneity of *L. garvieae* isolated from fish. The researchers have proposed biotyping schemes based on phenotypic characteristics (acidification of tagatose, ribose and sucrose) and have recognized three biotypes of *L. garvieae* (Ravelo et al., 2001). Some of these biochemical characteristics can differ depending on the strain. Over the last few years, the use of more discriminating molecular methods permitted to correlate the high degree of biodiversity to the geographical origin of the pathogen (Vela et al., 2000; Schmidtke & Carson, 2003; Eyngor et al., 2004; Michel et al., 2007).

The host range of *L. garvieae* is not limited to aquatic species. The pathogen has been found in domestic animals such as cows, from subclinical intramammary infections (Devriese et al., 1999) cat and dog tonsils (Pot et al., 1996), bullfrog (Mendoza et al., 2012). In addition, the agent has been isolated from humans as an opportunistic pathogen (Fihman et al., 2006; Russo et al., 2012) as well as a component of the intestinal biota (Kubota et al., 2010).

L. garvieae can also be isolated from several food matrices. One of the most important sources are artisanal cheeses made with goat and cow raw milk, where it is present sometimes as a major component (Fortina et al., 2003; Foschino et al., 2006; Fernández et al., 2010). *L. garvieae* has also been isolated from vegetables (Kawanishi et al., 2007) and meat (Santos et al., 2005). Despite the growing importance of *L. garvieae* in human and veterinary medicine and in the food sector, little research data are available on this pathogen in matrices other than fish products.

Various typing methods have been applied for studying genetic variation among *L. garvieae* populations, including random amplified polymorphic DNA (RAPD)-PCR (Ravelo et al., 2003), Sau-PCR and amplified fragment length polymorphism (AFLP) (Foschino et al., 2008), restriction fragment length polymorphism (RFLP) ribotyping and serotyping (Eyngor et al., 2004), pulsed-field gel electrophoresis (PFGE) typing (Kawanishi et al., 2005) and amplified rRNA gene restriction analysis (ARDRA) (Michel et al., 2007).

Another important differentiation in this species is the utilization of lactose (Lac) related to the presence of the phospho-β-galactosidase (*lac*G) gene. This tool was considered as marker for distinguishing between fish (Lac⁻/*lac*G⁻) and dairy isolates (Lac⁺/*lac*G⁺) of *L. garvieae* (Fortina et al., 2009). Recently, *L. garvieae* has been isolated from different ecological niches (Aguado-Urda et al., 2010); in these strains, *lacG* seemed heterogeneously scattered.

1.1.4. Antigenic characteristics of *L. garvieae*

Many studies are available about the pathogenicity of *L. garvieae* in fish. The serological characterization of this bacterium has been carried out in several studies identifying an antigen denominated KG from the cellular wall. There are two antigenic types of this pathogen, KG⁺ and KG⁻ type strains. The KG⁺ type strain agglutinates with antiserum of KG 7409 strain and the KG⁻ type strain possesses a specific envelope-like substance, which inhibits agglutination with anti-KG 7409 serum. The KG⁻ type strain was more virulent than the KG⁺ in causing infection in yellowtail (Kitao, 1983).

Barnes and Ellis (2004) compared 17 geographically distinct strains of *L. garvieae* isolated from diseased fish, using antiserum raised against the pathogen in rainbow trout. These results indicate that *L. garvieae* can be distinguished serologically into three different serotypes: an European capsulated serotype, a Japanese capsulated serotype and a non-capsulated serotype from both regions.

Several virulence experiments have been performed in order to determine the possible correlation between pathogenicity of *L. garvieae* for rainbow trout and the two antigenic profiles (KG⁻ and KG⁺). Its virulence seems to be associated with the ability to produce capsule, but it is unknown which

factors can influence the synthesis of this extramural layer, nor the genetic parameters that are responsible of the specific phenotypical expression. The results revealed that capsulated strains (KG⁻) were more virulent than non-capsulated (KG⁺) (Barnes et al., 2002).

1.1.5. Genome analysis of *L. garvieae*

With the increasing availability of genome sequences, comparison studies on the genetic content of different bacteria are easily feasible. Recently the complete genome sequence of 7 *L. garvieae* becomes available in databases: four clinical strains of *L. garvieae* isolated from yellowtail and trout (8831, Lg2, ATCC 49156, UNIUD074) (Aguado-Urda et al., 2011a; Morita et al., 2011; Reimundo et al., 2011), one human clinical strain (21881) (Aguado-Urda et al., 2011b), one dairy strain (IPLA 31405) (Floréz et al., 2012) and one strain isolated from mallard duck intestines (DCC 43) (Gabrielsen et al., 2012) have been published.

The analysis of the genome of a fish-isolated virulent strain (Morita et al., 2011) revealed the presence of a capsule gene cluster composed of 15 genes that was not found in a no virulent strain used for comparison. However, strains isolated from human with endocarditis, seemed able to show pathogenicity in human, even if they did not show the presence of genes encoding extracellular envelope. The comparative genome analysis of these strains indicates that the presence of a capsule gene cluster is not the only factor leads pathogenicity. Other potential virulence factors can be found in *L. garvieae* genome, such as adhesion surface proteins, haemolysins, NADH oxidase, resistance to antibiotics, that could play a more important role in the pathogenicity both in humans and animals. The real involvement of these genes in virulence remains to be elucidated as well as their association to strains isolated in specific environments and their specificity at the strain-level.

Moreover another study on comparative genome analysis (Miyauchi et al., 2012) did not permit to group strains associated with infection both in animals and humans. The data obtained suggest that this species is characterized by a high degree of genetic diversity, that also comprises distribution and functionality of genes related to virulence factors. Several information about preliminary genome comparison is reported in Table 1.1.

1. Aquaculture sector model: *Lactococcus garvieae*

Table 1.1. *L. garvieae* strains whose genome sequences are available in databases, with summaries information and references.

Strain	Lenght (bp)	N° of contigs	Genes	tRNA	% GC	Isolation	AN
ATCC 49156	1950135	1	1947	79	38.8	Diseased yellowtail	AP009332
Morita et al., 2011							
LG2	1963964	1	1968	62	38.8	Diseased yellowtail	AP009333
Morita et al., 2011							
UNIUD 074	2171966 (draft)	25	2101	>60	38.7	Diseased Rainbow trout	AFHF0000000
Reimundo et al., 2011							
21881	2164557 (draft)	91	2141	42	37.9	Human blood	AFCF00000000
Aguado-Urda et al., 2011b							
8831	2087226 (draft)	87	1969	48	38.0	Diseased Rainbow trout	AFCD00000000
Aguado-Urda et al., 2011a							
DCC 43	2244387 (draft)	68	2227	54	37.8	Muller duck intestines	AMQ501
Gabrielsen et al., 2012							

AN: Accession number

1. Aquaculture sector model: *Lactococcus garvieae*

1.2. AIM OF THE STUDY

This PhD thesis research project aims to improve the current systems of management and control of food safety, focusing the attention on the study of *Lactococcus garvieae* that represent a valid model of contaminating emerging pathogens in fish products.

This PhD thesis objectives are:

- Creation of a strains collection, through the research and specific selection of *L. garvieae* from different food habitat;

- Molecular typing, by using method that can assemble isolates in function of their source of isolation, to underline a specific molecular fingerprint for every ecotype and gave a detailed framework about the degree of biodiversity in the species;

- Research of extracromosomal DNA molecules;

- Genome sequencing of representative *L. garvieae* strains coming from different food matrices;

- Study of specific genes or genetic cluster encoding virulence and pathogenicity factors;

- Phylogenetic study through the research and the sequencing of particular conserved genes, called molecular clocks, and the use of bioinformatics software to building detailed phylogenetic tree.

1. Aquaculture sector model: *Lactococcus garvieae*

1.3. MATERIALS AND METHODS

1.3.1. Sampling, selection, culturing and identification of *L. garvieae* isolates

A total of 40 food samples purchased in different supermarkets or collected from different mills of Northern Italy, were analysed for the presence of *L. garvieae*. The products consisted of raw meat (beef, poultry and turkey), processed meat products (salami and sausages), several vegetables and cereals (wheat flour, wheat bran, soybean and barley) (Table 1.2). All samples were aseptically collected and transported in isothermal boxes to the laboratory. For *L. garvieae* isolation, food samples (25 g) were enriched in 1:9 (w/w) M17 broth (Difco, Detroit, Michigan, USA) supplemented with 1g/L glucose (M17-G) at 37 °C for 24 h. After enrichment, total DNA was extracted as reported below and the presence of *L. garvieae* was established through a species-specific PCR assay, as reported by Zlotkin et al. (1998). For each sample positive to the species-specific amplification, *L. garvieae* selection was attempted on M17-G agar. Appropriate dilutions in 0.1% peptone solution of positive enriched cultures were plated and incubated at 37 °C for 24 h; after incubation randomly selected colonies were purified and then submitted to taxonomic identification, as above reported. The new isolates were studied in comparison with representative dairy and fish *L. garvieae* ecotypes previously isolated. Stock cultures were maintained at -80 °C in M17-G with 15% glycerol.

1.3.2. DNA extraction and quantification

For strains grown in pure culture, DNA was extracted as previously described by Borgo et al. (2012). Plasmid DNA was extracted as previously described (Fortina et al., 2009). For the extraction of DNA from food samples, the Ultraclean™ Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA) was used according to the manufacturer's instructions. The concentration and purity of the DNAs were determined with a UV-Vis spectrophotometer (SmartSpec™ Plus, Biorad, Milan, Italy).

1.3.3. PCR amplification

PCRs were performed in a 25 μL reaction mixture contained 100 ng of bacterial DNA, 2.5 μL of 10x reaction buffer (Fermentas, Vilnius, Lithuania), 200 μM of each dNTP, 2.5 mM $MgCl_2$, 0.5 μM of each primer, and 0.5 U of *Taq* polymerase (Fermentas). After incubation for 2 min at 94 °C, samples were subjected to 35 cycles of 60 s at the annealing temperature (Table 1.3), followed by 1 min at 72 °C; the reaction was completed by 7 min at 72 °C and kept at 4 °C using a PCR-Mastercycler 96 (Eppendorf, Hamburg, Germany). Amplification products were separated on a 1.5% agarose gel stained with ethidium bromide in 1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) buffer and photographed.

1.3.4. Multi-locus restriction typing (MLRT)

Internal fragments of seven loci, *atp*A (α-subunit of ATP synthase), *tuf* (bacterial elongation factor EF-Tu), *dlt*A (D-alanine-D-alanyl carrier protein ligase), *als* (α-acetolactate synthase), *gap*C (glyceraldehyde-3-phosphate dehydrogenase), *gal*P (galactose permease), *lac*G (phospho-β-galactosidase) were amplified using primers and conditions reported in Table 1.3. Products from each amplified locus were tested to select a suitable discriminating restriction enzyme, i.e. a panel of two or five enzymes that cut frequently along each of the amplified fragments was examined in order to clearly identify allelic variations. Overnight restriction digestion was carried out at 37 °C in a 20 μL reaction mixture containing 4 μL of the PCR product, 2 μL of 10x incubation buffer and 10 U of each enzyme (Amersham Pharmacia Biotech., Milan, Italy). Restriction digests were subsequently analyzed by agarose electrophoresis (2% agarose gel).

1.3.5. Genomic fingerprinting of *L. garvieae* strains

L. garvieae strains were typed by combined analysis of repetitive element (REP) typing using primers (GTG)$_5$ and BOXA1R (Versalovic et al., 1994; De Urraza et al., 2000) and random amplification of polymorphic DNA-PCR (RAPD) typing with primer M13 (Rossetti & Giraffa, 2005). The PCR products were analyzed by electrophoresis and photographed as reported above. The digitized image

1. Aquaculture sector model: *Lactococcus garvieae*

was analyzed and processed using the Gel Compare software (Applied Maths, Kortrijk, Belgium). The value for the reproducibility of the assay, evaluated by analysis of repeated DNA extracts of representative strains was greater than 93%.

1.3.6. PCR-ribotyping

The genomic DNA of *L. garvieae* strains (ca 10 µg) was digested by incubation with 30 U of *Pst*I endonuclease (Fermentas) according to manufacturer's instruction. A 20 µL aliquot of the digestion mixture was combined with 5 µL of loading buffer and the preparation was electrophoresed on 0.8% (w/v) agarose gel at 100 V for 2 h. DNA fragments were subsequently transferred to a nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) by Southern blot. Hybridization was performed at 60 °C using the 16S rDNA of *L. garvieae* DSM 20684[T]. The probe was amplified using the universal primers: 16SF and 16SR. PCR cycle was 2 min at 94 °C, then 5 cycles of 45 s at 94 °C, 45 s at 50 °C, 1 min at 72 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min at 72 °C, with a 7 min final extension at 72 °C. The DIG DNA Labeling and Detection kit (Roche) was used for digoxigenin labeling of the 1513 bp fragment. Prehybridization and hybridization overnight were performed in 50% (w/v) formamide at 42 °C and stringency washes in 0.1x SSC buffer at 65 °C (10x SSC is 1.5 M NaCl, 150 mM sodium citrate). The probe was detected by chemiluminescent detection using CSPD (Roche), and the signals were visualized by exposure to X-ray film for 2 h.

1.3.7. Data analysis

Banding pattern similarity was evaluated by construction of dendrograms using the NTSYSpc software, version 2.11 (Applied Biostatics Inc., NY, USA), employing the Jaccard similarity coefficient. A dendrogram was deduced from a similarity matrix by using the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm. The faithfulness of the cluster analysis was estimated by calculating the cophenetic correlation value for each dendrogram.

1.3.8. Genomes analyses and comparison

Sequencing analyses were performed employing ABI Prism Big Dye Terminator Kit and the reaction products were analyzed with the ABI PrismTM310 DNA Sequencer. A whole-genome shotgun strategy with an Illumina Genome Analyzer Hiseq 1000 was used. Quality-filtered reads were assembled using the Velvet software (version 1.1.04). Open reading frames (ORFs) were predicted using Glimmer 3.02, functional annotation was done by merging the results obtained from the RAST (Rapid Annotation using Subsystem Technology) Server, BLAST, tRNAscan-SE 1.21, and RNAmmer 1.2. Genome alignments were performed using Mauve and the phylogenetic tree obtained was visualized in Splitstree v4.1 (Huson & Bryant, 2006).

1.3.9. Multi Locus Sequence Typing (MLST) loci amplification

Internal fragments of seven loci, *als*, *atpA*, *tuf*, *gapC*, *gyrB*, *rpoC* and *galP* were amplified as reported in section 1.3.3. Nucleo Spin Extract II (Macherey-Nagel GmbH & Co., Düren, Germany) was used to purify PCR products that were sequenced employing ABI Prism Big Dye Terminator Kit (Applied Biosystems, Foster, CA, USA). The reaction products were analyzed with the ABI PrismTM310 DNA Sequencer (Applied Biosystems). Sequence similarities searches were carried out using Basic Local Alignment Search Tool (BLAST, Altschul et al., 1997) on the EMBL/GenBank databases.

1.3.10. MLST Data analysis

Forward and reverse DNA sequences obtained from PCR amplification were trimmed and added with sequences from seven *L. garvieae* genomes deposited in database (8831, 21881, ATCC 49156, LG2, UNIUD074, IPLA 31405 and DCC43). After selecting most polymorphic regions of 800-850 bp, these were analysed using MEGA v5 (Takamura et al., 2011). Isolate dataset creation, arbitrary allele assignation were done using mlstdbNet software. Each unique allelic profile, as defined by the allele numbers of the seven loci, was assigned a Sequence Type (ST) number. The same ST number was used for more than one strain if they shared the same allelic profile. The number of segregating or polymorphic site (S), nucleotide diversity (π), Tajima's D, Fu & Li's D and F were calculated using

1. Aquaculture sector model: *Lactococcus garvieae*

DnaSP v5.10 (Librado & Rozas, 2009). π_{MAX} values were extracted from the squared similarity matrix calculated with the DNADIST program (D option set to "similarity table") in the PHYLIP v.3.69 package. For phylogenetic analysis, concatenated sequences were aligned and analyzed with MEGA v5. Genetic distances were computed by the Kimura two-parameter model, and the phylogenetic tree was constructed using the neighbor-joining method.

Strains relationships were analyzed using eBURST program (Feil et al., 2004) to identify potential clonal complexes and founders. eBURST analysis was performed using the default stringent (conservative) definition. To investigate the population structure, the Clonal Frame method was used. The recombination to mutation rate (r/m) was calculated as reported by Vos and Didelot (2009). For each dataset, two runs of the Clonal Frame MCMC were performed each consisting of 200000 iterations. The first half of the chains was discarded, and the second half was sampled every hundred iterations. Split decomposition trees were constructed with 1000 bootstraps replicates based on parsimony splits as implemented in SplitsTree v4.1. The standardized Index of Association (I^A_S) was calculated using LIAN 3.5, using a Monte Carlo randomization test with 1000 resampling.

1.3.11. Detection of virulence factors

Internal fragments of two loci, *hly* and *fbp*, identified through BLAST and codifying for an haemolysin and a cell wall adhesion protein respectively, were amplified using primers developed in this study and reported in Table 1.3. PCRs and electrophoresis were performed as reported in section 1.3.3.

1.3.12. RNA extraction and rewriting into cDNA

To study differential gene expression in different conditions, selected strains were grown in M17-G, as reference media for all experiments, M17-G with addition of 0.2 % bile salts (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (M17-GBS), Brain Heart Infusion (BHI) broth (Sigma) and Meat Simulation medium (MSM) broth (Verluyten et al., 2003). The exponentially growing cell cultures in M17-G (OD_{600} =0.9) were collected through centrifugation (9600 x *g* at 4 °C for 10 min), washed three times with phosphate saline buffer (PBS), resuspended in sterile water and distributed into the selected pre-warmed media. These conditions were applied for 1 h at 30°C for MSM and at 37°C for BHI and M17-GBS. Samples were taken subsequently, centrifuged and the pellets obtained used for RNA extraction. *L. garvieae* cells were washed three times with PBS buffer and resuspended in 1.5 mL RNase-free water (Mackery-Nagel, Germany). RNA was extracted using NucleoSpin RNA II (Mackery-Nagel), following the manufacturer's instructions. RNA concentration and purity were optically determined using a UV-Vis spectrophotometer (SmartSpecTM Plus, Bio-Rad) and RNA integrity was assessed by electrophoresis. Residual contaminating DNA was hydrolyzed with Dnase1 Rnase-Free Kit (Fermentas) at 37°C for 1h. Subsequently, 1 µg of RNA was rewritten into cDNA using RevertAid First Strand cDNA Syntheis Kit (Fermentas) according to the manufacture's instructions.

1.3.13. RT-PCR

A master mix containing, per sample, 7.5 µL EvaGreen (Bio-Rad), 0.38 µL water and 0,5 µM of each primer, was combined with 7 µL of each cDNA sample. The qPCR temperature procedure was performed according to the manufacture's recommendations and corresponds to an initial cycle of denaturation at 94°C for 5 min, followed by 40 cycles of denaturation 95°C for 30 sec, annealing and extension 60°C for 30 sec. Fluorescence acquisition was done at the end of each amplification. After real time PCR a melting curve analysis was performed by measuring fluorescence during heating from 72°C to 95°C at a transition rate of 0.5°C/s. Amplifications were performed in triplicate.

The *gyr*B gene was used as internal control to which all other virulence gene expression was normalized. The primers used are reported in Table 1.4.

Samples were examined for differences in gene expression using relative quantification in which relevant gene expression is normalized to a housekeeping gene and the relative expression related to specific environment. These values were calculated according with Pfaffl (2001) equations.

1. Aquaculture sector model: *Lactococcus garvieae*

Table 1.2. Prevalence of *L. garvieae* in different food products and strains analyzed in this study.

Origin	n	Frequency	Strains
Mastitic cow	nd	nd	DSM[c] 20684[T]
Raw meat	10	10 (100%)	
Beef	4	4	Bov1, Bov2, Bov3, Bov4
Poultry	2	2	Po1
Turkey	2	2	Tac1, Tac2
Meat products	5	5 (100%)	Sa113[d], Sml1, Sml2, Sml3, Smp1, Smp2, Smp3, Smp4
Vegetables	13	4 (31%)	
Salad	3	1	Ins1,Ins2
Broccoli	4	2	Br1, Br2, Br3, Br4
Celery	2	1	Sed1, Sed2
Cereals	12	1 (8%)	Far1
Dairy products[a]	nd	nd	G18, G20, G27, G32, G36, G6, G8, G03, G07, G9[d], G13, G15, G16, G01
Fish[a, b]	nd	nd	Lg9, Lg19, Lg20, Lg23, Lg28, Lg30, V32, V61, V63, V69, V72, V79

[a] strains previously isolated
[b] strains kindly provided by Dr Prearo (Experimental Institute for Zooprophylaxis, Torino, Italy) and by Dr. Amedeo Manfrin (Venetian Experimental Institute for Zooprophylaxis, National Reference Lab. for Fish Diseases, Legnaro, Italy).
nd=not determined
[c] DSM= Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
[d] synonymus of I113 and TB25 strains reported in Ricci et al., 2012, 2013.

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Table 1.3. PCR primers and conditions used for the detection of genes in *L. garvieae* strains.

Gene /Product	Primer pairs (5′-3′)	Annealing temperature (°C)	Amplicon (bp)
(GTG)$_5$	GTGGTGGTGGTGGTG	42	
BOXA1R	CTACGGCAAGGCGACGCTGACG	48	
M13	GAGGGTGGCGGTTCT	38	
16S	F: AGAGTTTGATCCTGGCTCAG R: CTACGGCTACCTTGTTACGA	Section 1.3.6	1500
hly (haemolysin)	F: ATGGAAAAGCCAGCTTCTCG[a] R: AACCATAGATGGAGAACCAC	56	640
fbp (cell wall adhesion protein)	F: ACAGACTTCCAAAATCCTCA[a] R: TCACCTTTACGACTGAGTTT	54	1236
*atp*A (α-subunit of ATP synthase) (Naser et al., 2005)	F: TAYRTYGGKGAYGGDATYGC R: CCRCGRTTHARYTTHGCYTG	56	1180
tuf (elongation factor EF-Tu) (Ludwig et al., 1993)	F: ATATGCGGCCGCCATYGGHCACGTBGACCA R: AAAATATGCGGCCGCTCNCCNGGCATNACCAT	56	1080
*dlt*A (D-alanine-D-alanyl carrier protein ligase) (Reimundo et al., 2010)	F: ATCCGGAGCAACAAAATACC[a] R: TTACCTCCAAGGACTTGCTT	58	1473
als (α-acetolactate synthase) (Menéndez et al., 2007)	F: ATTCGGCTCAGACTTAGTTG[a] R: TTCAGCTGCTTCAACATCAA	58	1076
*gap*C (glyceraldehyde-3-phosphate dehydrogenase)	F: AAGTTGGTATTAACGGTTTCG[a] R: AAGTGTACGAACGAGGTTAG	56	974
*gal*P (galactose permease) (Fortina et al., 2009)	F: TGGGGAAAATTTAAACCTTGG[a] R: ATCATCAGAACGGCTGGAAG	58	1070
*lac*G (phospho-β-galactosidase) (Fortina et al., 2009)	F: GCTACAGCTGCTTATCAAGC R: AGGATAAATAATCCAATCCCA	58	1014
*gyr*B (DNA gyrase β- subunit)	F: CATGCTGGTGGTAAATTTGG[a] R: GTCATCCATTTCTCCTAAACC	58	1464
*rpo*C (RNA polymerase β'-subunit)	F: TTGGTCCACAAAAGGACTGG[a] R: TCACGTCCTTTTGCTTCCAT	58	1377

[a] this study

1. Aquaculture sector model: *Lactococcus garvieae*

Table 1.4. RT-PCR primers and conditions used for the gene expression experiments, designed for this study.

Gene/product	Primer-pair (5'-3')	Annealing temperature (°C)	Amplicon (bp)
hly	F: TAGCACTTGTTTGGCTTTGTGC R: CCATAGATGGAGAACCACATCA	60	301
fbp	F: CGGTTCGTTCAGGAAGAACATC R: CGGTCATTGCCTACTTGCTCAA	60	181
*gyr*B	F: TGGTAAACTTGCGGACTGCTCT R: TCCACGTTCAAGATTTTACCACG	60	150

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1.4. RESULTS AND DISCUSSIONS

To contribute to the characterization of the natural variability of the species *Lactococcus garvieae*, we evaluated the genetic diversity of a collection of strains isolated from different sources. *L. garvieae* is mainly known for its presence in aquatic environments and as component of milk and many artisanal cheeses. In this work, we studied new isolates from other sources, in order to give a comprehensive indication of the diversity found within the species.

1.4.1. *L. garvieae* diffusion in food matrices

We focused our attention on food matrices not yet or poorly investigated for the presence of *L. garvieae*, particularly, meat, vegetables and cereals. Out of 40 food samples tested, 20 (50%) were found to contain *L. garvieae* (Table 1.2). Raw meat and meat products showed the highest prevalence of contamination with *L. garvieae*: all samples analyzed were positive for the presence of this bacterial species. A high rate of *L. garvieae* was also found in vegetables (31%), while cereals do not seem an important reservoir for the species. From these sources we selected 24 new ecotypes that were studied in comparison with previously isolated dairy and fish ecotypes (Table 1.2). All new isolates were properly identified by specific PCR, giving the expected amplification product of 1100 bp belonging to the 16S rDNA gene (Zlotkin et al., 1998).

1.4.2. *lac*G presence

The strains were screened for the presence of the *lac* operon. In previous studies (Fortina et al., 2007, 2009) carried out on dairy and fish isolates, we observed that only the isolates of dairy origin were able to utilize lactose, since they harbored a *lac* operon, which shares a high sequence homology to that found in *Lactococcus lactis*. As a conclusion, we hypothesized a gene gain by lateral gene transfer, which provided dairy *L. garvieae* strains of a key physiological property contributing to adaptation to milk/dairy niche. When *lac*G was tested on new isolates, we found that the ability to metabolize lactose was not exclusively related to dairy isolates, but was heterogeneously scattered among *L. garvieae* meat isolates. Indeed, three meat isolates (strains Smp2, Smp3 and Smp4) were positive for the presence of the *lac*G gene. The remaining strains from meat and the isolates from vegetables and cereals did not show any amplification signal. These results indicate that *lac* operon cannot be considered a suitable genetic marker for associating strains to their niche of isolation.

1.4.3. Plasmid profile

Another molecular characterization was performed studying the presence and distribution of plasmid DNA: a high degree of biodiversity was observed, with strains coming from fish, plasmid free and strains coming from other sources containing a variable number of plasmids, ranging from 2 to 8 molecules of different molecular weight, as reported in Figure 1.1.

These data show a genetic heterogeneity within *L. garvieae* population.

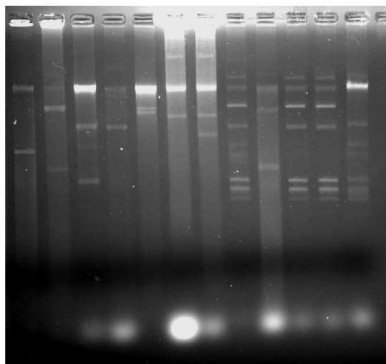# 1. Aquaculture sector model: *Lactococcus garvieae*

Figure 1.1. Plasmid profiles of *L. garvieae* strains.

1.4.4. Genomic fingerprinting

The genomic fingerprinting of *L. garvieae* strains was determined using RAPD and REP-PCR with BOXA1R and $(GTG)_5$ primers. These methods, which use primers targeting noncoding random or repetitive sequences interspersed throughout the genome are an established approach for delineation of bacteria at the species and strain-level (Randazzo et al., 2009). The discriminatory power of these primer sets was similar, with 20 different profiles obtained by BOXA1R and $(GTG)_5$ and 23 different profiles obtained by M13 for a collection of 49 strains. Although isolated at different times, some strains had identical fingerprints with all tested primers; on the contrary, most of the strains grouped at low similarity values. Independently from the primer used, the 49 strains grouped in two distinct clusters, which we named A_T and B_T (Figure 1.2): one cluster (A_T) contained all meat isolates (with the exception of BOXA1R experiment where the meat isolate Sa113 showed a unique fingerprint at a very low similarity value), whereas the other cluster (B_T) included all dairy isolates. Unexpectedly, four out of 12 strains isolated from fish (V32, V63, Lg23 and V79), always grouped with dairy isolates, whereas the others grouped with meat isolates. Likewise, strains isolated from vegetables allocated between the two main groups. The cluster analysis resulting from the combined profiles of the three primer sets employed, confirmed the existence of two major divisions, which were separated at a level of similarity of 0.13 (Figure 1.2), and did not coincide with the ecological niche of isolation. In particular, the low correlation value between the two clusters, suggested the existence of a marked genomic divergence.

1. Aquaculture sector model: *Lactococcus garvieae*
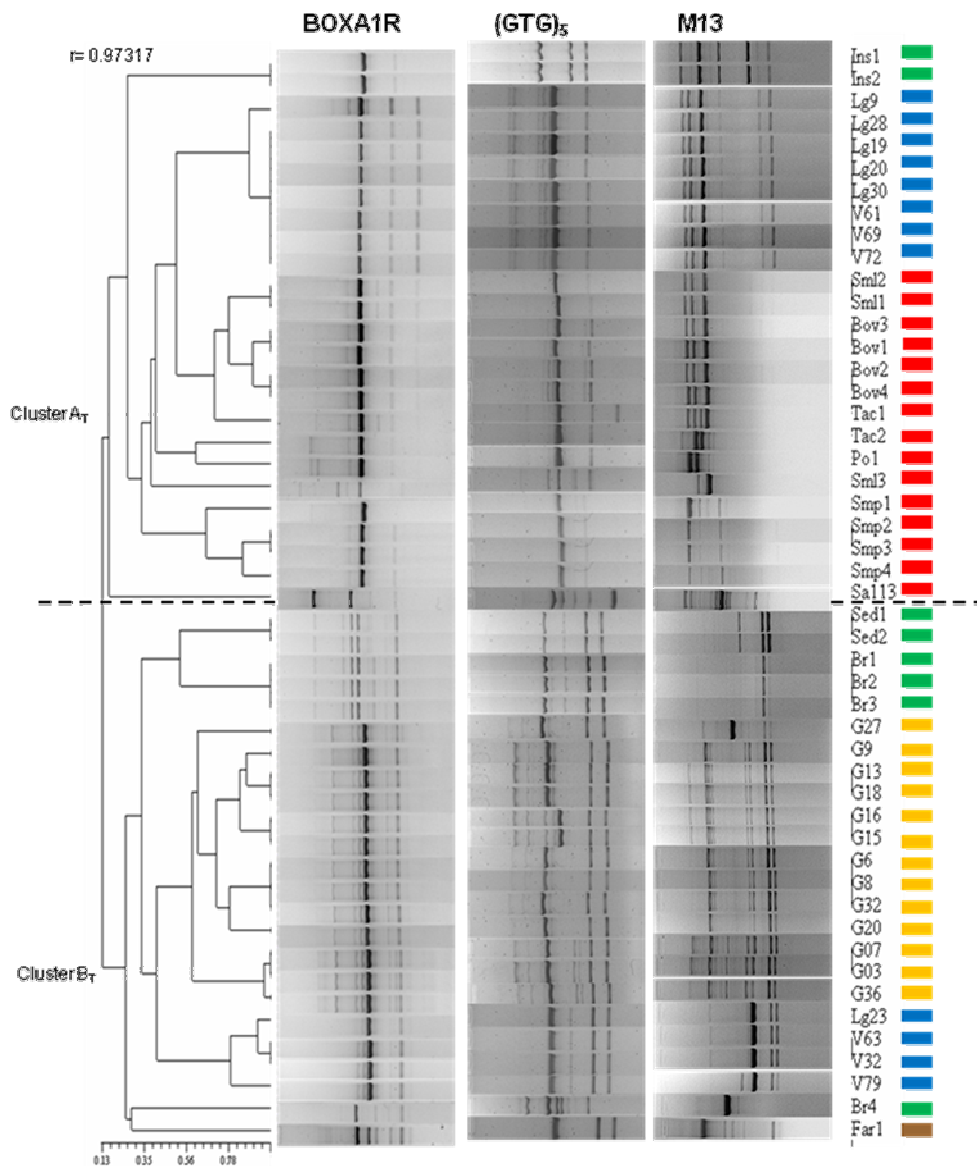

Figure 1.2. Cluster analysis of the *Lactococcus garvieae* strains using combined BOXA1R, (GTG)₅, and M13 fingerprints (UPGMA; NTSYSpc software, version 2.11 - Applied Biostatics Inc., NY, USA). From left-to-right: neighbour-joining tree of the 49 strains, strain name, strain origin (color code: green = vegetables, brown = cereals, red = meat, yellow = dairy, blue = fish).

1. Aquaculture sector model: *Lactococcus garvieae*

1.4.5. MLRT

When we tested several genes belonging to the core genome of *L. garvieae*, we observed again that all bacterial isolates can be shared out between two clusters, which are correlated to a low similarity level. Specifically, on the basis of conserved regions identified by sequence comparison of several housekeeping or functional genes in *L. garvieae*, we selected suitable primers to employ for PCR amplification (Table 1.3). The expected fragment length of the α-subunit of ATP synthase, elongation factor EF-Tu, D-alanine-D-alanyl carrier protein ligase, α-acetolactate synthase, glyceraldehyde-3-phosphate dehydrogenase and galactose permease amplicons was observed for all the 49 strains studied. Restriction analysis of each of the loci tested produced one to seven different patterns consisting of one to seven bands, depending on locus, restriction enzyme and strain examined (Table 1.5). The cluster analysis resulting from the combined restriction profiles of the six amplicons reported in Figure 1.3, revealed two distinct *L. garvieae* clusters at similarity level of approximately 0.12. Notably, the groups obtained were highly similar to PCR-fingerprinting clusters (A_T and B_T). In fact, also in this case, all meat isolates grouped together with the two salads and 8 fish isolates (cluster A_R), whereas dairy isolates grouped with cereal isolate and the remaining vegetables and fish isolates (cluster B_R). Within the various sub-clusters, further discrimination reflected the polymorphism revealed by restriction analysis of the tested loci (Table 1.5). *Gap*C gene resulted the most conserved among the tested strains; in fact, restriction analysis of the amplified fragment with different restriction enzymes did not reveal sequence variations among the strains, with the exception of isolate V79 from fish, which differentiated from the other strains when *Hae*III was employed (Identification profile in Table 1.5 = Ip 24). Restriction analysis of the *gal*P amplicon grouped the strains into two main clusters, within which the distribution of strains was always the same, even using different enzymes. One cluster (named "meat-group", Ip 1, 4, 9, and 12) contained all meat isolates (with the exception of Sa113), two salad isolates and 8 of the 12 fish isolates; the second cluster (named "dairy-group", Ip 3, 5, 8, and 10) included all dairy isolates and the remaining vegetables and fish isolates. The isolate from wheat flour always grouped with strains of dairy origin. Strain Sa113 from meat products showed a unique restriction profile (Ip 2, 6, 7, and 11). Restriction analysis of the *atp*A gene with *Rsa*I delineated the same two clusters obtained when *gal*P gene was tested; in this case, Sa113 grouped with meat isolates (Ip 16). Also using *Hpa*II the differentiation among strains was respected (meat-group, Ip14 – dairy-group, Ip 15) with an additional discrimination for four meat isolates (Smp1-2-3-4, Ip 13). The digestion of *tuf* gene with *Rsa*I grouped two meat isolates (Po1 and Tac2) with dairy-group (Ip 19), while the use of *Hha*I permitted the separation of Sa113 (Ip 20) and the differentiation of dairy isolates and Po1 and Tac2 (Ip 22) from the remaining meat, fish and vegetable isolates (Ip 21). Restriction analysis of the *dlt*A and *als* genes revealed further polymorphisms, and the possibility to discriminate the two salad (Ip 28) and the cereal isolates (Ip 42) from the other strains and to highlight two sub-groups within dairy isolates (Ip 32, 33).

1. Aquaculture sector model: *Lactococcus garvieae*

Table 1.5. Restriction profiles obtained for *L. garvieae* strains after digestion of PCR-amplified regions of six genes analyzed

Genes	Amplicon size (bp)	Restriction enzyme	Restriction pattern	Identification profile= Ip
*gal*P	1070	*Hpa*II	590, 340, 90, 50	1
			930, 90, 50	2
			980, 90	3
		*Rsa*I	1070	4
			420, 400, 250	5
			420, 300, 250, 100	6
		*Hha*I	1070	7
			880, 190	8
			380, 350, 190, 100, 50	9
		*Hae*III	700, 370	10
			550, 370, 150	11
			370, 360, 340	12
*atp*A	1180	*Hpa*II	1000, 180	13
			880, 180, 120	14
			880, 120, 120, 60	15
		*Rsa*I	400, 200, 170, 150, 100, 100, 60	16
			500, 200, 70, 150, 100, 60	17
tuf	1080	*Rsa*I	480, 210, 180, 130	18
			260, 220, 210, 180, 130	19
		*Hha*I	1080	20
			960, 120	21
			800, 160, 120	22
*gap*C	974	*Hae*III	560, 410	23
			560, 290, 120	24
*dlt*A	1473	*Hha*I	900, 570	25
			900, 400, 170	26
			700, 550, 220	27
			700, 400, 220, 150	28
		*Hpa*II	1470	29
			1070, 400	30
			1070, 370	31
		*EcoR*V	1470	32
			1200, 270	33
			780, 420, 270	34
als	1076	*Hha*I	500, 250, 220, 120	35
			500, 220, 200, 70, 60, 50	36
			500, 220, 200, 120, 50	37
		*Hinf*I	580, 500	38
			580, 280, 220	39
		*Hpa*II	680, 300, 100	40
			460, 400, 160, 50	41
			580, 300, 80, 70, 50	42
			680, 400	43
			460, 300, 220, 100	44
			720, 300, 50	45
			570, 30, 200	46

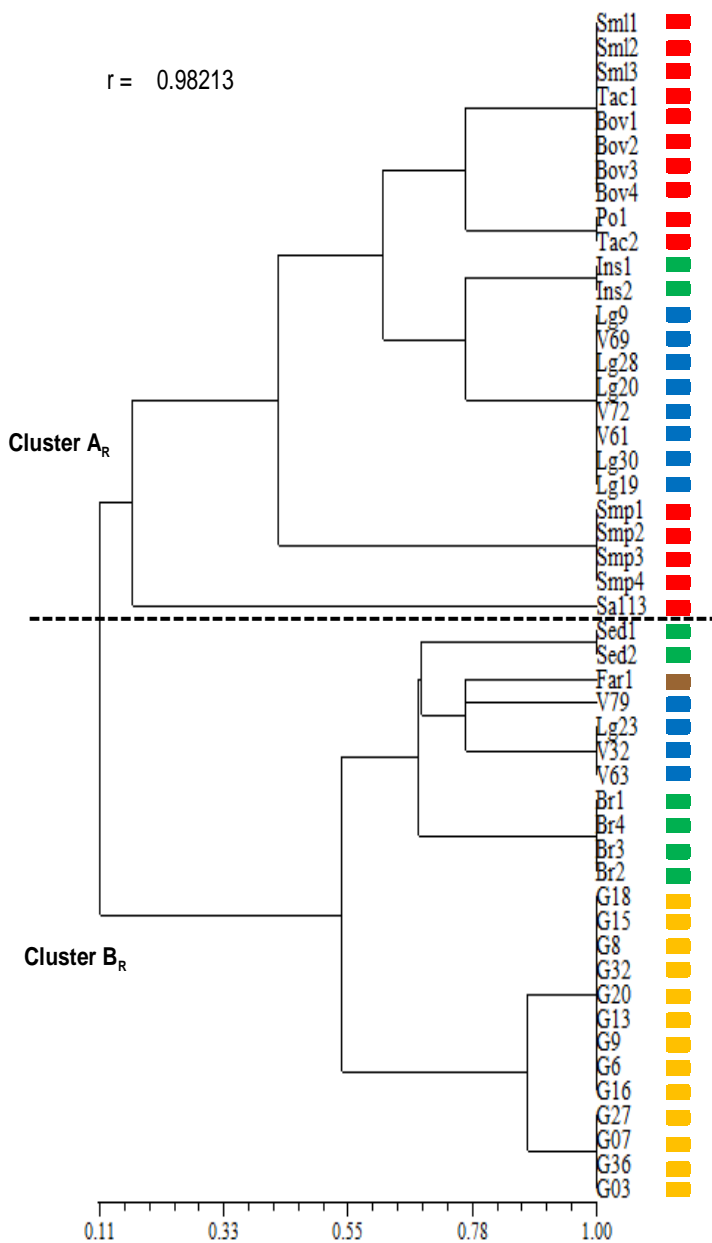# 1. Aquaculture sector model: *Lactococcus garvieae*

Figure 1.3. Dendrogram showing the relatedness of the *Lactococcus garvieae* strains based on analysis of their restriction profiles generated by MLRT analysis, constructed using the UPMGA algorithm in the NTSYSpc software, version 2.11 (Applied Biostatics Inc.), employing the Jaccard similarity coefficient. From left-to-right: neighbour-joining tree of the 49 strains, strain name, strain origin (color code: green = vegetables, brown = cereals, red = meat, yellow = dairy, blue = fish).

1. Aquaculture sector model: *Lactococcus garvieae*

1.4.6. PCR-Ribotyping

PCR-ribotyping generated by digestion of total DNA with *Pst*I, revealed the presence of nine different electrophoretic profiles, characterized by two to five bands of molecular weight varying from 4000 to >10000 bp (Figure 1.4A). The data obtained indicate an important heterogeneity both in the copy number and in the distribution of the ribosomal operons along the chromosomal DNA, as evidenced in the corresponding dendrogram (Figure 1.4B). Two main groups were distinguished, at a low similarity level (0.36). The distribution of the tested strains within the main groups differed in part from that previously observed. In this case, dairy isolates, cereal and six vegetable isolates (which always grouped together in the other experiments) and only one fish isolate (V79) showed the same ribotype, separated at similarity level of 0.80 with Sa113 from meat products and at minor similarity level with other two meat isolates. The remaining meat isolates grouped in different subgroups, all within group 2, which also included the remaining fish and salad isolates.

The data obtained in this step of the research support the hypothesis of an early separation of *L. garvieae* population into two independent genetic lineages. Subsequently, the environmental stimuli of a specific niche could have exerted a selective pressure favoring the emergence of several independent genotypes. It appears plausible that genomic flux within the dispensable genome, recombination events between genetically distinct strains during mixed colonization, and/or gene (in)activation, could have governed the bacterial adaptation to different habitats. Recently, we carried out the complete genome sequencing of representative *L. garvieae* stains. Whole-genome comparison among these and other *L. garvieae* available complete genomes, together with MLST experiments are reported below for a deeper understanding of the evolutionary history and the global complexity of this bacterial species.

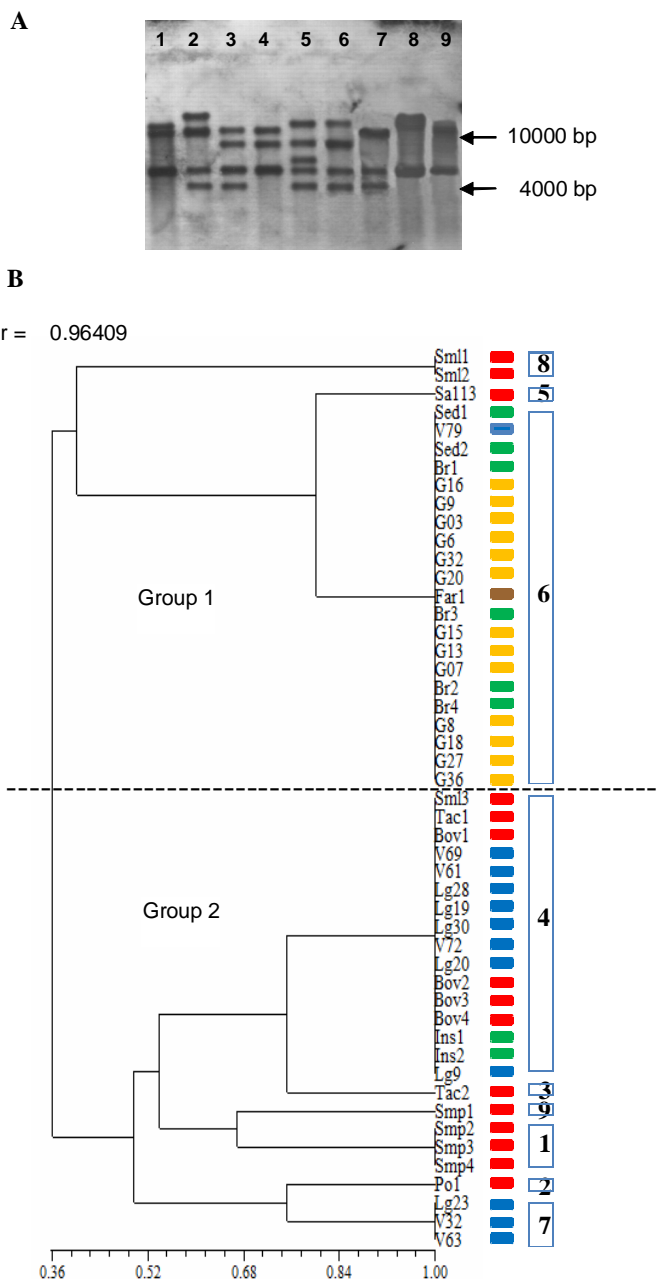# 1. Aquaculture sector model: *Lactococcus garvieae*

Figure 1.4. PCR-ribotyping analysis of *Lactococcus garvieae* strains. (A) PCR-ribotyping profiles identified among the tested strains, generated by genomic DNA digestion with PstI, lanes 1 to 9. (B) Dendrograms represented the relatedness of the strains, based on ribotyping data (UPGMA; NTSYSpc software, version 2.11 -Applied Biostatics Inc.). From left-to-right: neighbour-joining tree of the 49 strains, strain name, strain origin (color code: green = vegetables, brown = cereals, red = meat, yellow = dairy, blue = fish), PCR-ribotyping profiles.

1. Aquaculture sector model: *Lactococcus garvieae*

1.4.7. MLST

The MLST scheme developed in this study was designed to be technically robust, generating high amplicon yields for all genotypes, under the same PCR conditions for all seven loci. Chosen target genes were widely distributed around the chromosome, showed discriminatory power, and lacked insertions or deletions that caused changes in length.

Sequences from 26 *L. garvieae* strains from different environmental sources were classified in 18 unique Sequence Types (STs) in the MLST scheme, highlighting a significant heterogeneity in the strain collection. All loci were polymorphic (Table 1.6) and the number of allele assignation varied between 8 in *gap*C, the most conserved locus, and 14 in *rpo*C, suggesting a different evolution rate between different loci, equally distributed along the genome sequences. Indeed, the increasing availability of *L. garvieae* genomes in database allowed us to determinate the location of these target genes and verify their distribution; the minimum distance between loci was 18 kb.

To estimate the genetic diversity of *L. garvieae* we used the average nucleotide diversity π, but since this parameter could be influenced by the sample size, only one sample from each ST was analyzed. We also measured the π_{MAX}, defined as the number of nucleotide differences per site between the two most divergent sequences within the population. This value in fact is not directly sensitive to sampling size but only to the extreme values of sequence divergence (Passerini et al., 2010).

The average nucleotide diversity π of *L. garvieae* generated by the analysis of the concatenated DNA sequences of all loci was 0.0297 ± 0.0068, corresponding to 691 polymorphic sites (Table 1.7). This value of π was significantly higher than π for similar species, like *L. lactis* (Passerini et al., 2010) that appear monophyletic in comparison, suggesting a possible high variability in *L. garvieae* species. For single locus π ranges from 0.0074 ± 0.0032 for *gap*C to 0.0663 ± 0.0159 for *gyr*B, and these results are also confirmed by the determination of $\pi_{MAX,}$ supporting the hypothesis of different evolution rate of considered loci.

The phylogeny of the 26 *L. garvieae* strains was analyzed by constructing a neighbor-joining tree from the 5713 bp concatenated sequence of the seven loci (Figure 1.5). The tree revealed the presence of two main subgroups (S_A and S_B) as highlighted in previous work (Ferrario et al., 2012) and also in this case not coherent with the ecological niche of isolation.

The MLST experiments show the presence of a third branch in the phylogenetic tree (Subgroup C, S_C) composed by a meat isolate (Sa113) and a strain from mallard duck intestines (DCC43). This branch is totally separated from the other lineages. The phylogenetic tree from concatenated sequences analysis, was compared to the topologies of the seven tree constructed for each gene (data not shown). The general sample classification of the single locus tree was very similar to that of the concatenated one.

When sequences data were analyzed after stratification by subgroups, the number of polymorphisms and genetic diversity within each subpopulation were reduced (Table 1.7). The S_C subgroup was the exception, showing the highest π and number of polymorphic sites, also if compared to the total population. This suggests the presence of a barrier for genetic exchange between these *L. garvieae* subgroups. Moreover, the diversity of the subgroup S_C influences hardly the mean genetic diversity of the total population and could represent the origin of the species.

1. Aquaculture sector model: *Lactococcus garvieae*

Table 1.6. Origin of *L. garvieae* strains analyzed during this study and allelic profile at each locus.

Strain	Source	Sub group	*als*	*atp*A	*tuf*	*gap*C	*gyr*B	*rpo*C	*gal*P	ST	Clonal Complex (CC)
DSM20684[T]	Bovine mastitis	B	1	1	1	1	1	1	1	1	Singleton
Smp3	Meat products	B	2	2	2	2	2	2	2	2	Singleton
Po1	Poultry	B	3	3	3	2	3	3	3	3	1
Tac2	Turkey	B	3	3	3	2	3	3	3	3	1
Bov3	Beef	B	3	3	4	2	3	3	3	4	1
Sa113[a]	Meat products	C	4	4	5	3	4	4	4	5	Singleton
Ins1	Salad	B	5	5	6	2	5	5	5	6	Singleton
Sed2	Celery	A	6	6	7	2	6	6	6	7	Singleton
Br3	Broccoli	A	7	6	8	2	7	7	7	8	Singleton
Br4	Broccoli	A	7	6	8	2	7	7	7	8	Singleton
Far1	Wheat flour	A	8	7	9	2	8	8	8	9	Singleton
G27	Cow milk	A	9	7	3	4	7	9	9	10	3
G07	Cow chees	A	9	7	3	4	7	9	10	11	3
G9 [a]	Cow chees	A	9	7	3	2	7	10	9	12	Singleton
G01	Cow chees	A	9	7	3	4	7	9	9	10	3
Lg9	Rainbow trout	B	10	3	4	2	3	3	3	13	1
Lg19	Rainbow trout	B	10	3	4	2	3	3	3	13	1
V63	Trout	A	11	6	10	2	9	11	8	14	Singleton
V79	Trout	A	11	6	11	5	7	12	11	15	Singleton
8831	Rainbow trout	A	11	6	10	2	9	11	8	14	Singleton
21881	Human blood	A	9	7	3	4	7	9	9	10	3
ATCC49156	Yellowtail	B	12	8	6	6	10	13	12	16	2
Lg2	Yellowtail	B	12	8	6	7	10	13	12	17	2
UNIUD074	Rainbow trout	B	10	3	4	2	3	3	3	13	1
IPLA31405	Cow milk	B	3	3	4	2	3	3	3	4	1
DCC43	Mallard duck intestines	C	13	9	12	8	11	14	13	18	Singleton

ST: Sequence Type; [a]: synonymous of I113 and TB25 strains reported in Ricci et al., 2012, 2013.

1. Aquaculture sector model: *Lactococcus garvieae*

Table 1.7. Polymorphism at seven genes in *L. garvieae*.

Locus	Size (bp)	No. alleles	No. Polymorphic sites	π	π max	Tajima's D[a]	Fu & Li's D[a]	Fu & Li's F[a]	$I_A{}^S$
als	811	11	110	0.0396±0.0086	0.107	-1.068	-1.288	-1.407	
*atp*A	803	9	92	0.0358±0.0117	0.093	-1.133	-0.984	-1.147	
tuf	809	12	37	0.0122±0.0022	0.028	-1.163	-1.521	-1.626	
*gap*C	821	8	23	0.0074±0.0032	0.022	-1.6321[b]	-1.7136[b]	-1.886[b]	
*gyr*B	827	11	164	0.0663±0.0159	0.151	-0.703	-0.887	-0.954	
*rpo*C	830	14	79	0.0271±0.006	0.072	-0.701	-1.067	-1.111	
*gal*P	812	13	186	0.0616±0.0188	0.183	-1.413	-1.736	-1.889	
Conc	5713	18	691	0.0297±0.0068	0.091	/	/	/	0.127[c]
Conc S_A	5713	8	87	0.0063±0.0007	0.009	/	/	/	0.162[c]
Conc S_B	5713	8	110	0.0071±0.0008	0.011	/	/	/	0.549[c]
Conc S_C	5713	2	491	0.0859±0.0429	0.086	/	/	/	/

Conc: concatenated sequences of seven loci
S_A= Subgroup A, S_B= Subgroup B, S_C= Subgroup C.
[a]: Statistical significance: Not significant, P > 0.10
[b]: Statistical significance: Not significant, 0.10<P<0.05
[c]: linkage disequilibrium detected
[d]: Ferrario et al., 2012-11-26
[e]: developed in this study
/: not determined

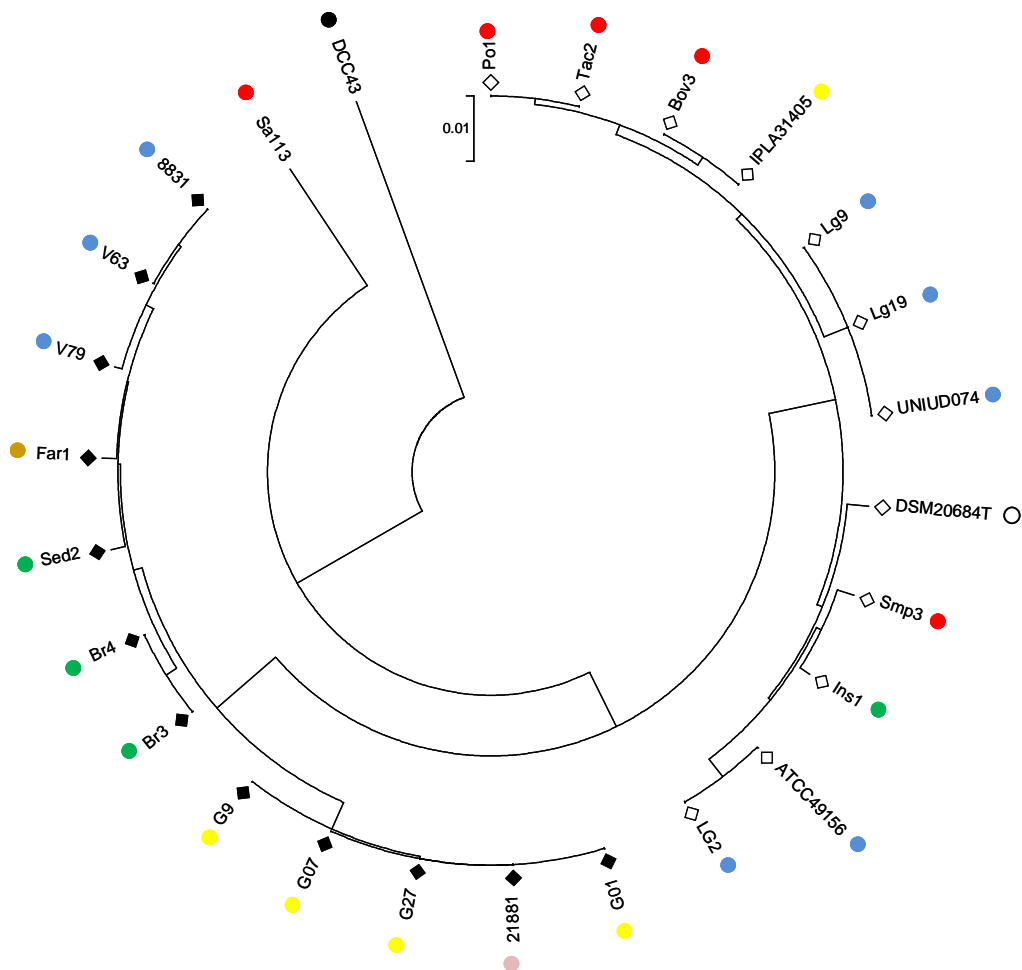# 1. Aquaculture sector model: *Lactococcus garvieae*

Figure 1.5. The unrooted neighbor-joining tree (bootstrap 1000, Kimura 2-parameter model) of *L. garvieae* was constructed from the 5713 bp concatenated DNA sequences of the seven loci, fo the 26 strains investigated. In figure are indicated also the subgroup belonging (■ for Subgroup A, □ for Subgroup B, nothing for Subgroup C) and the strain origin (color code: green = vegetables, brown = cereals, red = meat, yellow = dairy, blue = fish, pink= human, black= animal intestine, white = mastitic cow).

1. Aquaculture sector model: *Lactococcus garvieae*

The analysis of allelic profiles can reveal micro evolutionary relationship among genotypes. In this contest, clonal groups are defined as groups of strains linked by a single allele mismatch. Within *L. garvieae* population, the correlationship was defined using the eBURST algorithm that defines Clonal Complexes (CCs) by single-locus variants. As reported in Table 1.6, 50% of strains analyzed were clustered in CCs, sharing the trend of closed related genotypes in this species. Three CCs were identified. CC1 included 7 strains from meat products and fish, grouped in ST3, ST4, ST13. The correlation among these strains is also showed in the neibor-joining tree reported in Figure 1.5. CC2 grouped ST16 and ST17, corresponding to strains ATCC49156 and LG2, isolated from diseased trout, whose genomes were compared by Morita et al. (2011), revealing an high degree of sequence identity. CC3 grouped four strains belonging to ST10 and ST11, three isolated from dairy products and one from a case of human septicemia.

The Clonal frame analysis suggests that the two main subgroups appeared at approximately the same time, while S_C strains represent the ancestors from whom S_A and S_B have been originated (Figure 1.6). The r/m ratios were calculated for the entire population and for the two main subgroups, to evaluate whether the high genotypic diversity could be due to recombination events. The r/m was 0.978 for the total population, 0.925 for S_A and 1.203 for S_B. This fact might underline that these two subgroups have distinct inclinations and adaptive abilities to environments: S_B seems respond to high selective pressure increasing the recombination rate. It is interesting to note that despite the highest recombination rate of S_B, this does not affect its nucleotide diversity; it is similar to the π of S_A, and lower if compared to the one of the total population. One possible explanation is that members of S_B recombines only with other members of the same subgroup, so recombination does not introduce novel polymorphisms into subgroup and consequently the nucleotide diversity is not affected.

Presence of recombination in *L. garvieae* population was also investigated using the SplitsTree program, with the splits decomposition methods on the concatenated sequence of the total population, and for subgroups (Figure 1.7). We can notice the presence of interconnected network of phylogenetic relationships, resembling a parallelogram in shape, in all the splits graphs reported, especially in S_B which could be read as a consequence of a major recombinational effect. As a confirmation of the neighbor-joining method, the distribution of the clusters previously identified was visible.

The same analysis was also performed using *L. lactis* subsp. *lactis* IL1403, KF147, CV56 (respectively ANs: NC_002662, NC_013656 and NC_017486) and *L. lactis* subsp. *cremoris* A76, SK11 and MG1363 (respectively ANs: NC_017492, NC_008527 and AM406671.1), phylogenetically related (Morita et al., 2011). The split graph resulted showed the same subdivision of *L. garvieae* population, with the strains Sa113and DCC43, interconnected with *L. lactis* species by a recombinational event (data not shown).

Tajima's D, Fu & Li's D and F tests of neutrality were used to identify the evolution model of each target gene. All three tests gave values that did not significantly deviate from 0 (p> 0.10; for *gap*C locus, 0.10<P<0.05; Table 1.7), indicating that the seven loci evolved by random genetic drift.

The intergenic recombination was calculated by estimating the linkage disequilibrium between loci, using the standardized index of association statistic, I_A^S. Only one sample from every ST was analyzed, to avoid introduction of linkage disequilibrium by sampling bias. Significant linkage disequilibrium was detected considering either the 18 STs of the collection (see Table 1.7), or the two subgroups S_A and S_B. I_A^S are not significantly different from 0, even if subgroup B shows a higher value, suggesting that the recombination in this cluster have experienced a recent expansion of the population size.
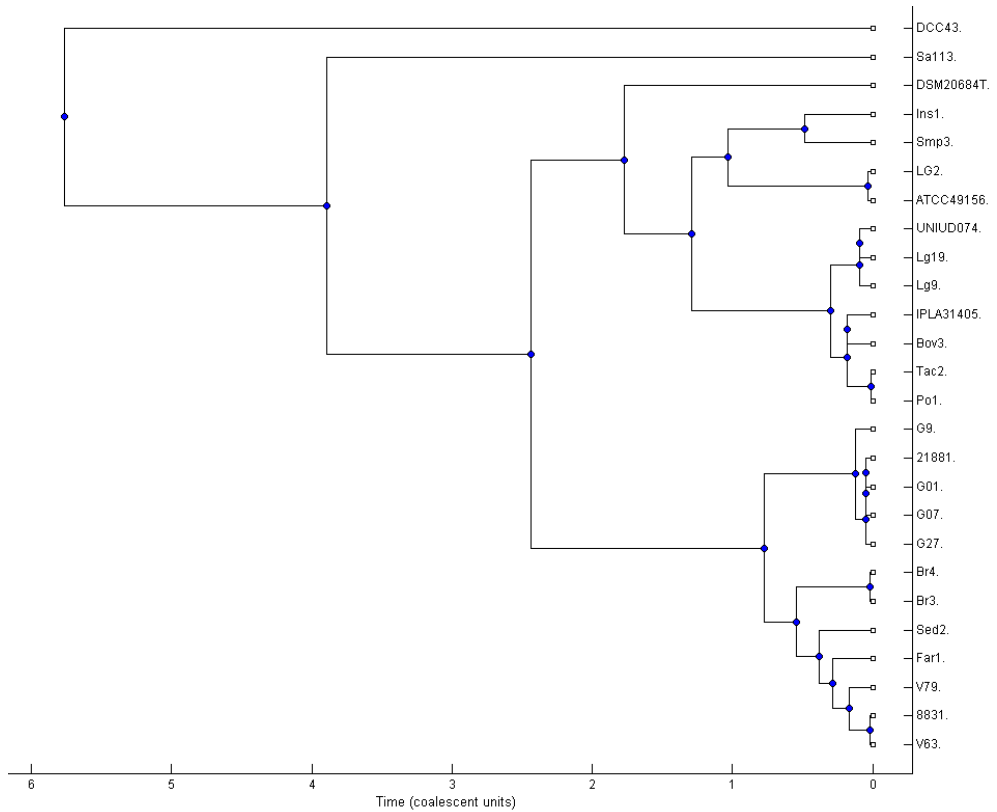
1. Aquaculture sector model: *Lactococcus garvieae*

Figure 1.6. Major rule consensus tree generated by Clonal Frame analysis of concatenated sequences of all loci, for the total population.

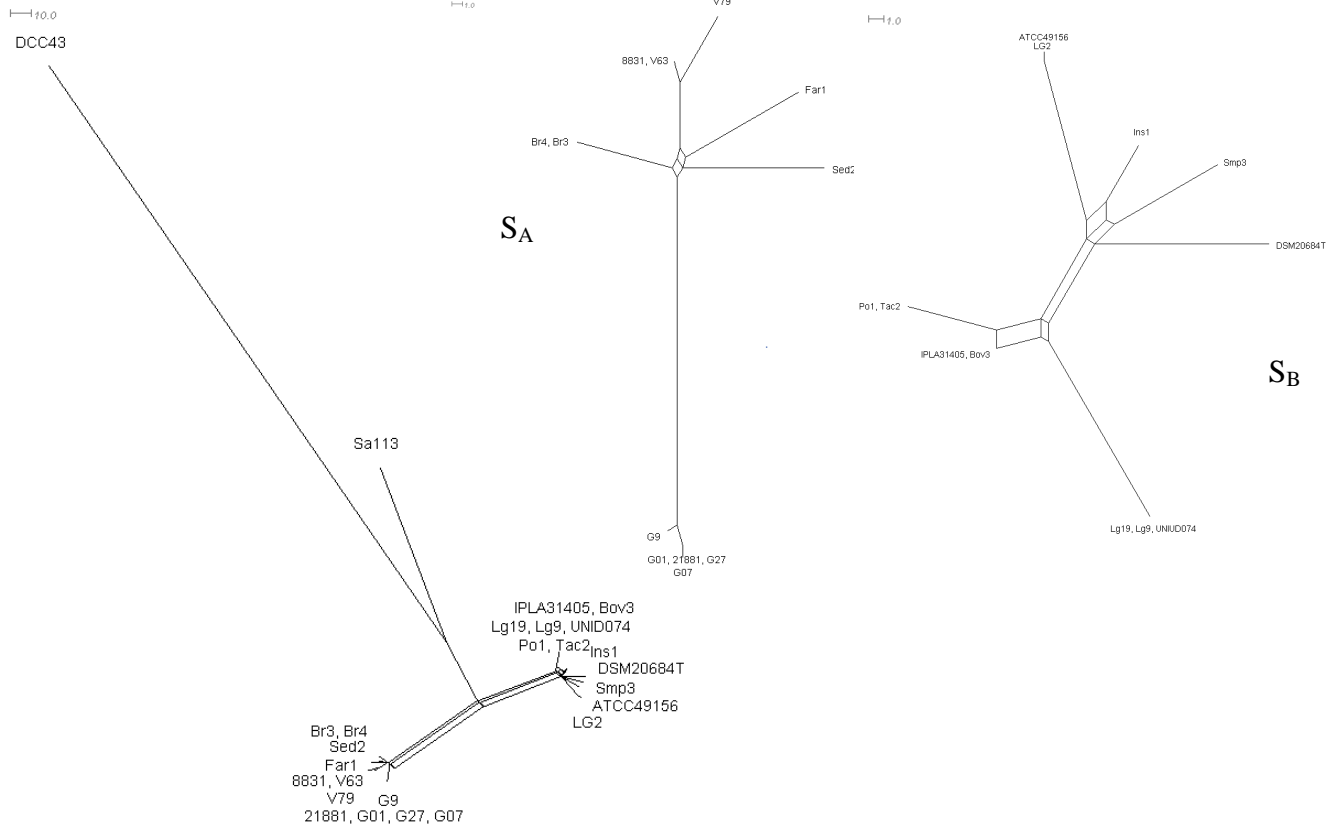**1. Aquaculture sector model:** *Lactococcus garvieae*

Figure 1.7. Splits decomposition analysis of the concatenated sequences of all loci for the total population of *L. garvieae* and for subgroups SA and SB.

1. Aquaculture sector model: *Lactococcus garvieae*

This step of the study confirms the significant genetic heterogeneity and the presence of two principal evolution lineages in *L. garvieae*, that could be originated from a common ancestor. All the performed analysis suggest the presence of a barrier for genetic exchange between *L. garvieae* subgroups, but with high recombinational rate inside subpopulations, suggesting recombination events only with other members of the same subgroup.

The availability of a larger strain collection and whole genome sequence comparisons could better explain phylogenetic structure and evolutionary history of *L. garvieae* .

1.4.8. Preliminary genomes comparison

We obtained the whole genome sequence of one strain coming from dairy product, one from diseased fish and two isolated from meat products, like turkey meat and pork sausage (Ricci et al., 2012, 2013). Whole genome comparison among these and other 7 *L. garvieae* available complete genomes, was carried out. Origin of isolation and other interesting information of these strains were reported in Table 1.8.

The simultaneous comparative analysis of these eleven genomes seemed to confirm our previous results. The chromosome of all strains have about the same length (2×10^6 bp), are characterized by the presence of about 2000 genes. They could be grouped in three distinct subgroups, that reflect the subgroups obtained through MLST analysis, (named S_A, S_B and S_C, Table 1.8) on the basis of nucleotide similarity and % GC. Within each subgroup the nucleotide similarity was high (99%), while the nucleotide similarity found among the subgroups obtained was lower (94-95%). Subgroup S_A comprised an heterogeneous group of isolates, coming from fish, cheese and human sources, showing a % GC value of 38.0. Subgroup S_B was characterized by the presence of four fish isolates showing a mean % GC value of 38.7. Subgroup S_C grouped the two strains, Sa113 and DCC43, that grouped separately from the others.

The alignment of *L garvieae* genomes with and without related genomes of *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *cremoris* MG1363 sequences is shown in Figure 1.8. *L. garvieae* shows the three branches previously described S_A, S_B and S_C. Subgroup S_C showed the least genetic distance to the two main subgroups. This subgroup seems to serve as evolutionary intermediate among *L. garvieae* and *L. lactis*.

A preliminary comparison of the genome content of the eleven *L. garvieae* strains indicates that the core genome (composed by genes present in all strains) contains, in additions to genes codifying for the main metabolic pathways, also genes related to virulence factors such as adhesins and hemolisins. Genes related to carbohydrate fermentation seemed part of the dispensable genome (comprising genes specific of a single strain or of a group of strains). It is the case of *lac*G, codifying for a phospho-β-galactosidase responsible of the lactose metabolism in *L. garvieae*. This gene was present only in the genome of two strains (G9 and IPLA31405) coming from dairy product. Also genes involved in sucrose fermentation were only present in a few genomes, in particular in genomes of three strains (G9, 21881 and 8831) belonging to subgroup S_A. In these strains three genes were detected *scr*A, *scr*B and *sac*K, codifying respectively for a sucrose specific IIABC component (PTS system), a sucrose-6-phosphate hydrolase and a fructo kinase. Upstream these genes, was located a sucrose repressor (Figure 1.9).

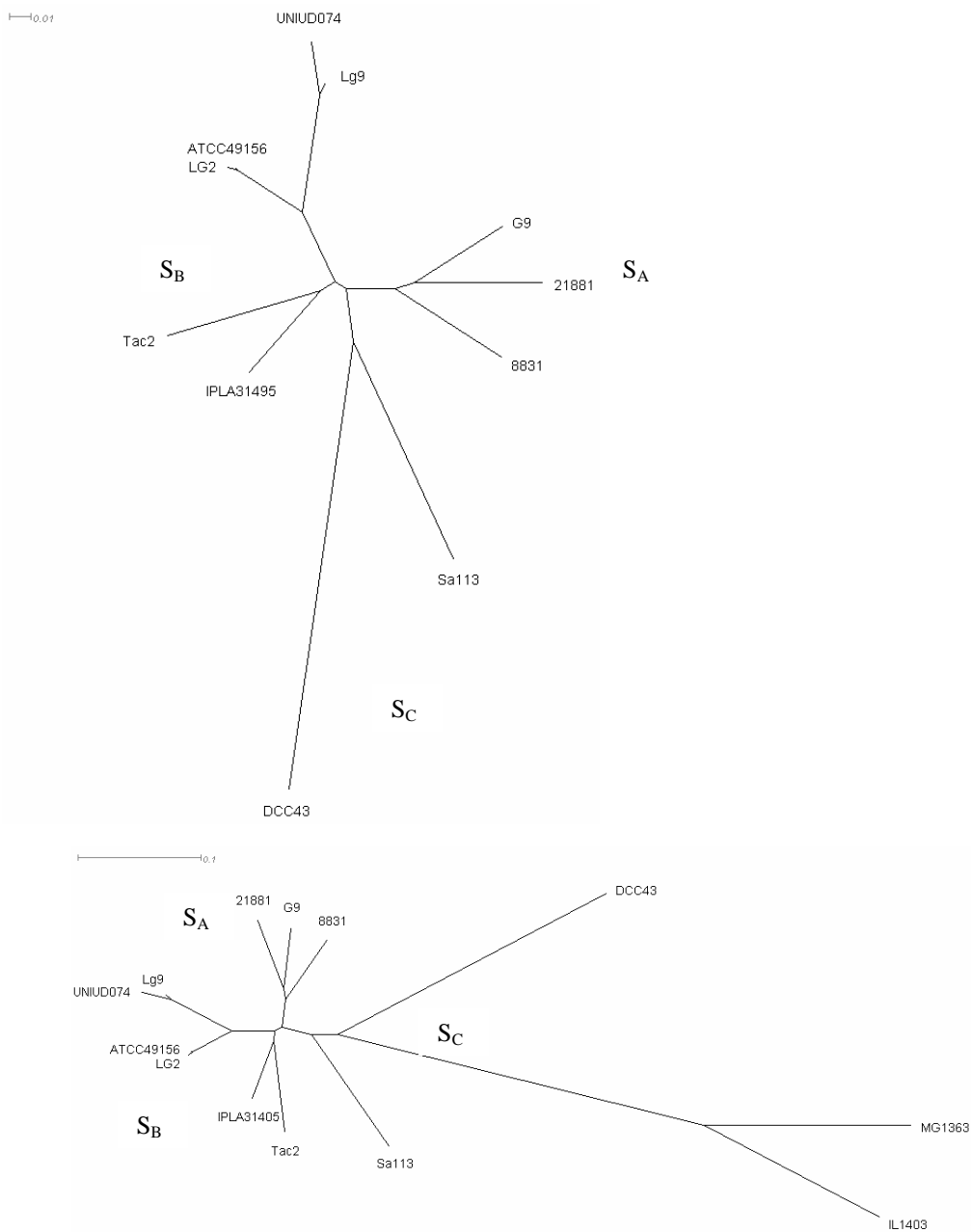# 1. Aquaculture sector model: *Lactococcus garvieae*

Figure 1.8. Above, Consensus Network graph obtained after allineament of *L. garvieae* genome sequences available in datatbases, with Mauve. Under, Consensus network graph obtained aligning *L garvieae* and *L. lactis* subsp. *lactis* IL1403 and *L. lactis* subsp. *cremoris* MG1363 genome sequences. Stratification in subgroups S_A, S_B, S_C is reported.
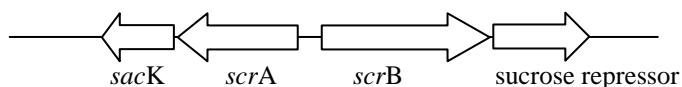
1. Aquaculture sector model: *Lactococcus garvieae*

Figure 1.9. Schematic representation of the sucrose fermentation pathway in strain G9.

Only one strain of subgroup S_B (specifically Tac2 strain) showed the presence of this gene cluster, even if the sequence homology was lower (60-70%). All strains of subgroup S_B, in place of genes directly related to sucrose utilization, showed the presence of a putative sucrose phosphorylase (*spl*R), able to hydrolyze sucrose and convert it to glucose-1-phospate and fructose.

The comparative analysis of genomes of a virulent strain LG2 and a non-virulent strain ATCC49156 of *L. garvieae*, performed by Morita et al. (2011) revealed that the two strains shared a high degree of sequence identity, but LG2 had a 16.5-kb capsule gene cluster that is absent in ATCC 49156. The capsule gene cluster was composed of 15 genes, of which eight genes are highly conserved with those in exopolysaccharide biosynthesis gene cluster often found in *Lactococcus lactis* strains. The research of this capsule gene cluster in the other 9 *L. garvieae* genomes gave negative results. So, also genes related to capsule biosynthesis are part of the dispensable genome and other virulence factors in a-capsulated strain should be studied.

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1. Aquaculture sector model: *Lactococcus garvieae*

Table 1.8. *L. garvieae* strains used for genome sequence analysis. Cluster identifies three lineages

Strain	Accesion number	Isolation	Lenght (bp)	N° contigs	Genes	% GC	Subgroup
ATCC 49156 Morita et al., 2011	AP009332	Yellowtail	1950135	1	1.947	38.8	B
LG2 Morita et al., 2011	AP009333	Yellowtail	1963964	1	1.968	38.8	B
Lg9 Ricci et al., 2012	AGQY01000000	Rainbow trout	2087705 (draft)	141	2.029	38.5	B
UNIUD074 Reimundo et al., 2011	AFHF0000000	Rainbow trout	2171966 (draft)	25	2.101	38.7	B
Tac2 Ricci et al., 2013	AMFE01000000	Turkey meat	2242863 (draft)	97	2153	38.23	B
21881 Aguado-Urda et al., 2011b	AFCF00000000	Human blood	2164557 (draft)	91	2.141	37.9	A
8831 Aguado-Urda et al., 2011a	AFCD00000000	Rainbow trout	2087226 (draft)	87	1.969	38.0	A
G9 [a] Ricci et al., 2012	AGQX01000000	Italian cheese	2014328 (draft)	92	1.977	38.1	A
Sa113 [a] Ricci et al., 2013	AMFD01000000	Pork sausage	2178733 (draft)	49	2124	37.9	C
DCC 43 Gabrielsen et al., 2012	AMQ501	Mallard duck intestines	2244387 (draft)	68	2227	37.8	C

[a]: synonymous of I113 and B25 strains reported in Ricci et al., 2012, 2013.

1. Aquaculture sector model: *Lactococcus garvieae*

1.4.8. Virulence Gene detection

The research of hypothetical virulence factors carried out by RAST server showed in all genomes tested the presence of genes that could be involved in pathogenicity, as candidate genes encoding haemolysins and cell surface adhesin proteins.

In particular, we found an ORF that exhibited significant similarity to the known sequences of haemolysin genes (*hly*). This ORF was present in all genomes tested and its nucleotide sequence seemed highly conserved among the genomes (93-100%). *L. garvieae* hypothetical hemolysin exhibited the highest levels of similarity to a hemolysin like protein of *L. lactis* (70% identity) (accession number BAL50414). It also exhibited significant similarity to hemolysin III of *Enterococcus faecium* (ZP03948925) and *Bacillus cereus* (EJP90039) (57 and 48% identity, respectively). We referred to this ORF as the *L. garvieae hly* gene.

Among the candidate genes encoding cell surface adhesins, we found an ORF codifying a protein that exhibited significant similarity to the known sequences of fibronectin binding proteins (FnBPs) belonging to the family of the FnBP A N-terminus (FbpA) of *L. lactis* (81%) (YP_809382), *Streptococcus mitis* (65%) (ZP12558846), *S. sanguinis* (64%) (ZP07887906) and *S. pneumoniae* (63%) (AF181976_1). Also in this case this ORF was present and highly conserved in all genomes tested (nucleotide similarity ranging from 92 to 100%): we referred to this ORF as the *L garvieae fbp* gene. Most of the understanding of bacterial FnBPs has emerged from the study of the proteins of *Staphylococcus aureus* and *Streptococcus pyogenes* (Terao et al., 2001; Speziale et al., 2009). The binding of FnBPs to fibronectin mediates not only the adherence of these pathogens to extracellular matrices but also to the surface of a number of host cell types, including endothelial and epithelial cells. Recently, several studies revealed that a wide range of bacteria possess adhesin-like proteins with characteristics very different, but able to bind to fibronectin, such as proteins containing the FbpA motif.

PCR experiments carried out with primers designated on *hly* and *fbp* conserved sequences, employing a collection of *L. garvieae* strains coming from different sources (Table 1.6), showed that these genes could be considered as part of the core genome of the *L. garvieae* species. Moreover, the high levels of similarity found with the corresponding genes of *L. lactis*, also reported for other genes/operons, (Fortina et al., 2009; Morita et al., 2011) could suggest a co-evolution of these related species, accompanied by active processes of gene exchange via HGT (Horizontal Gene Transfer) and/or a recent divergence from a common ancestor.

Since the distribution of these genes among the 11 genomes is homogeneous, and they were described as important tools able to lead pathogenicity in human, their differential expression in *L. garvieae*, grown in conditions simulating environmental conditions and infections, was analyzed by reverse transcriptase PCR. In specific we used M17-G as reference medium for all experiments, brain heart infusion (BHI) due to association of *L. garvieae* in different cases of endocarditis, meat simulation medium (MSM) and M17 with addition of 0.2 % bile salts to simulate meat products and the digestive tract respectively.

After RNA extraction and retrotranscription to cDNA, gene expression experiments were performed. Analyses were done using cells of two *L. garvieae* strains (Lg9 and G9) representative of the two main clusters previously identified by molecular typing, ribotyping, MLRT, MLST and genome comparison. The gene expression values obtained by Expression ratio equation (Figure 1.10), were substantially different between the two strains considered. In general, it is possible to note that strain coming from dairy product (G9) showed higher level of expression, than Lg9, isolated from fish, in all conditions tested. In G9 strain, *hly* and *fbp* were significantly overexpressed after exposure to bile salts (Expression Ratio, ER: 9.65 for *hly* and 12.14 for *fbp*); a minor degree of expression in the other environmental conditions tested was detected (in MSM, ER 4.84 and 2.41 respectively; in BHI, ER values of 1.11 and 1.44). Also for strain Lg9, the expression of both genes was positively influenced by the presence of bile salts. The isolate from fish expressed *hly* and *fbp* at lower level compared to the dairy isolate (Figure 1.10).

Expression Ratio $= \dfrac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}}$
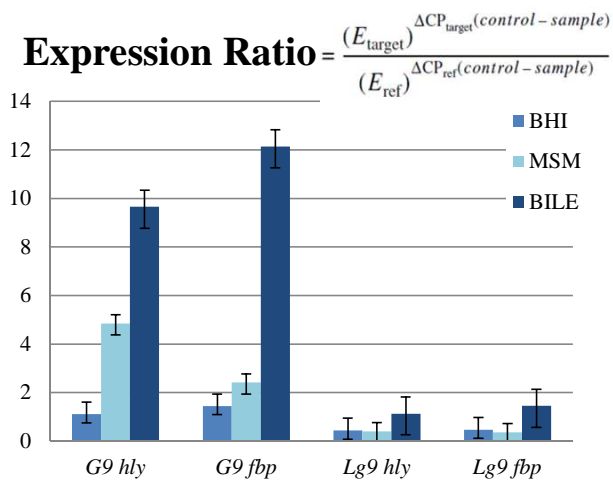
Figure 1.10. Expression values calculated by Expression ration equation (Pfaffl, 2001), where Cp is the crossing point at which the level of fluorescence starts to exponentially increase above any background fluorescence, and E (determined by the equation E=10[-1/slope]) is the efficient of the reaction.

1. Aquaculture sector model: *Lactococcus garvieae*

1.5. CONCLUSIONS

According to the objectives reported in section 1.2, the following conclusions were drawn:

- *L. garvieae* seems widely distributed in all food matrices, particularly in dairy and meat products;

- *L. garvieae* ability to metabolize lactose was not exclusively related to dairy isolates, but was heterogeneously scattered among *L. garvieae* meat isolates;

- DNA fingerprinting, MLRT and PCR-ribotyping results support the hypothesis of an early separation of *L. garvieae* population into two independent genetic lineages, not coherent with the niche of origin of the strains;

- MLST study confirmed the separation of *L. garvieae* population in two main subgroups and also reveals the presence of a third lineage which includes two isolates that could be considered the ancestors strains from which the others have been originated;

- Comparative analysis of 11 *L. garvieae* genomes permitted a first elucidation of the history of this species, through the research of genes belonging to core/dispensable genome;

- Experiments carried out to evaluate the expression of virulence-related genes in *L garvieae* represent the first approaches necessary to clarify the pathogenicity potential of this species, especially for food safety.

Publications related to this part of the PhD project:
o Ferrario C, Ricci G, Borgo F, Rollando A, Fortina MG, 2012, Genetic investigation within *Lactococcus garvieae* revealed two genomic lineages. FEMS Microbiol Lett 332:153-161.

o Ricci G, Ferrario C, Borgo F, Rollando A, Fortina MG, 2012, Genome sequences of *Lactococcus garvieae* TB2.5, isolated from Italian cheese, and *Lactococcus garvieae* LG9, isolated from Italian rainbow trout. J Bacteriol 194:1249-50.

o Ferrario C, Borgo F, Ricci G, Fortina MG, 2012, Variability at gene and genome levels revealed different genomic lineages in *Lactococcus garvieae*. Presented at 23rd International ICFMH Symposium: Food Micro 2012, Istanbul, Turkey.

o Ricci G, Ferrario C, Borgo F, Eraclio G, Fortina MG, 2013, Genome sequences of two *Lactococcus garvieae* strains Isolated from meat. Genome Announc, 1: in press.

1. Aquaculture sector model: *Lactococcus garvieae*

1.6. REFERENCES

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2. FISHING SECTOR MODEL: *Morganella morganii*

2. Fishing sector model: *Morganella morganii*

2.1. STATE OF THE ART

2.1.1. Health hazards in seafood

Fish and fish products are one of the most important foods with a high nutritional value. It has a limited shelf life as a consequence of microbial growth and activity, causing various changes in the sensory characteristics (Gram & Huss, 1996). Seafood-related diseases have been frequently reported. In fact, an increase in seafood-related illnesses has been documented (Ababouch, 2006) and there is now greater interest in seafood safety worldwide. A large proportion of outbreaks are caused by microorganisms.

Fishery products can be carriers of health hazards such as human pathogens *Salmonella* spp. and *Vibrio* spp., in addition to parasites, natural toxins, heavy metals and other pollutants (Olsen et al., 2000). For these reasons, the maintenance of acceptable quality of fishery products necessitates development of means for precise and rapid quality evaluation. Regarding safety aspects, one of the most important parameters to be taken in consideration is the biogenic amines (BA) content of the product. During decomposition of seafood, especially during storage at elevated temperatures, various amounts of selected biogenic amines are usually produced, depending on the fish species (Figure 2.1). The most common biogenic amines in seafood associated with spoilage are histamine, tyramine, putrescine and cadaverine (Lehane & Olley, 2000). They are formed by bacteria naturally present in decomposed fish that decarboxylate the corresponding free amino acids. The reason for the monitoring of selected biogenic amines in seafood is twofold: as indices of decomposition and to prevent potential toxicity on human health.
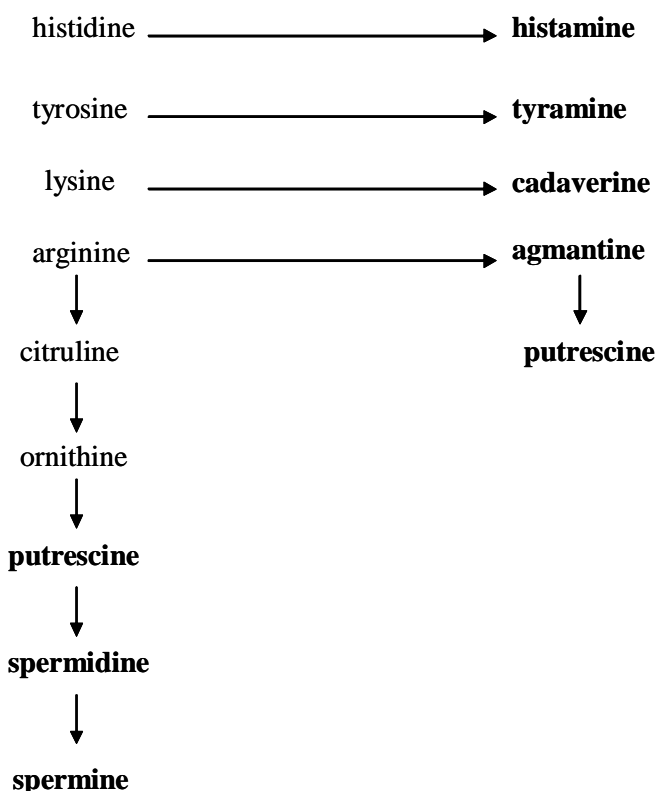

Figure 2.1. Biosynthesis of biogenic amines (Prester, 2011).

2. Fishing sector model: *Morganella morganii*

2.1.2. Histamine Fish Poisoning

Among BA, histamine regulates and modulates a variety of functions by binding to four subtypes of receptors on membranes in various tissues such as respiratory, gastrointestinal, cardiovascular, haematological and immunological system tissues, and the skin (Maintz & Novak, 2007).

Histamine is potentially hazardous from the toxicological point of view: it is the causative agent of HFP, also known as Scombroid poisoning, since caused by the ingestion of histidine-rich fish such as tuna, bonito, mackerel, belonging to the families *Scomberesocidae* and *Scombridae* (the so-called scombroid fish) (Lehane & Olley, 2000). HFP was firstly reported in 1828 (Henderson, 1830) and since then, it has been described in many countries. Nowadays one of the most relevant forms of seafood-borne disease (Hungerford, 2010).

HFP is described as a food hypersensitivity associated with the consumption of decomposed fish with toxic histamine levels (4500 mg/kg). The symptoms are variable and include peppery or metallic taste, oral numbness, headache, dizziness, palpitations, rapid and weak pulse (low blood pressure), difficulty in swallowing, and thirst. Other symptoms are allergy-like such as hives, rash, flushing and facial swelling. Less specific symptoms are also reported: nausea, vomiting, abdominal cramps and diarrhea (Specht, 1998).

Histamine production in fish is related to the histidine content of the fish, the presence of the enzyme histidine decarboxylase (HDC) and to environmental conditions. Histamine in fish can be produced rapidly by bacterial decarboxylases. Only free histidine can be decarboxylated (Figure 2.2).
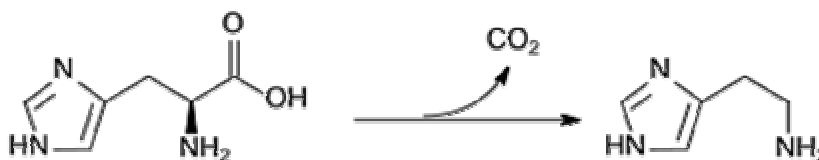

Figure 2.2. Decarboxylation of free histidine to histamine**.**

However, the decarboxylation of histidine to histamine is not the only route of the histidine metabolism, and the occurrence of this pathway in fish spoilage is quite limited. The pathway favoured is the catabolic one, in which the first step is the loss of ammonia to histidine, resulting in the formation of urocaic acid and then to glutamate (Lehane & Olley, 2000).

The globalization of seafood in the last decade has greatly amplified all food safety challenges, leading to huge increases in international trade such as popular scombroid species, like tuna (Constance & Bonanno, 2009). Improvements in seafood safety and quality can be traced to the application of risk analysis, establishment of international standards, and use of risk analysis plus hazard analysis and critical control point (HACCP) principles.

Since histamine poisoning is one of the most important seafood diseases, as previously reported, data for worldwide outbreaks of HFP are usually updated. The last update was the project BIOCOM, Biogenic amines in seafood – assessment and management of consumer exposure studies, which results were reported by Dalgaard et al. (2008). Together with ciguatera, HFP continues to account for the majority of finfish-borne illness. It is not only one of the most prominent seafood intoxications, scombroid poisoning accounted for 38% of all seafood associated outbreaks in the United States and in England and Wales for 32% in the 1990s. Rates of seafood consumption did not correlate with outbreak rate. Further, for the countries with the highest (reported) outbreak rates, the numbers ranged from 2 to 5 outbreaks/year/million people like in Denmark, New Zealand, France and Finland.

2. Fishing sector model: *Morganella morganii*

2.1.3. Regulatory limits and analytical procedures

Histamine is the most widely amine investigated in fishery products, and is the only one with the established legal limits for human consumption.

According to the European Commission, based on nine samples, the maximum average histamine content of the species belonging to the S*combridae, Clupeidae, Engraulidae, Coryfenidae, Pomatomidae* and *Scombresosidae* families, is 100 mg/kg. No more than two samples can have histamine concentrations between 100 and 200 mg/kg, and no fish sample may have histamine level higher than 200 mg/kg. The same European Union regulations also established the critical levels of histamine of 200 mg/kg to fish that had undergone enzyme maturation treatment in brine, manufactured from species associated with a high amount of histidine. No more than two samples out of nine can have histamine concentration between 200 and 400 mg/kg and no sample may have a histamine level higher than 400 mg/kg. The analysis of biogenic amines must be performed by HPLC (European Commission, 2005).

In Italy, according to the D.L. n 531/1992, the Reg. CE 2073/2005 is accepted and implemented without changing.

The US Food and Drug Administration (FDA) set the stricter acceptable histamine value of 50 mg/kg for scombroid-like fishes based on data collected from numerous outbreaks. The same directive recommends the determination of other biogenic amines associated with fish decomposition. Furthermore, histamine levels above 500 mg/kg are considered toxic and dangerous for human health (FDA, 2001).

The number and variety of methods developed for histamine determination in fish and fish products is impressive. In literature are frequently reported methods for the detection of histamine using different approaches ranging from a simple and inexpensive colorimetric reaction to resource-intensive and more powerful LC-MS methods.

Reference methods should be proven thoroughly in interlaboratory studies in which multiple labs demonstrate that the method is rugged. The most widely used and officially accepted method to detect histamine in fish is a batch fluorescence method (AOAC, 1977). Although it is time consuming, requires manual manipulations, timing and ion exchange cleanup. Whereby, numerous other methods were developed, ranging from the easiest colorimetric method (Patange et al., 2005), followed by the thin layer chromatography (TLC) procedures, to continue with reversed-phase high performance liquid chromatography (HPLC) with pre-column derivatization (Petridis & Steinhart, 1995). Other popular separation-based methods include ion chromatography, capillary electrophoresis, paper electrophoresis, and gas chromatography-mass spectrometry (Hungerfort, 2010).

2.1.4. Bacterial origins of histamine in fish

Histamine in food is formed mainly through the decarboxylation of histidine by exogenous histidine decarboxylases (HDC) released by the microbial species present; although some are present in live fish tissues, most seem to be derived from post harvesting contamination on board fishing vessels, at the processing plant or in the distribution system.

Histidine decarboxylase is not widely distributed among bacteria, in particular only about 1% of the surface microflora of live fish represents histamine producers (Leane & Olley, 2000). Despite this fact, species of many genera are capable of decarboxylate histidine. However, the ability of bacteria to decarboxylate amino acids is highly variable and depends not only on the species, but also on the strain and the environmental conditions (Marcobal et al., 2006). Gram-positive bacteria are characterized by a pyruvoyl-dependent HDC enzyme thoroughly studied in different organisms, producing histamine in fermented foods, such as cheese, wine and beer (Lucas et al., 2005). For Gram negative bacteria, the HDC enzyme required pyridoxal 5′-phosphate (PLP) as essential coenzyme.

Most frequently isolated prolific histamine-formers from fish under controlled storage conditions are mesophilic enteric bacteria, such as *Morganella morganii*, *Raoultella planticola*, *Proteus vulgaris*, *Hafnia alvei*, and *Citrobacter freundii* (Bjornsdottir et al., 2009). Spoilage, ammonia production and

2. Fishing sector model: *Morganella morganii*

BA production by these bacteria are enhanced at elevated storage temperatures. However, once a large bacterial population has been established, residual enzyme activity continues slowly at refrigeration temperatures, although bacterial growth ceases (Lehane & Olley 2000). Once formed, histamine is resistant to commonly used food preservation methods, including freezing and cooking (Prester, 2011). For these reasons, the quantification of histamine in foods is a crucial task, as well as the detection of spoiling bacteria able to produce amines in order to establish the potential risk of histamine generation.

For the identification of histamine producing bacteria, many different analytical procedures have been developed. Commonly used traditional methods for the detection of spoiling bacteria, based on differential growth media and enzymatic pathways (Tao et al., 2009) are often tedious and unreliable. Molecular methods detecting the gene responsible for production of HDC are becoming more widely accepted as an alternative to traditional culture methods (Takahashi et al., 2003; Bjornsdottir-Butler et al., 2011). PCR techniques are fast, reliable and can be successfully used for the routine detection of bacterial strains potentially producers of BA.

2.1.5. *Morganella morganii* as a model of histamine producing bacteria

Several enteric bacteria, such as *Morganella morganii*, *Raoultella planticola*, *Enterobacter aerogenes*, *Photobacterium phosphoreum* and *P. damselae* subsp. *damselae* are reported to be dominant histamine-producing bacteria in fish (Kanki et al., 2007). Among these species, *M. morganii* is considered the most prolific histamine former, and the main responsible of histamine accumulation during storage of fish belonging to the family of *Scombridae* (tuna, mackerel) (Bjornsdottir et al., 2009).

The genus *Morganella* comprises two species, *M. morganii* and *M. psychrotolerans*. The first one includes two subspecies (*M. morganii* subsp. *morganii* and *M. morganii* subsp. *sibonii*), which can be distinguished from one another on the basis of trehalose fermentation (Jensen et al., 1992). *M. morganii* is often found in the intestines of humans and animals. It is also associated to urinary tract infections (O'Hara et al., 2000). It grows between 4° and 45 °C and has the ability to produce toxic concentrations of histamine in seafood (Janda & Abbott, 2005).

The *hdc* gene coding for the *M. morganii* HDC was expressed in *E. coli* (Vaaler & Snell, 1989). The enzyme contains 378 amino acids, and the active sites present were reported below: the lysine that binds pyridoxal phosphate (PLP) is the residue 233 and the conserved region Ser – Gly – His – Lys were residues 230 – 233.

```
MTLSINDQNKLDAFWAYCVKNQYFNIGYPESADFDYTNLERFLRFSINNCGDWGEYCNYL          60

LNSFDFEKEVMEYFADLFKIPFEQSWGYVTNGGTEGNMFGCYLGREIFPDGTLYYSKDTH         120

YSVAKIVKLLRIKSQVVESQPNGEIDYDDLMKKIADDKEAHPIIFANIGTTVRGAIDDIA         180

EIQKRLKAAGIKREDYYLHADAALSGMILPFVDDAQPFTFADGIDSIGVSGHKMIGSPIP         240

CGIVVAKKENVDRISVEIDYISAHDKTITGSRNGHTPLMLWEAIRSHSTEEWKRRITRSL         300

DMAQYAVDRMQKAGINAWRNKNSITVVFPCPSERVWREHCLATSGDVAHLITTAHHLDTV         360

QIDKLIDDVIADFNLHAA   378
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Figure 2.3. Amino acid sequenze of the HDC of *M. morganii*. Conserved residues Ser – Gly – His – Lys were underlined; residue 233 that binds pyridoxal phosphate (PLP) is in bold.

2. Fishing sector model: *Morganella morganii*

The amino acid sequences of the PLP dependent histidine decarboxylase has been studied in other histamine Gram negative producers, but only for two of them, studies for the determination of *hdc* related genes and their expression were carried out.

Morii et al. (2006) reported that the histamine-forming genetic cluster of *P. phosphoreum* was composed by and histidine decarboxylase *hdc,* an hypothetical amino acid permease gene (*ydj*E), and a putative histidine-tRNA synthetase gene (*his*S) (see Figure 2.4).

For *P. damselae* subsp. *damselae* three open reading frames were identified: the *hdc*A gene encodes the PLP dependent histidine decarboxylase, the *hdc*T gene is assumed to be a histidine ∕ histamine antiporter, and the *his*RS gene is considered to be a histidyl-tRNA synthetase (Figure 2.4). Through northern-blot analysis and RT-PCR experiments, induction of the three genes was revealed under conditions of low pH and histidine excess (Kimura et al., 2009).
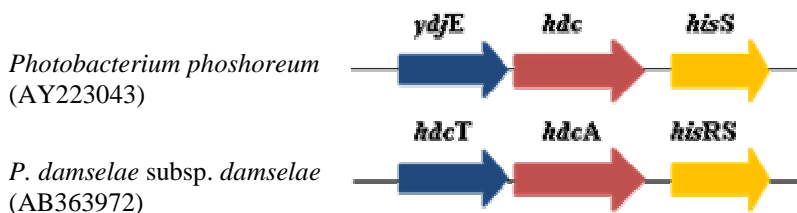
Figure 2.4. Schematic representation of the histidine decarboxylase cluster of *P. phosphoreum* and *P. damselae*. Arrows indicate ORFs. As reported in figure, genes coding for HDC are represented in red, putative antiporters or amino acid permeases are colored in blue, while histidyl tRNA sinthetase are yellow. Accession numbers are reported in brackets.

Any studies were conducted for *E. aerogenens* and *R. planticola hdc* related genes, but the sequences of their HDC enzymes were studied by Kamath et al. (1991). HDCs were isolated, sequenced, and expressed in *E. coli* under control of the lac promoter, and the overproduced enzymes were purified to homogeneity from the recombinant host. The two enzymes showed 85% sequence identity. The availability of several genome sequences of these two species in the National Centre for Biotechnology Information (NCBI) Genome database (http://www.ncbi.nlm.nih.gov/genome/) and their analysis could help us to understand the gene organization of these DNA regions.

For *M. morganii* no genome sequencing project was available up to now. Other information about the HDC enzyme were provided from Kanki et al. (2007). In this study was demonstrated that the HDC of this species was more stable than the other Gram negative histamine producers HDCs, in a temperature range of 4° to 40°C. This result could be important if related to the fact that *M. morganii* is often implicated in HFP cases due to an improper temperature/time abuse during storage (Kim et al., 2001).

2. Fishing sector model: *Morganella morganii*

2.2. AIM OF THE STUDY

The aims of the present study were several: to study and monitoring the development of microbial spoilage associations in tuna fish, to evaluate the diffusion of *Morganella morganii* and to develop a methodological polyphasic approach suitable for the obtainment of a quality and safety indicator, specific at strain- and/or metabolite- level.
These objectives were developed through different steps reported below.

- Evaluation of quality of filleted tuna fish, through the study of the relationship among bacteriological quality, histamine content and occurrence of histamine-forming bacteria;

- development of a specific molecular tool for the quantification of *M. morganii,* suitable for both classic and real time PCR;

- functional expression in *E. coli* of *M. morganii* DSM30164[T] histidine decarboxylase;

- sequencing of the *hdc* gene cluster and comparison with other Gram negative histamine producing bacteria;

- transcriptional analysis of the identified genes of *M. morganii* simulating different environmental conditions;

- molecular typing of strains coming from fish products, in comparison with clinical isolates of *M. morganii*. These experiments were applied to understand the effect of different ecological sources in the species variability.

2. Fishing sector model: *Morganella morganii*

2.3. MATERIALS AND METHODS

2.3.1. Bacterial strains, plasmids and growth conditions

Nineteen *M. morganii* strains, including 13 isolated from filleted tuna fish samples and six clinical isolated (kindly provided from Professor Rossolini of Laboratory of Physiology and Biotechnology of Microorganisms, Department of Biotechnology, University of Siena), were studied in comparison with the type strain of the species *M. morganii* DSM30164[T] (purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). In addition, 32 relevant strains belonging to species other than *M. morganii* were also analyzed, as negative controls for *M. morganii* specific amplification (Table 2.1). Gram negative strains were grown in Tryptic Soy Broth/agar (TSB/TSA) plates; coccoid and spore-forming bacteria were grown in Nutrient Broth/agar plates; lactic acid bacteria were grown in MRS broth/agar plates or M17 broth/agar plates. Strains were incubated at 30°C for 24 h. All media and reagents were purchased from Difco (Difco Laboratories, Detroit, USA).

Escherichia coli DH5αF'[F'/endA1 hsdR17(rk-mk+) supE44 thi-1 recA1 gyrA(- Na1r) relA1 Δ(lacIZYA-argF)U169 deoR (Φ80dlacΔ(lacZ)M15); Promega, Madrid, Spain] was used for all DNA manipulations. *E. coli* BL21 (DE3) pLysS (Promega) competent cells, were used for expression in pURI3 vector. *E. coli* strains were grown in LB medium at 37 °C by shacking at 200 rpm. When required ampicillin was added to the medium at 100 μg/mL. For the sequencing of the flanking regions of the *hdc* gene, a DNA library was build, using the vector pUC19 (Takara Bio Inc., Shiga, Japan).

For the protein expression we used plasmid pURI3 that encodes expression of a leader sequence consisting of a N-terminal methionine followed by three spacer amino acids, a six histidine affinity tag, a spacer glycine residue, and the five amino acid enterokinase (EK) recognition size, under control of the T7 RNA polymerase φ promoter, which can be induced at high levels of isopropyl-β-D-thiogalactopyranoside (IPTG) (Curiel et al., 2011).

All these strains were conserved at -80°C in glycerol stock 15 %. Plasmids were conserved at -20°C.

2.3.2. Fish sampling

Different filleted yellowfin tuna fish (*Thunnus albacares)* were purchased from wholesale fish market (WM) and different local supermarkets (SM) in Milan, Italy. The study was conducted between April and June 2010, visiting the markets during their usual working hours (WM, 4 to 6 a.m.; SM, 7 to 10 a.m.). For each SM tested, two filleted tuna sampled at different times, were analyzed, while for WM samples, two different sellers were considered (Table 2.3). All fillets acquired, were immediately transported to the laboratory in refrigerated bags and processed within 1 h. Each tuna fillet was aseptically cut into three parts (about 30 g) removed from adjacent areas to ensure uniformity in initial values of histamine content and microbial population. Samples were placed in sterile bags. The first piece was immediately used for histamine determination and microbiological tests. The second, has been preserved to 6-8 ° C for 1 day to simulate storage in a home refrigerator. The third piece was temperature-abused for 3 h at 25 °C and then stored at 6-8 °C for 1 day, to mimic the house-supermarket journey of a common consumer. At the end of these steps, microbiological changes during storage were determined.

2.3.3. Microbiological examinations and isolation of bacteria

Tuna pieces were homogenized with ten volumes of sterile saline solution (SS; 0.9% NaCl) in a laboratory blender (Stomacher 400, Seward, London, UK). The homogenates were serially diluted with sterile SS and 1 mL aliquots were inoculated into aerobic Plate Count Agar (PCA; Difco). Plates were incubated at 25 °C for 72 h, to recover both psychrotrophic and mesophilic bacteria, and then colonies were counted. *Enterobacteriaceae* were enumerated on violet red bile glucose agar (VRBG, Sigma-Aldrich, GmbH, Steinheim, Germany) incubated for 24 h at 37 °C. All red colonies with a diameter ≥ 0.5 mm were considered as *Enterobacteriaceae*. For all performed experiments, bacterial numbers were expressed as CFU/g of tuna piece. The experiments were carried out in triplicate. Selection of colonies for further studies was restricted to *Enterobacteriaceae* that, as reported before, includes species known as histamine producing bacteria in fish products. Randomly selected colonies were

2. Fishing sector model: *Morganella morganii*

picked from VRBG, pure-cultured and characterized by cell morphology, motility, Gram staining, catalase and oxidase production (Oxidase identification sticks, Oxoid, Milan, Italy), glucose fermentation, production of gas from glucose, indole and acetoin production (Voges-Proskauer). These standard tests were performed as described by Barrow & Feltham (1999).

The ability of the selected bacteria to produce histamine was measured in tuna fish infusion (TFI) supplemented with 1% glucose, at pH 5.7, as reported by Taylor et al. (1979). The isolates were pre-incubated in TSB (Difco) containing 1 mg/mL of histidine (Sigma) at pH 6.8 and incubated for 16 h. Then, 2 mL of culture were inoculated into 50 mL TFI broth and incubated at 25 °C under aeration. Aliquots of 1 mL of the culture broth were analised at different incubation times for histamine quantification, as described below.

2.3.4. Chemical analyses

The histamine content was quantified by HPLC, as described by Gosetti et al. (2007). For the quantification of the biogenic amine directly in tuna samples, pieces of 10 g were homogenized in 50 mL of methanol (100%), incubated at 60 °C for 20 min and filtered. The filtrate was subjected to the derivatization reaction in tubes: 0.5 mL of the obtained solutions were mixed with 5 mL of 21 g/L $NaHCO_3$, 1 mL of 5.4 g/L dansyl chloride and acetone up to 10.5 mL. Hermetically sealed tubes were kept at 60 °C for 20 min and then refrigerated to 25 °C before analysis. Histamine production by bacterial isolates was measured using 0.5 mL aliquots of TSI culture supernatants, obtained as described above; these aliquots were subjected to derivatization as previously reported. Each sample was analyzed in duplicate. The chromatographic system consisted of a Hitachi L-7400 HPLC instrument with UV-VIS detector (Hitachi, Tokyo, Japan). The stationary phase was a Purospher Star RP-18 LichroCART HPLC-Cartridge (5 µm, 250 x 4.6 mm) (Merck, Darmstadt, Germany). The mobile phase contained methanol 65% and water 35%, with a flow rate of 0.7 mL/min. Derivatives were detected spectrophotometrically at 254 nm. The concentration of histamine was calculated by interpolation in the corresponding linear calibration curve (amine peak area/amine concentration) between 0.05 and 1.00 mg/mL.

2.3.5. DNA extraction and amplification

DNA was extracted using a phenol-chloroform extraction procedure (Ferrario et al., 2012a). For each strain, DNA was extracted from 100 µL of an overnight broth culture in TSB. For tuna samples, pieces of 1 g were homogenized in 2 mL of TSB, and different aliquots were analysed at different times: 100 µL of homogenate was immediately subjected to DNA extraction (t=0). The remaining homogenate was incubated at 30 °C. After 8 and 16 h of incubation aliquots of 100 µL were taken for DNA extraction (t=8 and t=16).

The concentration and purity of the DNAs were determined with a UV-Vis spectrophotometer (SmartSpec[TM] Plus, Biorad, Milan, Italy).

PCR reactions were conducted in 25 µL mixtures: 100 ng of template DNA were added with 2.5 µL 10 X reaction buffer, 200 µM of each dNTP, 0.5 µM of primer forward and reverse, and 0.5 U *Dream Taq* DNA polymerase (Fermentas, Vilnius, Lithuania). Amplifications were carried out in a PCR-Mastercycler 96 (Eppendorf, Hamburg, Germany), with an initial denaturation of 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, selected annealing temperature (Ta) for 1 min, and 72 °C for 1 min. All reactions ends with a final extension of 72 °C for 7 min. Primers sequences, annealing temperature used and applications were reported in Table 2.2. Different amplification protocols were reported where necessary. All amplification products were separated in 1.5% (w/v) agars gel in 1 x TAE buffer (7 g/L Tris-acetate, 0.3 g/L EDTA, pH 8.0). Gene-Ruler DNA ladder mix (Fermentas) was used as a size marker.

2. Fishing sector model: *Morganella morganii*

2.3.6. *hdc* and *M. morganii* detection and 16S rDNA sequencing analysis

For amplification of the bacterial 16S/23S rRNA internal transcribed spacer (ITS) region, primer set G1 and L1 (Jensen et al., 1993) was used. The *hdc* gene was amplified using primers designed by Takahashi et al. (2003), using the primer set hdc-fw and hdc-rv. For specific *M. morganii* detection, the 16S rDNA-targeted primers Mm208F and Mm1017R (Kim et al., 2003) were used. In this last case amplification cycles were 30. To identify representative isolate from tuna samples and for DNA sequence analysis a 500 bp region of the 16S rRNA gene was sequenced. Amplification was performed as previously reported (Fortina et al., 2003), using the primer 16SFw and 16SRv. Briefly, the cycle applied is as follow: 2 min at 94 °C, then 5 cycles of 45 s at 94 °C, 45 s at 50 °C, 1 min at 72 °C, and then 30 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min at 72°C.

Nucleo Spin Extract II (Macherey-Nagel GmbH, Düren, Germany) was used to purify PCR products that were sequenced using the dideoxy chain-termination principle (Sanger et al., 1977), employing ABI Prism Big Dye Terminator Kit (Applied Biosystems, Foster City, CA). The reaction products were analyzed with the ABI PrismTM310 DNA Sequencer. The database searches were performed by using the Basic Local Alignment Tool (BLAST) programs (Altschul et al., 1997) from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Multiple-sequence alignments were constructed by using ClustalW software (Thompson et al., 1994). All primer sequences were reported in Table 2.1.

2.3.7. Primer design and PCR assay for *Morganella morganii* detection and quantification

The 16S rRNA gene sequences obtained for *M. morganii* strains DSM30164^T and MMlx were compared with those obtained from Genbank database for other *M. morganii* strains (Accession numbers DQ358146, DQ885260, DQ358145, DQ358144, DQ358131, AB089244) and other closely related enteric bacteria (*Providencia stuartii* NR024848, *Raoutella planticola* GU329705, *Proteus vulgaris* EU373433, *P. mirabilis* GQ205673, *Yersinia enterocolitica* HM007567, *Serratia fonticola* NR025339, *S. marcescens* AB571066, *Hafnia alvei* AY572428, *Erwinia carotovora* GU936989, *Citrobacter braaki* NR028687, *C. freundii* FN997609, *Enterobacter aerogenes* GQ337696, *E. cloacae* HM030748, *Pantoea agglomerans* HM130697, *Escherichia coli* HM486679, *Salmonella enterica* HM099659).

Primers Mm453F and Mm631R were designed on the basis of variable regions observed from the alignment and were synthesized by PRIMM (Milan, Italy). The specificity of the primer set was determined using BLAST and testing a total of 32 different Gram positive and negative bacteria (Table 2.2). PCR was performed as reported above, with 30 cycles and an annealing temperature of 65°C.

2.3.8. Quantitative PCR assay

Quantitative PCR was performed by using the SYBR Green chemistry in a CFX96 Real-Time PCR thermocycler (Biorad). The primer set Mm453F and Mm631R described above was used to develop the real-time assay. The qPCR reaction mixture was prepared in 15 µL: 7.5 µL of 2x SsoFast™ EvaGreen® Supermix (Biorad), 0.36 µL of each primer (0.3 µmol/L) and 5 µL of DNA. The reaction conditions were initial denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, and annealing at 65°C for 30 s. A positive control composed by DNA extracted from a pure culture of *M. morganii* DSM30164^T and a negative control as sterile, PCR-grade DNA-free water (Sigma-Aldrich), were used in each run. The identity of the PCR product was confirmed by performing a melting curve analysis comparing its melting temperature (T_m) with T_m of the product from the positive control. The T_m peaks of the products were calculated based on initial fluorescence curves by plotting negative derivative of fluorescence over temperature, versus temperature. The PCR product was also verified by sequence analysis of the obtained amplicons.

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2.3.9. Sensitivity studies with pure cultures

DNA extracted from an overnight *M. morganii* DSM30164[T] culture grown in TSB (9 x 10^7 CFU/mL) was decimally diluted from 563 ng to 0.056 pg/10 µL in a sterile Tris-EDTA buffer (TE), pH 8.0 and stored at -20 °C until further use. DNA was also prepared from a 10 fold dilution series of *M. morganii* cells in a concentration range of 9 to 9 x 10^6 CFU/mL. The number of CFU for each dilution was obtained by the standard plate count method using TSA plates. Standard curves were constructed plotting CFU versus threshold cycle (C_T) produced for the target gene. The correlation coefficient (R^2) and the efficiency of the amplification were calculated. All experiments were done in duplicate series and repeated three times to evaluate the repeatability of the real-time PCR assay. Results were obtained using the Biorad CFT Manager™ software (Biorad).

2.3.10. Detection studies with seeded and naturally contaminated fish samples

Portions of a filleted tuna fish sample (5 g), confirmed *M. morganii* free by conventional PCR methods was used for the seeding experiments. The sample was blended with 5 mLof buffered peptone water (Difco) in a blender for 2 min. After homogenization, each mixture obtained was seeded with 500 µL of 10^1 to 10^6 CFU/mL of *M. morganii* grown overnight in TSB. One uninoculated portion of fish sample was used as a negative control.

For naturally contaminated tuna fillets, samples were purchased from super markets in the Lombardy region. 10 g of each sample were aseptically cut, mixed with 10 mLof buffered peptone water and homogenized as above.

Five mLof the homogenate samples of seeded and naturally contaminated tuna were subjected to DNA extraction qPCR. The C_T obtained from seeded samples were used to determine the standard curve. C_T obtained from naturally contaminated samples, were used to calculate the CFU/mL present in each tuna fillets. Amplicons were confirmed by agarose gel electrophoresis and by sequence analyses.

2.3.11. DNA manipulation and library

To sequence the flanking regions of the *hdc* gene of *M. morganii* DSM30164[T], a plasmid library from *E. coli* DH5α was constructed.

10 µg of both the *M. morganii* DNA and the vector pUC19 were digested with different restriction enzymes and incubated at the optimal condition for the enzyme activity as described by suppliers: *Hind*III, *Pst*I, *Xba*I, *Sac*I, *Eco*RI (Roche, Madrid, Spain), *Bbu*I (Promega), *Sal*I, *Sma*I (Takara), *Bam*HI (BioLabs, Madrid, Spain). After 4 h of incubation, all the reactions were inactivated at 80 °C for 20 min. Digested DNA fragments and vector were incubated with T4 DNA ligase (USB) at room temperature for 16 h, inactivated at 70 °C and conserved at 4 °C. Ligation mixes were used for PCR amplifications. PCR was performed using AmpliTaq Gold DNA polymerase (Roche) in 50 µL, amplification reaction mixture by using an initial denaturation step at 95 °C for 10 s, followed by 30 cycles of 95 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, followed by a final elongation step at 72 °C for 10 min. All the amplifications were done by using a primer annealing into the plasmid and a primer located at the end of the known region to determine the nucleotide sequence surrounding *hdc* known region. Resulted bands were visualized in agarose gel and cut with bisturi. After purification using the Quiakit gel extraction kit (Quiagen, Madrid, Spain), DNA was resuspended in 30 µL of TE, and sequenced. Multiple sequence alignment and calculation of the phylogenetic relationships were performed as reported in 2.3.6 section.

2.3.12. Heterologous expression of *hdc* in *E. coli*

To clone into the pURI3 expression vectors, the histidine decarboxylase gene from *M. morganii* was amplified by PrimeSTAR HS DNA polymerase (Takara) DNA polymerase using the oligonucleotides 1046 and 1047 reported in Table 2.1. The amplification was made as described before, with an annealing step at 50 °C for 1 min.

After amplification, the 1.2 kb PCR products were gel-purified and inserted into pURI3 vectors by using the enzyme restriction- and ligation-free cloning strategy described previously for pURI3 vectors (Curiel et al., 2011). Briefly, the purified PCR products were used as homologous primer pair in a PCR

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reaction using the corresponding expression vectors as template. The product of this PCR was digested with *Dpn*I, that exclusively restrict methylated DNA and, later, with *Not*I, an enzyme that only cuts the original copies of pURI vectors. *E. coli* DH5α cells were transformed directly with the digestion product. The pURI3 expression plasmid carrying the *M. morganii hdc* gene (pURI3hdc) was sequenced to verify the presence of the gene. The plasmid was then transferred, for protein production, to the host *E. coli* BL21 (DE3).

2.3.13. Protein production, purification and activity
E. coli strain BL21 (DE3) carrying pURI3hdc was grown at 37 °C in LB medium containing 100 µg/mL ampicillin. To evaluate the best condition for the HDC protein production, cells were induced by adding IPTG (0.4 mM final concentration) when the culture reached 0.5 OD_{600nm}. After this, the culture was incubated at different temperatures (16, 22, 37 °C) for different times (4, 16 h). Cells were harvested by centrifugation (7000 rpm for 15 min at 4 °C), washed with 50 mM phosphate buffer (PB, pH 6.5) and mechanically disrupted by three French Press passages (1000 psi). The insoluble fraction was separated by centrifugation (12000 rpm, 30 min, 4 °C), and the supernatant was quantified using the Bio-Rad protein assay (Biorad). The proteins extracts were then run on SDS gel to check the cleavage, at the different condition tested.

For protein purification *E. coli* BL21 pURI3hdc was incubated at the best production condition. Culture was harvested, resuspended with 50 mM phosphate buffer, NaCl 300 mM (PBS, pH 6.5) and disrupted. The soluble fraction, was applied to a Talon Superflow metal affinity column (Clontech, Torrejon de Ardoz, Spain), equilibrated with PBS. The bound enzyme was eluted by applying a continuous gradient of imidazole concentration, from 10 mM to 150 mM. Fractions containing the eluted HDC were pooled and the protein was then dialyzed against PBS (pH 7.0). Samples were run on SDS gel.

The assay to determine HDC activity was performed in PB, in presence of 5 µM histidine and 400 µM PLP. The reaction was incubated at 37 °C for 10 min and 1 h. Afterward, the histamine formed was derivatized and detected by TLC as described by García-Moruno et al. (2005).

2.3.14. RNA isolation
Different growth parameters were chosen to simulate several environmental conditions, for the evaluation of the gene expression of *M. morganii*: nutrients concentration (medium LB or Tx), temperature (37 or 15°C), pH (7.0 or 4.5) and presence or absence of 5 mM histidine (LBH or TxH). LB broth was used as the basic environmental condition to which the gene expression in all other conditions was compared.

Aliquots (500 µL) of overnight *M. morganii* cultures in LB were inoculated into 50 mL of selected medium and conditions, in a shaker set at 200 rpm, until the cultures reached 0.3 OD_{600nm}. Cells were centrifuged at 4°C for 10 min, at 6500 rpm, washed with PBS (pH 7.4), and resuspended in 20 mL of the medium buffered at the chosen conditions, for 3 h. Cells from these cultures were harvested by centrifugation and washed with PBS. Total RNA was isolated using the NucleoSpin RNA II (Macherey-Nagel GmbH) according to manufacturer's protocol. Once mRNA concentration and purity were optically determined measuring the adsorbance at 260 and 280 nm with a UV-Vis spectrophotometer, mRNA integrity was verified by visualization of the two bands (16S and 23S RNA) by gel electrophoresis. RNA was finally treated with DNase1, Rnase-Free kit (Fermentas) at 37°C for 1h, and after addition of EDTA 50 mM to stop the reaction, was incubated at 65°C for 10 min. RNA was stored at -80°C and subsequently used for cDNA synthesis.

2.3.15. RT-PCR experiments
DNase-treated RNA was subjected to reverse transcription into cDNA with RevertAid First strand cDNA Synthesis Kit (Fermentas) in accordance with manufacturer's instructions. In order to quantitatively measure the expression of the *M. morganii* genes, amplification of an endogenous control gene was performed simultaneously, and the relative expression level between genes, and the endogenous control gene was assessed. The quantity of 16S rRNA in each sample was used for the

endogenous control, assuming that the expression of 16S rRNA is constant for all cells used in this analysis. Specific primer pairs were designed on the nucleotide sequence of the DNA region detected around the *hdc* gene of *M. morganii* and reported in Table 2.1. Amplifications were performed in triplicate in a final volume of 15 µL, using 2 µL of appropriate dilution of cDNA template, 0.3 µmol/L each of the corresponding primers, and 7.5 µL of the SsoFast_ EvaGreen_Supermix (Bio-Rad). The reaction conditions were the following: initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing at 60 °C for 30 s. The identity of the PCR product was confirmed by performing a melting curve analysis. As a control, PCR of DNase-treated RNA was performed with the same primers to check for any DNA contamination. Results were analysed using the Bio-Rad CFT Manager_software (Bio-Rad) for relative quantification.

2.3.16. Genomic fingerprinting of *M. morganii* strains

M. morganii strains isolated from different ecological niches were typed by combined analysis of repetitive element (REP) typing using primers (GTG)$_5$ and BOXA1R (Versalovic et al., 1994; De Urraza et al., 2000) and random amplification of polymorphic DNA-PCR (RAPD) typing with primer M13 (Rossetti & Giraffa, 2005). Annealing temperatures of 42°, 48° and 38 °C were used respectively for (GTG)$_5$, BOXA1R and M13. The PCR products were analyzed by electrophoresis and photographed as reported above. The value for the reproducibility of the assay, evaluated by analysis of repeated DNA extracts of representative strains was greater than 93%.

Banding pattern similarity was evaluated by construction of dendrograms using the NTSYSpc software, version 2.11 (Applied Biostatics Inc., NY, USA), employing the Jaccard similarity coefficient. A dendrogram was deduced from a similarity matrix by using the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm. The faithfulness of the cluster analysis was estimated by calculating the cophenetic correlation value for each dendrogram.

2.3.17. Statistical analysis

All microbial counts were converted to log CFU per g of sample before analysis. Significant differences ($P<0.05$) among means were determined with a long-way analysis of variance. Relationship between microbial count and histamine level was then assessed using Pearson correlation matrices. A Pearson coefficient (r) from 0 to 0.3 was considered a weak correlation, moderate if it was included between 0.3 and 0.7, strong if $r > 0.7$.

2. Fishing sector model: *Morganella morganii*

Table 2.1. Bacterial strains analysed in this study

Bacterial strain	Code	Origin
Morganella morganii	MoMo	DSM30164[T, a]
	MMlx, MMp1, MMp3, MMp5, MMp7, MMp11, MMq1, MMq3, MMq4, MMq6, MMq9, MMq11, MMq13,	Our collection[b]
	MR210, MR234, MR462, MR798, MR894, VA1342/03	Our collection[g]
Aeromonas hydrophila	AeHy	Our collection
Bacillus subtilis	BaSu	DSM10[T]
B. thuringiensis	BaTh	Our collection
Brevundimonas vesicularis	BrVe	Our collection
Chryseobacterium indologenens	ChIn	Our collection
Chryseomonas luteola	ChLu	Our collection
Citrobacter freundii	CuFr	Our collection
Enterobacter aerogenes	EnAe	Our collection
Enterococcus faecalis	EnIs	BCCM/LMG[c] 19456[T]
E. faecium	EnUm	ATCC[d] 19434[T]
Escherichia coli	EsCo	CNRS[e] JM101
Hafnia alveii	HaAl	Our collection
Klebsiella pneumoniae	KlPn	Our collection
Kocuria marina	KoMa	Our collection
Lactobacillus fermentum	LaFe	Our collection
L. helveticus	LaHe	ATCC15009[T]
L. paracasei	LaPa	DSM5622[T]
Lactococcus garvieae	LaGa	DSM20684[T]
L. lactis	LaLa	Our collection
Moellerella wisconsiensis	MoWi	Our collection
Proteus vulgaris	PrVu	Our collection
Providencia alcalifaciens.	PrAl	Our collection
Pseudomonas aeruginosa	PsAe	DSM939
P. fluorescens	PsFl	NCDO[f] 1524
P. fragi	PsFr	Our collection
P. paucimobilis	PsPa	Our collection
P. putida	PsPu	ATCC12633[T]
Ralstonia pickettii	RaPi	Our collection
Sphingomonas paucimobilis	ShPa	Our collection
Staphylococcus epidermidis	StEp	Our collection
Stenothrophomonas maltophila	StMa	Our collection
Streptococcus salivarius	StSa	Our collection

[a] DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
[b] Our collection-Bacteria isolated from food products
[c] BCCM/LMG-Belgian Co-ordinated Collections of Microorganisms
[d] ATCC- American Type Culture Collection
[e] CNRS-Centre National de la Recherche Scientifique
[f] NCDO-National Collection of Dairy Microorganisms
[g] kindly provided from Professor Rossolini of Laboratory of Physiology and Biotechnology of Microorganisms, Department of Biotechnology, University of Siena

Table 2.2. Primer sets and correlated information used in this study

Primer	Sequence (5' – 3')	Ta (°C)	Application	References
hdc-fw	TCHATYARYAACTGYGGTGACTGGRG	58	*hdc* gene detection	Takahashi et al., (2003)
hdc-rv	CCCACAKCATBARWGGDGTRTGRCC	58	*hdc* gene detection	Takahashi et al., (2003)
Mm208F	CTCGCACCATCAGATGAACCCATA	62	*M. morganii* detection	Kim et al., (2003)
Mm1017R	CAAAGCATCTCTGCTAAGTTCTCTGGATG	62	*M. morganii* detection	Kim et al., (2003)
G1	GAAGTCGTAACAAGG	54	ITS	Jensen et al., (1993)
L1	CAAGGCATCCACCGT	54	ITS	Jensen et al., (1993)
16SF	AGAGTTTGATCCTGGCTCAG	Section 2.3.6.	16S	Lane (1991)
16SR	CTACGGCTACCTTGTTACGA	Section 2.3.6.	16S	Lane (1991)
1046	CATCATGGTGACGATGACGATAAGATGACTCTGT CTATCAATGATCAAA	50	Cloning in pURI3	This study
1047	AAGCTTAGTTAGCTATTATGCGTATTATGCCGCG TGTAAGTTAAAATCC	50	Cloning in pURI3	This study
1044	GTCATAAAGTTGTAGCCAATA	50	DNA library	This study
1054	CGTCAGAACGCGTCTGGAGAGAAC	50	DNA library	This study
1233	ACGGGATAACAATTTCACACAGGA	50	DNA library	This study
1224	CGCCAGGGTTTTCCCAGTCACGA	50	DNA library	This study
1053	GCAGTGATAATGGCAGTGGTATC	50	DNA library	This study
1064	CGCCACTCTGAGCCTGATTG	50	DNA library	This study
1069	CTGGAACATATTTTAATGCAG	50	DNA library	This study
1070	GGTACGCTGATATTTATTATTG	50	DNA library	This study
1	TATGATGCCAGTGCGTCGCATC	50	DNA library	This study
Mm453F	TTTCAGTCGGGAGGAAGGTG	65/60	qPCR, RT-PCR	Ferrario et al., (2012b)
Mm631R	GGGGATTTCACATCTGACTC	65/60	qPCR, RT-PCR	Ferrario et al., (2012b)
hdcRT fw	ACTCAATCGGTGTTTCCGGC	60	RT-PCR	This study
hdcRT rv	TGTGACCGTTACGTGAACCG	60	RT-PCR	This study
hisRS RT fw	CGGTGAATCCACCGATATCG	60	RT-PCR	This study
hisRS RT rv	TGAGTGAACTGACGCAGACG	60	RT-PCR	This study
(GTG)$_5$	GTGGTGGTGGTGGTG	42	Molecular typing	Versalovic et al., (1994)
BOXA1R	CTACGGCAAGGCGACGCTGACG	48	Molecular typing	De Urraza et al., (2000)
M13	GAGGGTGGCGGTTCT	38	Molecular typing	Rossetti & Giraffa (2005)

2. Fishing sector model: *Morganella morganii*

2.4. RESULTS AND DISCUSSIONS

Usually, fresh tuna has a shelf life of about 11 days when stored at 4°C. Important factors contributing to the microbiological complexity of seafood are temperature, water activity, pH. This kind of food is typically spoiled by aerobic Gram negative bacteria belonging to the genera *Pseudomonas, Moraxella, Acinetobacter, Shewanella, Flavobacterium, Vibrionaceae* and *Aeromonadaceae* (Gram & Huss, 1996). Before studying directly our bacterial model *M. morganii*, we started exploring the bacterial population of fresh tuna samples and evaluating the correlation between bacterial counts and histamine content.

2.4.1. Microbiota in fresh filleted tuna and microbiological changes during storage

Tuna fish samples were purchased from two different suppliers. The WM is a large distributor of fish and seafood products in Milan that supplies many local retail markets and restaurants. SM tested are part of large distribution chains that have their own trading, transportation and processing centers.

A total of 14 filleted tuna fish samples were collected during three months: 6 samples were purchased from WM and 8 from different local SM (Table 2.3). In WM, fishes were filleted *in situ* and then displayed on ice beds, while at SM, filleted tuna were placed on crushed ice in refrigerated container during sale hours.

On the basis of bacterial enumeration in PCA and VRBG (Table 2.3), unacceptable tuna samples were 6/14, that correspond to the 46% of total samples; these filleted tunas showed values of total viable bacterial count higher than recommended microbiological limits in fish ($<10^6$ CFU/g). Among these, unacceptable samples M, N, P, Q samples, were purchased at different times from the same wholesaler. Here several possible risk factors were observed, such as mishandling with cross-contamination from the environment, and handling in non-refrigerated conditions during fillet preparation.

After a temperature abuse of 3 h at 25 °C and 1 day storage at 6-8 °C to simulate the transport and the household storage conditions, high levels of total and enterobacterial counts (with an increase that could reach 3 log cycles) were reached by these marketed fish. Values of 9.5-10.6 log CFU/g and 4.4-6.5 log CFU/g were reached for respectively PCA and VRBG counts. Finally, also during a controlled storage condition (overnight at 6-8 °C), the total count reached values >9 log CFU/g within 1 day. For samples purchased at different supermarkets two samples showed high values of viable total count and *Enterobacteriaceae*, as reported in Table 2.3. The remaining six SM tuna fish samples showed good quality, with an initial low number of viable microorganisms (Table 2.3) and with reasonable values during storage at the different time/temperature conditions tested (PCA count ranging from 5.2 to 8.0 log CFU/g; VRBG counts ranging from 2.6 to 4.8 log CFU/g).

2.4.2. Histamine content in fish

All tuna fish samples had low histamine levels (Table 2.3). Samples from SM showed histamine level <1 ppm, except for sample FT1 with 3.2 ppm. Fish from WM have histamine levels of 1 to 10 pp. Although these histamine levels do not represent poor quality fish, according to FDA and EU guidelines (50 and 100 ppm respectively) (FDA/CFSAN 2001; EC 2005), it should be noted that the highest histamine content was found in WM samples that also showed high initial bacterial count.

Table 2.3. Overview of the main results obtained on tested filleted tuna fish

Sellers	Samples	PCA counts (log CFU/g)	VRBG counts (log CFU/g)	No of isolates[a] (*hdc* positive strains)	ITS clusters	Identification cluster representative[b]	Histamine (ppm)
WS1	I	5.5 ± 1.5	< 2	9 (0)	NT[d]	NT	<1[c]
	L	5.2 ± 0.3	< 2	8 (0)	NT	NT	1.0 ±0.2
WS2	M	7.8 ± 1.1	2.5 ± 0.5	15 (12)	A	*Citrobacter koseri Enterobacter* spp.	5.6 ± 0.5
	N	8.3 ± 0.5	3.5 ± 0.1	15 (13)	A	*C. koseri Enterobacter* spp.	7.8 ± 0.4
	P	7.4 ± 1.9	2.5 ± 1.5	10 (5)	B	*Morganella morganii*	8.8 ± 0.7
	Q	8.0 ± 0.1	3.5 ± 0.2	11 (10)	B	*M. morganii*	9.7 ± 0.3
SM1	A	5.0 ± 0.2	< 2	6 (2)	C D	*Hafnia alvei* *Moellerella winsconsensis*	<1
	B	5.5 ± 2.9	< 2	7 (0)	NT	NT	<1
SM2	C	5.7 ± 1.5	<2	6 (0)	NT	NT	<1
	E	6.7 ± 0.7	2.9 ± 0.7	9 (0)	NT	NT	<1
SM3	G	5.6 ± 0.3	< 2	9 (0)	NT	NT	<1
	H	5.3 ±0.4	2.0 ± 0.1	8 (0)	NT	NT	<1
SM4	FT1	6.4 ± 0.2	4.1 ± 0.2	11 (1)	E	*M. morganii*	3.2 ± 0.1
	FT2	2.8 ± 0.1	2.0 ± 0.1	17 (0)	NT	NT	<1

[a] selected from VRBG plates
[b] the isolates were identified by 16S rDNA sequence analysis
[c] tested but not detected
[d] NT, not tested

2. Fishing sector model: *Morganella morganii*

2.4.3. Isolation and identification of potential histamine-forming bacteria

With the aim to isolate histamine-forming bacteria, 141 colonies were randomly selected from different VRBG plates obtained by the analysis of fresh and conserved tuna samples, as above reported. 68 colonies were selected originating from WM and 73 from SM. Preliminary physiological study reported in Materials and Methods section, were performed to assess the belonging to the *Enterobacteriaceae* family (data not shown). After this, new isolates were subjected to PCR amplification tests. Forty-three strains, representing 30.5% of total pure cultures screened, were positive for the presence of *hdc* gene. The incidence of histidine-decarboxylating bacteria appears low, if estimated on all isolates. However, it is relevant to observe that 40 of the 43 histamine-forming bacteria came from WM samples; in contrast, only 3 of the 73 strains isolated from SM samples were *hdc* positive (Table 2.3).

For a rapid identification of the strains, an ITS amplification analysis was carried out, to group strains in clusters characterized by the same electrophoretic profile. ITS analysis is an effective technique for a first clusterization step of a great quantity of different isolates. For many known bacterial groups its response is often species-specific. In this way, further experiments, such as species-specific probes, or sequencing of 16S rRNA gene, could be carried out only on representative strains of each cluster. Through ITS analysis we obtained 5 different clusters, as reported in Figure 2.3.
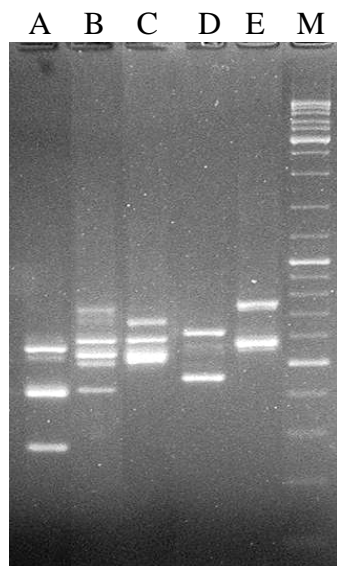
Figure 2.3. ITS profiles of representative strains of each cluster obtained: A, B, C, D, E. M: Marker.

Cluster A grouped 58% of the total isolates, followed by cluster B (35%). The remaining clusters (C, D, and E) were characterized by the presence of one or two isolates (Table 2.3).

For the genotypic identification, 16S rRNA sequence analysis of representative strains of each cluster was obtained (results reported in Table 2.3). Strains grouped in cluster B and E were identified as *M. morganii* despite they showed different ITS profiles. This fact indicates the presence of a genotypic intra-species heterogeneity, not yet investigated, that required further attention. With regard to the main cluster (A), 16S rRNA sequencing showed a high sequence homology (>98%) with different species, *C. koseri/C. diversus, E. hormaechei/E. ludwigii.* These results underscore the taxonomic confusion that dominates within the group of *Enterobacteriaceae*. Indeed these bacteria have undergone frequent, sometimes confusing changes in nomenclature with several reorganizations of genera and validation of species, most of which were later discarded. It is the case of *C. diversus*, now considered the former name of *C. koseri*, while *E. hormaechei* and *E. ludwigii* are described as

2. Fishing sector model: *Morganella morganii*

different species, even if they are phylogenetically closely related (Hoffmann et al., 2005). This confusion makes it difficult to clearly differentiate particular species, including our applied molecular methods.

2.4.4. Histamine formation by bacterial isolates

Prevalent histamine formers detected in the filleted tuna fish were *Citrobacter/Enterobacter* isolates. They represent the major portion of total *hdc* positive strains and the dominant population of samples M and N. These species are usually isolated from various species of scombroid fish and are considered weak histamine-formers, with the exception of *E. aerogenes* (Bjornsdottir et al., 2009). In our experiments, the ability of these strains to produce histamine was variable, producing from 81 to 259 ppm of histamine. The 16 *M. morganii* strains, representing the main *hdc* positive species in samples P and Q, produced from 250 to 700 ppm of histamine and could be considered prolific histamine-formers.

Despite this, the low histamine accumulation in fresh tuna indicates that these bacteria constituted a minor portion of the bacterial population. However, it is important to observe that if the products are kept for longer time under temperature abuse, fish could represent a potential danger for consumption, due to a rapid accumulation of histamine, following an increase of histamine-forming bacteria.

2.4.5. Fish quality evaluation by PCR

A direct extraction of DNA from fish homogenates, followed by PCR amplification of *hdc* gene did not reveal positive signals in our samples. These results are in agreement with other published results regarding the high detection limit of the PCR assay (about 10^4 CFU/mL) (Bjornsdottir-Butler et al., 2011). However, after a 16-h enrichment step of the fish homogenates at 30 °C, positive signals were detected in M, N, P and Q samples. The possibility to carry out rapid and specific PCR assays on food matrices represents an interesting tool for a rapid evaluation of safety and quality of foods. In this context, the detection of *hdc* gene in fish products can be used as indicator.

2. Fishing sector model: *Morganella morganii*

In order to develop an effective control system it is necessary to have reliable detection and enumeration methods, able of monitoring the presence of undesired microorganisms rapidly, according to the short time of preparation, distribution and marketing of perishable food products.

For *M. morganii* there are not rapid and selective cultural methods able to unequivocally discriminate this species from other related species within enteric bacteria. Niven's medium, which is capable of detecting amino acid decarboxylation in enteric bacteria, is used to enumerate histamine-producing bacteria in fish (Niven et al., 1981). However, the procedure is not suitable for the detection of specific species. The use of conventional selective media for *Enterobacteriaceae* also requires further biochemical tests for a reliable species identification (O'Hara et al., 2000).

Today molecular methods are preferred for a rapid and specific detection of bacterial species and numerous conventional PCR assays are now employed to evaluate the presence/absence of many microorganisms. Most recently, qPCR assays for the detection and quantification of important food pathogens have been developed. qPCR provides a means of detecting and quantifying DNA targets by monitoring PCR product accumulation, measured by increases fluorescence during cycling, and a number of different approaches have been developed to generate the fluorescence signal (SYBR Green, FRET, TaqMan) (Postollec et al., 2011).

Regarding *M. morganii*, a conventional PCR based on 16S rRNA specific primers has been developed (Kim et al., 2003). However, for a sensitive and specific detection of this species in food samples, PCR assay was coupled with Southern hybridization. Moreover, a specific molecular tool for the quantification of *M. morganii* is not yet available. In the present study, a simple, specific and reproducible real-time PCR method based on SYBR Green fluorescence for detection and enumeration of *M. morganii* from fish samples is reported.

2.4.6. Specificity of primers for *M. morganii* identification

Conventional PCR and qPCR assays for *M. morganii* detection was developed using a new primer set targeted to the 16S rRNA gene. For this purpose, the 16S rRNA sequences of *M. morganii* type strain and strain MMlx obtained in this study were comparatively analysed with other sequences of *M. morganii* available in Genbank (Figure 2.5).

Figure 2.5. Sequence alignment of 16S rRNA region in *M. morganii*. The sequences that were considered as primer targets in this study are underlined in black.

2. Fishing sector model: *Morganella morganii*

Subsequently, sequences of 16S rRNA genes of other related enteric bacteria were also compared (Figure 2.6). These alignments allowed to find two regions, specifically V3 and V4 regions of 16S rRNA gene, highly conserved in all *M. morganii* strains tested, that showed significative differences with the most related enteric bacteria. From these data, we designed two primers (Mm453F and Mm631R, reported in Table 2.2) within V3 and V4 regions (Figure 2.6), characterized by useful sequence variations in two or more base pairs located at the 3' end, allowing the amplification of a 179 bp fragment of the 16S rRNA gene.

```
            Species              V3 region                    V4 region

                              460       470              620       630
M. morganii DSMZ 30164ᵀ  ACTTTCAGTC GGGAGGAAGG TGTCAA   TGAGTCAGAT GTGAAATCCC CGGG
M. morganii MM1x         .......... .......... ......   .......... .......... ....
Y. enterocolitica        ........CG A......... CA.A..   .A........ .......... ..C.
S. marcescens            ........CG A......... ..GTG.   .A........ .......... ....
S. fonticola             ........CG A......... GT..TG   .A........ .......... ..A.
S. enterica              ......CACG ....A...A. G.GTGT   CC....C..A .G........ ....
R. planticola            ....C..... .......... C..TG.   .A.-.T.-.. .......... ....
P. vulgaris              ........CG .......... ..AT..   .A........ ......G... ..A.
P. mirabilis             ........CG .......... ..AT..   .A........ ......G... ..A.
P. carotovora            ........CG A......... C.GT..   .A...TG... .......... ....
P. agglomerans           ........CG A......... C.GTG.   .A........ .......... ....
H. alvei                 ........CG A......... CA.T..   .A........ .......... ..A.
E. coli                  ........CG .......... GAGT..   .A........ .......... ....
E. cloacae               ........CG .......... ...TGT   CA....G... .......... ....
E. aerogenes             ........CG A......... ...TG.   CA....G... .......... ....
C. freundii              ........CG A......... ...TGT   CA....G... .......... ....
C. braakii               ........CG A......... .A.TGT   CA....G... .......... ....

                              ──────→                      ←──────
                              Mm453F                       Mm631R
```

Figure 2.6. Sequence alignment of a 16S rRNA region in *M morganii* and other related enteric bacteria. The sequences that were considered as primer targets in this study are underlined in black.

During this step, we found that primers based on 16S rRNA gene reported in the literature (Kim et al., 2003) lacked specificity and could yield false-positive results, such as *Providencia* spp. and *Moellerella* spp. (Figure 2.7A).

The specificity of this new primer set was tested in conventional PCR assays using as target DNA extracted from different *Enterobacteriaceae* species (Figure 2.7B). Furthermore, a set of bacteria commonly found in foods was also used (Table 2.2). Several conditions of amplifications, focusing mainly on the annealing temperature and the number of thermal cycles were tested. The conditions described in the Materials and Methods resulted to be specific for *M. morganii*. The high specificity of the new primers and their high annealing temperature (65°C) allowed us to develop a qPCR assay for the quantification of *M. morganii*.

2. Fishing sector model: *Morganella morganii*
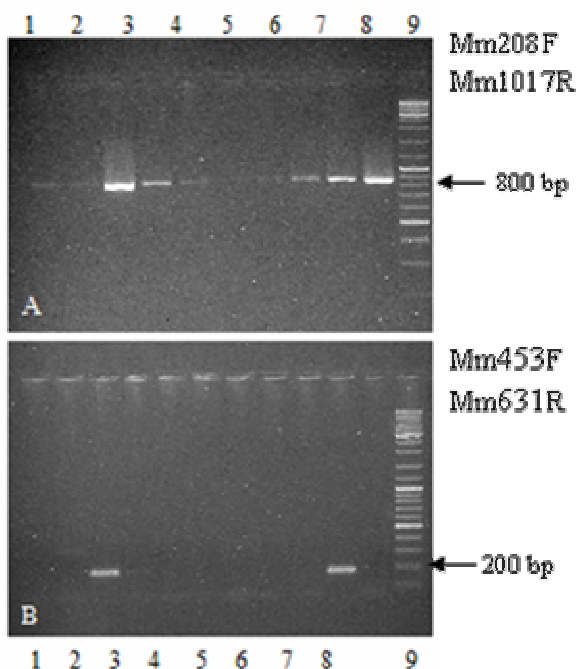
Figure 2.7. Conventional PCR assay for the identification of *Morganella morganii*. A) PCR products obtained after amplification with primer set Mm208-Mm1017 (Kim et al., 2003b); B) PCR products obtained after amplification with primer set Mm453-Mm631 obtained in this study. Lanes: 1. *M. morganii* MMlx; 2. *Providencia alcalifaciens* PrAl; 3. *Proteus vulgaris* PrVu; 4. *Citrobacter koseri* CiKo; 5. *Hafnia alvei* HaAl; 6. *Citrobacter freundii* CiFr; 7. *Moellerella wisconsiensis* MoWi; 8. *M. morganii* DSM30164[T]; 9. Marker.

2.4.7. Development of qPCR assay

The qPCR assay was developed by using the SYBR Green chemistry, that provides a simple and economical format for detecting and quantifying PCR products (Panicker et al., 2004; Nam et al., 2005). To evaluate the specificity of primers in qPCR, extracted DNA from cultures of *M. morganii* strains and closely related strains (*Provindencia alcalifaciens*, *Hafnia alvei*, *Citrobacter freundii*, *Moellerella wisconsiesis*) were examined as templates. All *M. morganii* strains tested were positive in the real-time PCR assay and produced mean C_T values of 21.76 ± 0.5. Amplification of DNA from all non-*Morganella* strains did not result in any PCR products. Specific PCR products were identified by melting curve analysis and a reproducible distinct melting point (T_m) of 84°C was observed for all *Morganella* amplicons (Figure 2.8). Negative controls and samples confirmed negative by both real-time and conventional PCR assays did not show peaks in T_m that corresponded to 84°C. Specificity of primers and absence of unspecific products or primer dimers were tested by analyzing the reaction in agarose gel and by sequence analysis of the obtained amplicons. The molecular weight of the amplified product was as expected, and no other bands were visible. Moreover, sequence analysis of the amplicons obtained showed a 100% sequence homology with the 179 bp fragment of the 16S rRNA gene of *M. morganii* species.
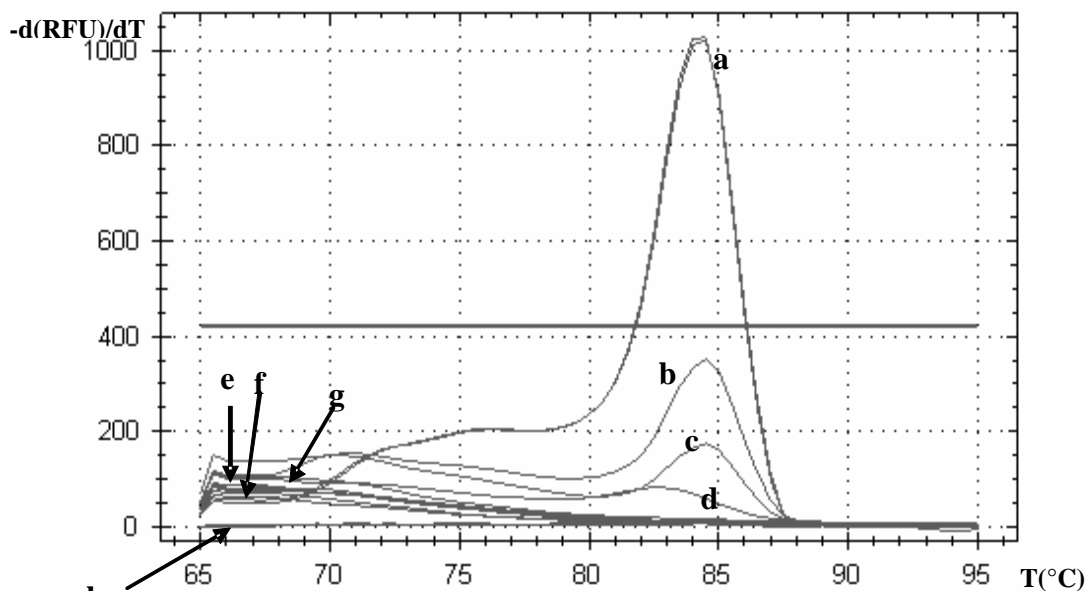
Figure 2.8. Melting curve analysis of real-time PCR products. Shown are the results for different *M. morganii* DNA concentrations (a,b,c), *P. alcalifaciens* (d), *H. alvei* (e), *C. freundii* (f), *M. wisconsiesis* (g), negative control (h).

2.4.8. Sensitivity and detection limits in pure culture

DNA obtained from a *M. morganii* culture at 9 x 10^7 CFU/mL was serially diluted tenfold to determine the DNA standard curve. The minimum detection sensitivity was 0.563 pg of pure DNA, with a C_T value of 31.96 ± 0.21. This value corresponds to DNA extracted from 9 cells of *M. morganii*. The linear range of detection spanned from 6 log cycles of DNA ranging from 56.3 ng to 0.563 pg (Figure 2.9). The correlation between the Log Starting quantity and C_T was linear ($R^2 = 0.992$).

A linear plot of C_T values against the log concentration (9 to 9 x 10^6 CFU/mL) in water revealed that the cell detection was linear up to 9 CFU/mL, with a C_T value of 30.38 ± 0.1. The regression coefficient ($R^2 = 0.910$) obtained indicates a good efficiency of the DNA extraction method irrespective of the initial number of cells in the sample.

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2. Fishing sector model: *Morganella morganii*

$$y = -3.3822x + 35.196$$
$$R^2 = 0.992$$
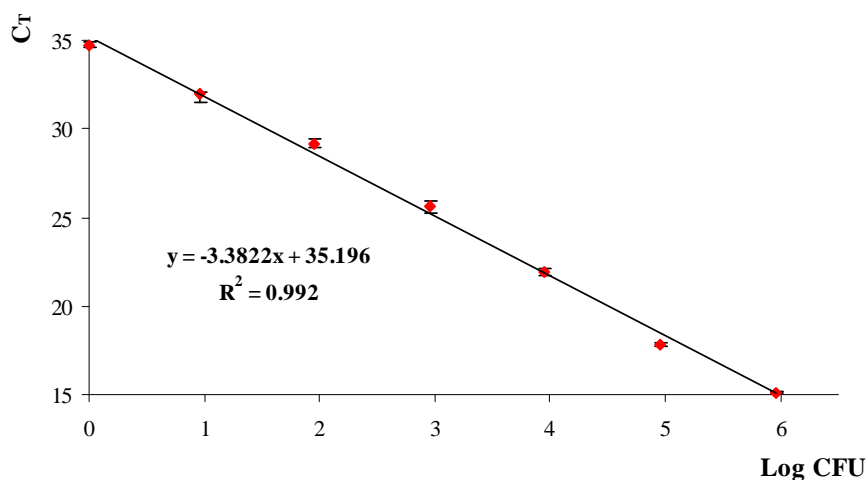
Figure 2.9. Standard curve showing the linear relationship between CT values and Log CFU for serially diluted DNA obtained from an overnight *M. morganii* culture (9 x 10^7 CFU/mL).

2.4.9. Quantification of *M. morganii* in seeded and naturally contaminated fish
When carrying out the real-time assay using the DNA extracted from seeded fish samples (10^1 to 10^6 CFU/mL), the results showed a linear plot of C_T values against a cell range from 10^2 to 10^6 CFU/mL (Figure 2.10), with regression coefficient (R^2) of 0.947. The quantification limit was in the order of 10^2 CFU/mL. The maximum threshold C_T value for the seeded fish samples was determined at 32.25 ± 0.38. No signals were detected for the samples not inoculated.
The qPCR based protocol was applied to detect and quantify *M. morganii* in 11 filleted tuna fish samples, taken from different super market in the Lombardy region, in the Northwest of Italy. When qPCR analysis was performed on DNA extracted at the time of sampling (t=0), all samples were negative. After an overnight enrichment (t=16), two of the 11 samples gave a positive signal by qPCR. The results were confirmed by agarose gel electrophoresis and by sequence analysis of the amplicons.
These data indicate that a *M. morganii* population below the quantification limit was present in two samples and it was able to produce a signal in real-time after enrichment.

This method can improve the microbiological control of this pathogenic microorganism during the fish production and distribution, in order to minimize histamine formation during storage.

2. Fishing sector model: *Morganella morganii*

$$y = -2,682x + 35,549$$
$$R^2 = 0,947$$
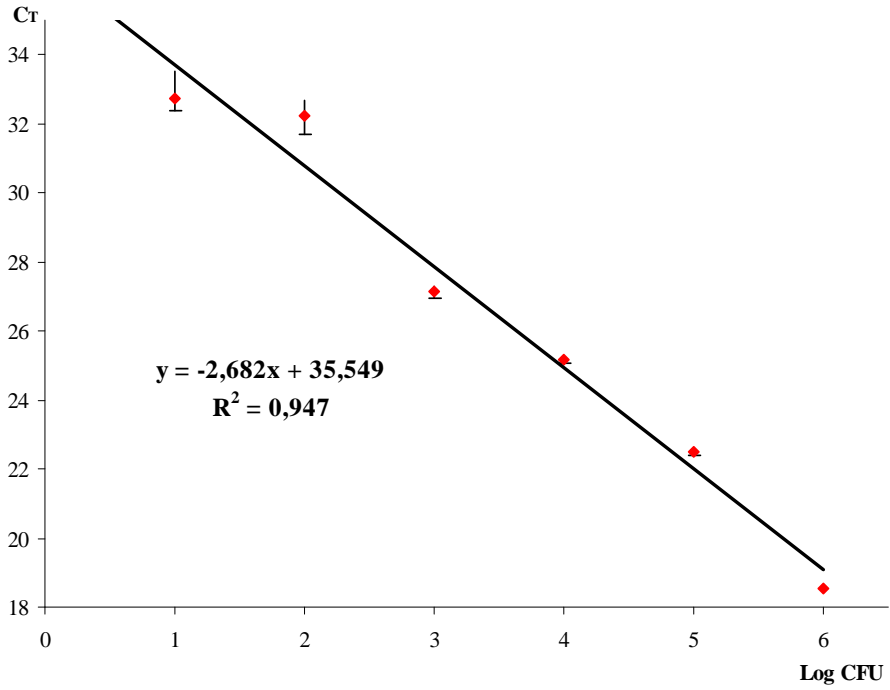
Figure 2.10. Detection of *M. morganii* in seeded fish homogenates using qPCR.

2. Fishing sector model: *Morganella morganii*

The ability of *M. morganii* to produce an active histidine decarboxylase is one of the causes of histamine accumulation in fish naturally rich in free histidine (Kanki et al., 2007). The responsible of this decarboxylation is the enzyme HDC. Little information are available for *M. morganii hdc* genetic cluster, since no genome sequences are available up to now. The aim of this PhD thesis part is to improve the knowledge of *M. morganii* HDC, both at genotypic and phenotypic level, for a better control of the histamine in fish products.

2.4.10. Sequence analysis of the *hdc* cluster of *M. morganii*

To improve the knowledge at genome level and to study the expression of the PLP dependent *hdc* of *M. morganii*, a DNA library of the type strain was constructed. The obtained region of 7681 bp including the *hdc* gene, showed the presence of other three open reading frames (ORFs); we designated these ORFs as genes related to histidine decarboxylation. In Figure 2.11, the gene cluster organization obtained was compared with other Gram negative histamine producers.
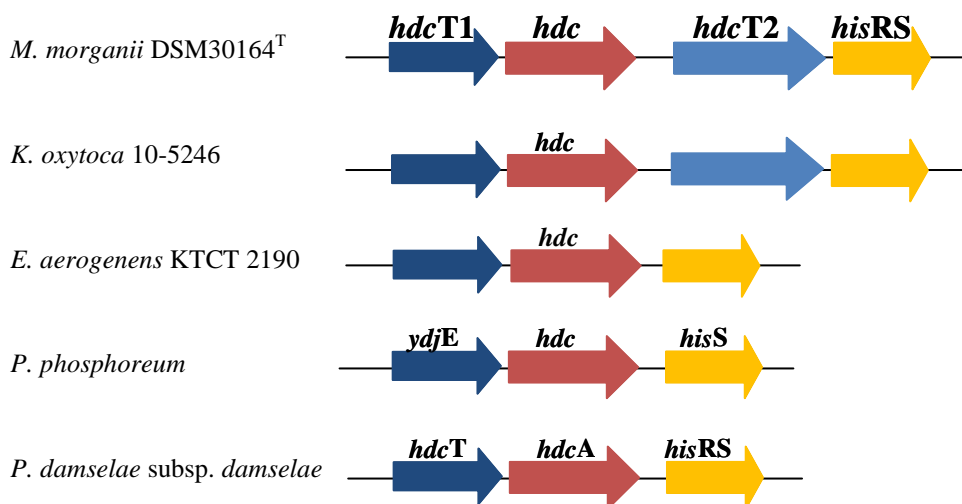
Figure 2.11. Schematic representation of the histidine decarboxylase cluster of *M. morganii* DSM30164[T], *K. oxytoca* 10-5246, *E. aerogenens* KTCT 2190 (CP002824), *P. phosphoreum* (AY223843) and *P. damselae* (AB363972). Arrows indicate ORFs. Genes coding for HDC are represented in red, putative antiporters or amino acid permeases are coloured in blue, while histidyl-tRNA sinthetase are yellow.

The best similarity was detected with the genome sequence of *K. oxytoca* 10-5246 (Bioproject: PRJNA52145, Accession: AGDM00000000). These two bacteria are the only that show the presence of the second histidine/histamine antiporter.

Analysing the sequence obtained, this region showed the presence of two genes (*hcd*T1 and *hdc*T2) codifying for basic aminoacid/polyamine transporter (antiporter) family, the first located 50 bp upstream the *hdc* and the second one 206 bp downstream, as shown in Figure 2.11. These genes were not homologous, but they were different in length (1361 and 1443 bp respectively). *hdc*T1 is similar to an hypothetical protein of *K. oxytoca* (subsequently identified as a putative antiporter trough BLAST analysis) and to an aminoacid permease of *E. aerogenens,* both with an identity (ID) of 82 % (coverage, Cov 98%). A minor degree of ID (73%, Cov 78%) was observed with an histidine/histamine antiporter (*hdc*T) of *P. damselae*. *hdc*T2 shows the most significant similarity with another hypothetical protein of *K. oxytoca* (ID 60%, Cov 89%; identified as an antiporter again), and at a lower similarity with an arginine/agmantine antiporter of *P. damselae* (ID 52%, Cov 90%), an aminoacid permease of *E. aerogenes* (ID 55%, Cov90%), and with the *hdc*T of *P. damselae* (ID 51%,

Cov 72%). Sequences alignments of HdcT1,2 with other amino acid/amine antiporters (HdcC of *Lactobacillus buchneri*, HdcP of *Staphylococcus capitis*, and HdcT of *P. damselae*) identified several conserved residues of histidine/histamine antiporter proteins. Regarding *hdc*, in comparison with sequences of other Gram negative bacteria, a high degree of similarity was found. In particular, the highest ID was obtained with the sequence of *hdc* of *R. planticola* (86%, Cov 99 %). A 1272 bp ORF was found 10 bp downstream the stop codon of *hdc*T2 (Figure 2.11). The deduced aminoacid sequence showed significant similarity to histidyl-tRNA synthetase gene (*his*RS) of the others gram negative histamine producing bacteria, such as *K. oxytoca*, *E. aerogenes* and *P. damselae*, ranging from 81 to 78% amino acidic identity. These sequences were compared and conserved motifs of class II aminoacyl-tRNA synthetase were identified (Figure 2.12), as already reported for *E. coli* (Guth & Francklyn, 2007).

```
M_mo     -MEKIQSIRGMRSVLPEETPVWQWLENRIRNITSRYGYQEVRLPILEPVALFERAVGEST 59
K_oxy    -MNKIQAIRGMRDVLPDETPMWQWLERKLHHMAFRYGYQEMRLPLLEPVTLFERAVGEAT 59
E_aer    MSAKLKAIRGMRDVLPAETPIWQWLENKFRRLTYRYGYQEIRLPLLEPVALFERAVGEAT 60
P_dams   --MDIKSIRGMKDILPTETPIWQWVEKVLRKATKNYGYKEIRLPILEPIELFQRAVGEAT 58

M_mo     DIVSKEMYNFLDKSGEHITLRPEGTSGCVRTVIENNMCYNTTQRLWYQGPMFRYERPQKG 119
K_oxy    DIVSKEMYDFTDKSGEHITLRPEGTSGCMRAVLENNMCYNKSQRLWYQGPMFRYERPQKG 119
E_aer    DIVAKEMYNFTDKSGEQITLRPEGTSGCMRAVLENNLCYNKTQRLWYQGPMFRYERPQKG 120
P_dams   DIVSKEMYDFIDKSNEHITLRPEGTSGCVRSVIENNLCYNNTQRLWYQGPMFRYERPQKG 118

M_mo     RLRQFTQFGVETFGMPGADVDAELIFMVKDIFKALGVDKHVRLEINSLGTPEERSEHRQQ 179
K_oxy    RLRQFTQFGIEAFGMSGADVDAELIFMVRDLFEDLNITQNVRLEINSLGTSAERQAHREE 179
E_aer    RLRQFTQFGAEAFGMAGPDVDAEMIFMVRDLFQELGLTPHVRLEINSLGTPDERRAHKAA 180
P_dams   RLRQFNQFGVETFGMSNADIDSELIYLSNEIFSALGLTKHINLEINSLGTIEERLEHRKA 178

M_mo     LVNYFMQHKALLDEDSLRRLETNPLRILDSKNPDMQEMIEAAPRLLDFLGEESQQHFDDL 239
K_oxy    LVKWFTQHAAMLDEDSVKRLAINPLRILDSKNPDLQNVIEHAPRLLDFLGEESRQHFDTL 239
E_aer    LVDWFNAHREQLDEDSLKRLETNPLRILDSKNPTMQPMLEQAPRLLDYLSEESRHHFSLL 240
P_dams   LINYFKKHQDLLDEDSLIRLEKNPLRILDSKNPQMQTMLENAPKLLDYLTEESLDHFKTV 238

M_mo     RRLLDSENIAYVVNPRLVRGLDYYTRTVFEWITEELGSQGTVCGGGRYDGLVELFSGKQL 299
K_oxy    RTILDNAGITYQINPRLVRGLDYYSRTVFEWVTDDLGSQGTVCGGGRYDGLAELFSGKKL 299
E_aer    CELLDKADIPYTINPRLVRGLDYYTRTVFEWVTDELGSQGTVCGGGRYDGLAELFNPKAL 300
P_dams   CSNLDQVDIKYTINPRLVRGLDYYTRTVFEWTTDQLGSQSTVCGGGRYDGLVALFSGQSL 298

M_mo     PASGFAIGIERLLLLIQTLGLDKDIVNQ--PDIVVTYEDPAQNVEALLLANTLRHQLPQY 357
K_oxy    PACGFAIGIERLLLLLQTVCDGQNPQWQSAPDIAITSELNDGGIRAQMLACQLRRDLPGL 359
E_aer    PACGFAIGIERLLLLIQTVSQKDNPLWHFHPDVVITSECQDDGLRAQMLAELLRQRIEKL 360
P_dams   PASGFAIGIERLILLLEAVN--ITPPHDSSKKIIFTCEDKLDTNKALILAKNLRDTFPQL 356

M_mo     KILNDFTSAKLKRQHSNALKSGCRYIVTLNRDGQVGLWDLAANTNETLTADTLANAFLNK 417
K_oxy    NVLTDCSGAKLKRQHQNALKSGCQYILTINYDENLRLWNLQNNQQESMSIEQVKLQISEI 419
E_aer    SVMVDCSGNKLKRQHQTALQSQCRAIITLNTDNEIRLWDLASSRQMTLEEKALIDWLQQH 420
P_dams   EVYSDFSGSKLKKQHQRALKESADIVVTLNVDHTLGIWSISDNDKLVINKEEFLDHIQIF 416

M_mo     TITAMA 423
K_oxy    SHK--- 422
E_aer    CQ---- 422
P_dams   IKE--- 419
```

Figure 2.12. Alignment of the HisRS sequences of *M. morganii* DSM30164[T] (M_mo) and histydyl-tRNA synthetase of *K. oxytoca* 10-5246 (K_oxy), *E. aerogenens* KTCT 2190 (E_aer) and *P. damselae* (P_dams). Significantly conserved domains of class II aminoacyl-tRNA synthetase protein are boxed. Grey and black boxes indicate domains related to the interaction of histidine substrate with histidyl-tRNA synthetase in *E. coli*. Black dots indicate important residues for specificity of histidyl-tRNA synthetase to histidyl-tRNA molecules in *E. coli* (Guth & Francklyn, 2007).

2. Fishing sector model: *Morganella morganii*

Meanwhile, the genome of *M. morganii* has been sequenced and deposited in a public database (BioProject: PRJNA78681, Accession: ALJX00000000). The comparison of our sequences with these obtained from the Genome Database, confirmed the results reported above.

The sequence of the *hdc* and presence of identified related genes were also researched for other *M. morganii* strains available in our collection. Twelve histidine decarboxylase sequences obtained were aligned, showing a nucleotide identity of the 100%, as a result of how this gene is highly conserved in this species.

2.4.11. Expression of *hdc* gene in *E. coli*

To verify the production of the histamine decarboxylase protein, the *hdc* gene was cloned into *E. coli*, and the correct sequence and insertion of *hdc* into recombinant plasmid pURI3 was verified by DNA sequencing. *E. coli* pURI3hdc was grown under different growth conditions (changing temperatures and incubation times). Protein extracts from *E. coli* pURI3hdc recombinant cells were used to detect the presence of an hyperproduced protein by SDS–PAGE analysis. *E. coli* pURI3hdc grown in LB with ampicillin, at 37 °C after 4 h of induction with IPTG, overproduced histidine decarboxylase (Figure 2.13A). Affinity chromatography was used to purify the histidine decarboxylase protein from these extracts. After purification, the ability of the HDC protein to decarboxylate the histidine, was assayed. Figure 2.13B showed the histamine spot on TLC plates, indicating that the histidine decarboxylase is a functional protein, after derivatization with dansyl chloride and in presence of histidine.
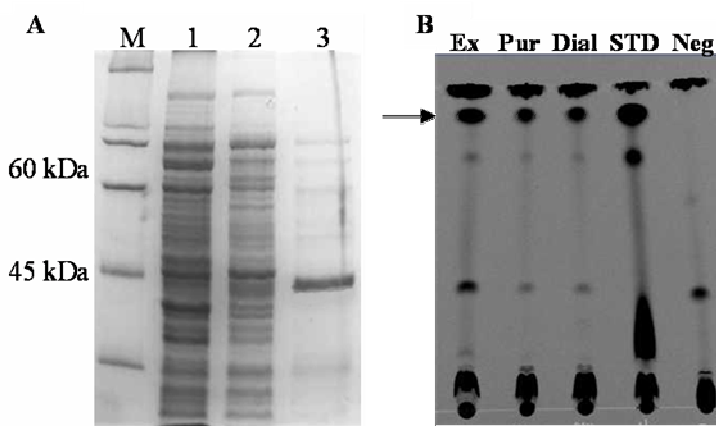
Figure 2.13. A) SDS gel of the purified HDC protein. Lanes: M, marker; 1, *E. coli* pURI3 negative control; 2, *E. coli* pURI3hdc; 3, purified HDC. B) Histamine detection by TLC after dansylation: cell extract (Ex), purified (Pur) and dialyzed (Dial) protein, Histamine standard (STD) and a negative control (Neg). The arrow indicates the histamine spot.

2. Fishing sector model: *Morganella morganii*

2.1.12. Expression of the histamine biosynthetic locus

The effect of the histamine content, nutrient concentration, temperature and pH on the expression of the genes located in the histidine decarboxylase locus of *M. morganii* was determined by quantitative RT-PCR analysis. The results obtained from the different growth conditions applied, were compared with those obtained during the growth in control conditions (LB medium, 37 °C), and normalized using the 16S rDNA endogenous control.

The expression of *hdc* and *his*RS genes was induced under the conditions of low pH, as reported in Figure 2.14.
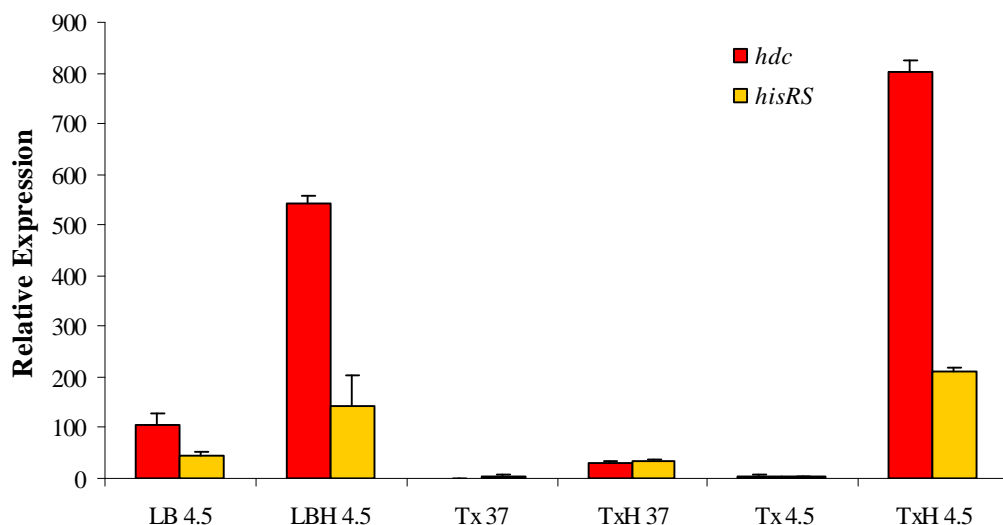
Figure 2.14. Quantification of mRNA extracted from cells of *M. morganii* under different culture conditions: LB medium at pH 4.5 (LB 4.5) with histidine addiction (LBH 4.5), minimal medium Tx at 37 °C (Tx 37) with histidine (TxH 37) and Tx at pH 4.5 (Tx 4.5) with histidine (TxH 4.5).

Growth in LB medium at pH 4.5 induced an increase of 140- and 60-fold, for *hdc* and *his*RS respectively, as compared to growth in LB at normal pH (Relative Expression, RE 103.82 and 43.71). In the same acidic condition, the presence of 5 mM histidine, further increased the expression of these genes 10- and 6-fold (RE 540.92 and 144.33). The greatest expression of *hdc* and *his*RS was obtained when *M. morganii* cells were grown in the minimal medium Tx, at pH 4.5 and in presence of histidine. In this case, the relative expression increased 146 and 48 times (RE 803.22 and 210.34). In the other growth conditions, low values of expression were detected during growth. In LB at pH 7.0, with or without histidine supplementation, and in the experiments in which cells were grown in the minimal medium at 15°C, also in the presence of histamine, the transcription of these gene was always detected, even if a low values (RE 0.5 – 5.4). These results suggest a costitutive minimal basal expression of *hdc* and *his*RS genes, that are up-regulated under conditions of low pH or histidine supplementation.

As reported from other authors (Tanaka et al., 2008; Kimura et al., 2009), bacterial amino acid decarboxylases are hypothesized to play a role in controlling pH to countermeasure the acidity resulting from fermentations. This mechanism is generally considered related to the decreasing of intercellular proton concentration trough the decarboxylation reaction and to the improvement of the microenvironment by excretion of a basic molecule, the amine. According to the models proposed, the amino acid is quickly decarboxylated to biogenic amine and CO_2 which can freely escape outside. The reaction eliminates a large quantity of H^+ ions from the cell. We can assume that the histidine

2. Fishing sector model: *Morganella morganii*

decarboxylation reaction in *M. morganii* may play a role in acid survival, since the transcription of the histamine biosynthetic genes were significantly induced under acidic conditions.

As reported for other histamine producing species, *M. morganii* shows a mechanism of histamine formation that involves two specific enzyme: a putative antiporter take the histidine from outside and excretes histamine from the cell, while the HDC operate the decarboxylation (as reported in Figure 2.15). The expression level studies indicate that in *M. morganii hdc* gene was expressed costitutively, but highest level of expression were detected under acidic conditions, especially in presence of an histidine excess. This fact corroborates the hypothesis that the histidine decarboxylation reaction in *M. morganii* may play a role in acid survival.
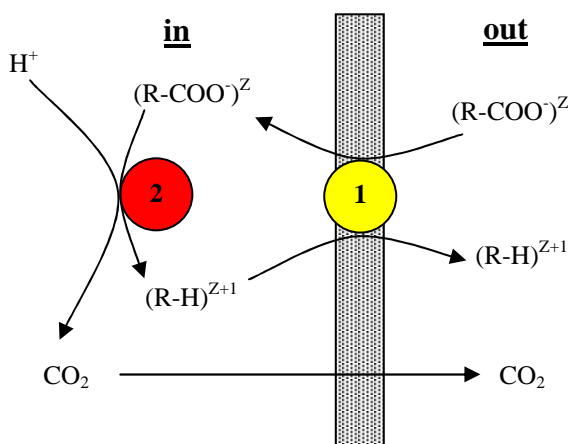
Figure 2.15. Generalized scheme of decarboxylation and electrogenic antiporter. R-COO⁻ and R-H are te carboxylic acid and the decarboxylated product, respecively. The particles which are recognized and exchanged by the antiporter are the carboxylic acid with charge z and the decarboxylated product with charge z+1. A net charge is transported to the outside, corresponding to the proton consumed in the cell.

2. Fishing sector model: *Morganella morganii*

Finally, with the aim to prepare a significant collection of different *M. morganii* strains from different ecological niches, we selected several clinical biotypes, in addition to strains isolated from fish. The study of these different strains was performed through the comparison of fingerprinting profiles obtained from REP and RAPD PCR analysis.

2.4.13. Molecular fingerprinting

This preliminary investigation on genetic variability of *M. morganii* was performed using RAPD PCR with primer M13, and REP-PCR with primers BOXA1R and $(GTG)_5$. These methods, which use short arbitrary primers or primers targeting short repetitive sequences interspersed throughout the genome are an established approach for delineation of bacteria at the species and strain level.

After preliminary analysis on *M. morganii* fish strains, analysis was carried out using only representative profiles. Interesting results were obtained and reported below.

With the M13 primer, 9 different profiles were detected (Figure 2.16), and the dendrogram obtained from NTSYS analysis allowed us to separate our collection in two principal cluster (1 and 2), related to the ecological niche of isolation (Figure 2.16). The two clusters have a low similarity coefficient (12 %) that means a high degree of diversity. In the first cluster (Cluster 1) grouped all the clinical strains of *M. morganii* that show high heterogeneity: only two strains show identical profile (similarity coefficient 100%). All other strains shown similarity coefficient from 40 to 90%. The second cluster includes all the fish strains (Cluster 2). Only three kind of profile were detected here. In fact, all the strains grouped with a similarity coefficient of 100% except strains Q1 and Q6 that show a correlation coefficient respectively of 50 and 90 % with the other identical strains.

2. Fishing sector model: *Morganella morganii*
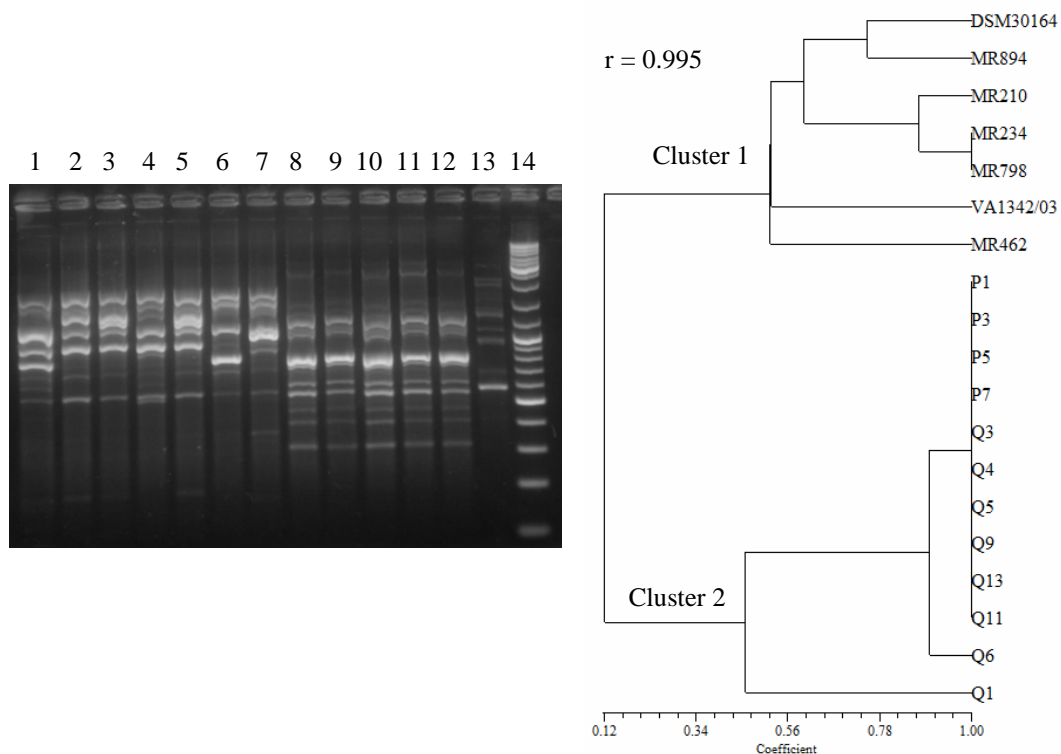
Figure 2.16. On the left, M13 profiles identified among the representing strains of *M. morganii*. Lanes: 1 DSM30164[T]; 2 MR210; 3 MR234; 4 MR462; 5 MR798; 6 MR894; 7 VA1342/03; 8 P1; 9 P3; 10 Q1; 11 Q6; 12 Q11; 13 negative; 14 Marker. On the right, dendrogram showing the relatedness of studied strains based on M13 fingerprint, costructed using the UPGMA algorithm in the NTSYSPC software, version 2.11 (Applied Biostatics Inc., NY).

From the profiles obtained using primer (GTG)$_5$, we can observe that this typing method is not able to discriminate fish isolates of *M. morganii* (Cluster 2, 100% of similarity). The similarity coefficient with the clinical isolates cluster is of 23%. In this cluster (Cluster 1) the correlation is about the 60% (Figure 2.17) with the presence of two subclusters.

2. Fishing sector model: *Morganella morganii*
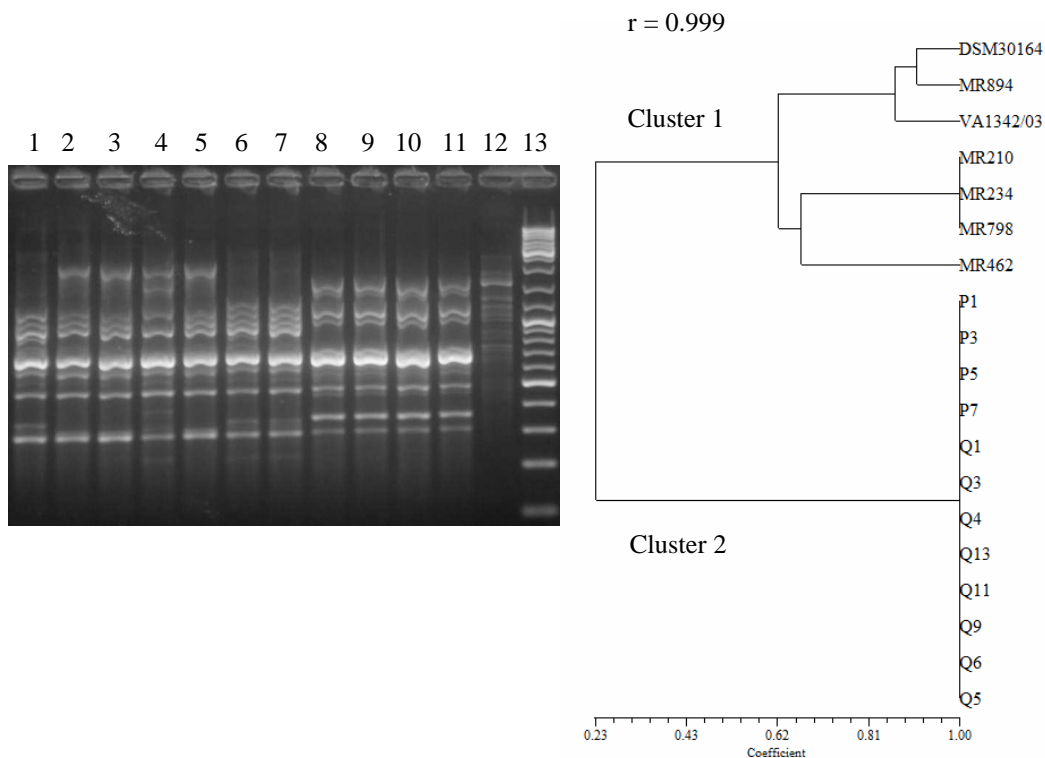
Figure 2.17. On the left $(GTG)_5$ profiles identified among the representing strains of *M. morganii*. Lanes: 1 DSM30164[T]; 2 MR210; 3 MR234; 4 MR462; 5 MR798; 6 MR894; 7 VA1342/03; 8 P1; 9 P7; 10 Q4; 11 Q5; 12 negative; 13 Marker. On the right, dendrogram showing the relatedness of studied strains based on $(GTG)_5$ fingerprint (UPGMA algorithm, NTSYSPC software, version 2.11 - Applied Biostatics Inc., NY).

With the third used primer BOXA1R, the discrimination power is higher than in previous experiments. In this last case, as reported in Figure 2.18 all the fish isolates profiles grouped with a similarity coefficient of 100 % (Cluster 2). Unlike before, two clinical strains, MR462 and MR798 joined the fish cluster with a correlation of 40%. The similarity % of these two strains with other clinical ecotype it is only about 18%.

The Cluster 1 that grouped the remaining five clinical strains is quite heterogeneous. In this group, only two strains show a 100% of similarity, while other strains were correlated with a coefficient ranging from 35 to 50%.

2. Fishing sector model: *Morganella morganii*
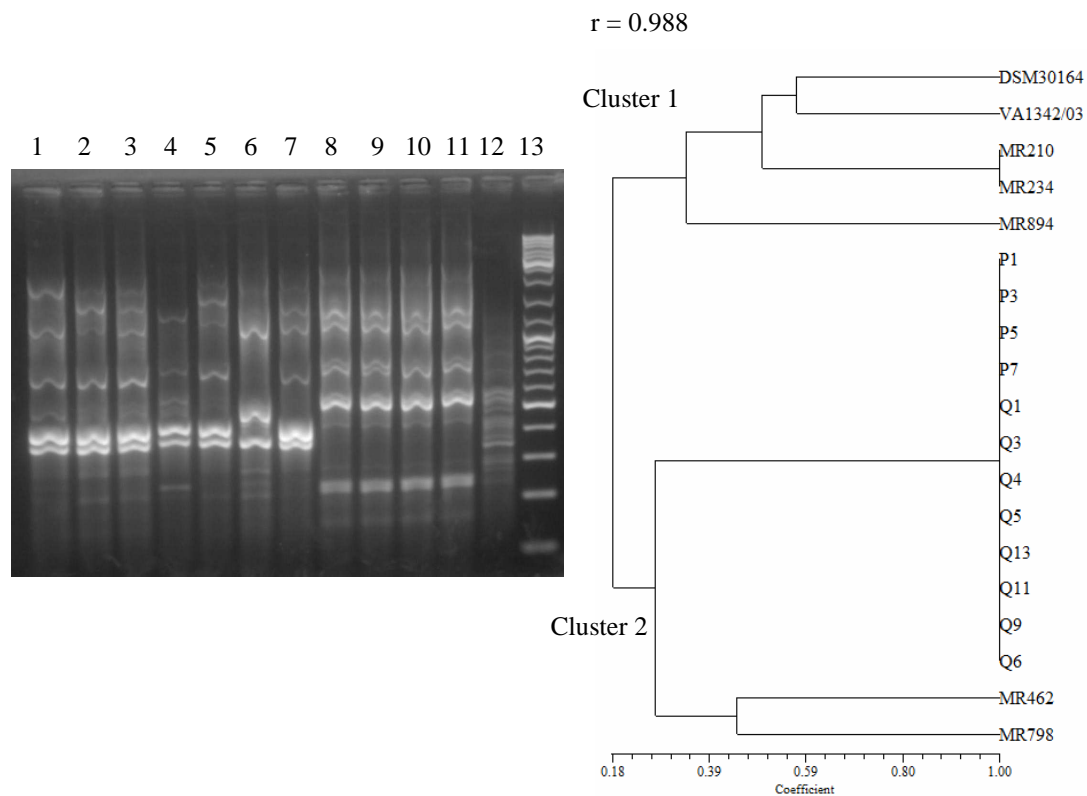
Figure 2.18. On the left BOXA1R profiles identified among the representative *M. morganii* strains. Lanes: 1 DSM30164^T; 2 MR210; 3 MR234; 4 MR462; 5 MR798; 6 MR894; 7 VA1342/03; 8 P1; 9 P5; 10 Q4; 11 Q11; 12 negative; 13 Marker. On the right, dendrogram showing the relatedness of studied strains based on BOXA1R fingerprint using the UPGMA algorithm (NTSYSPC software, version 2.11 of Applied Biostatics Inc., NY).

Combining M13, (GTG)$_5$ and BOXA1R fingerprints, the dendrogram obtained is reported in Figure 2.19. The cluster analysis confirmed the existence of the two main clusters related to the habitat of isolation, which were separated at a level of similarity of 19%. For clinical strains (Cluster 1) the minimum similarity coefficient was about 40% with a maximum of 95%.

Fish isolates (Cluster 2) show a high similarity with a correlation coefficient of 100% with the exception of strains Q1 and Q6, as reported above for M13 primer.

These results could indicate a significative influence of the habitat on the adaptive capability of the strains.

2. Fishing sector model: *Morganella morganii*
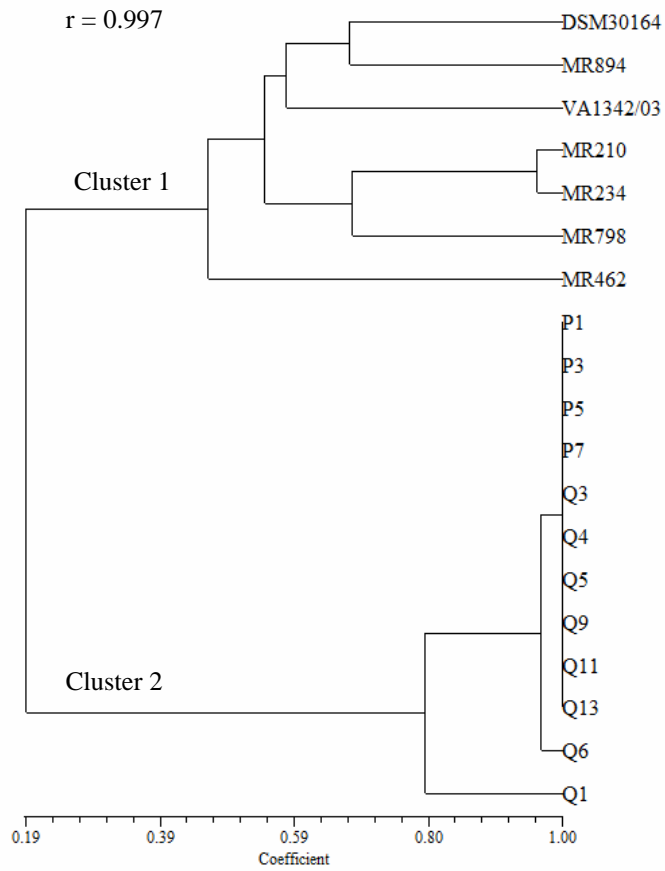
Figure 2.19. Dendrogram showing the relatedness of *M. morganii* strains based on combined results of M13, (GTG)$_5$, and BOXA1R fingerprints (UPGMA algorithm, NTSYSPC software, version 2.11 - Applied Biostatics Inc., NY).

2. Fishing sector model: *Morganella morganii*

2.5. CONCLUSIONS

According to the tasks described in the section 2.2, the following conclusions were drawn:

- the incidence of *M. morganii* strains in different tuna samples was of 37%;

- the development of a specific molecular tool for the detection and quantification of *M. morganii* could be useful to prevent the presence of this undesiderable species in the food chain;

- as reported for other histamine producing species, *M. morganii* shows a mechanism of histamine formation that involves two specific enzymes;

- the expression level studies indicate that in *M. morganii hdc* gene was expressed costitutively, but highest level of expression were detected under acidic conditions, especially in presence of an histidine excess. This fact corroborates the hypothesis that the histidine decarboxylation reaction in *M. morganii* may play a role in acid survival;

- strains grouped in relation to their niche of origin, suggesting a significative influence of the habitat on the adaptive capability of the strains.

Publications related to this part of PhD thesis are reported below:

o Ferrario C, Pegollo C, Ricci G, Borgo F, Fortina MG, 2012a, PCR detection and identification of histamine-forming bacteria in filleted tuna fish samples. J Food Sci 77:115-120.

o Ferrario C, Ricci G, Borgo F, Fortina MG, 2012b, Species-specific DNA probe and development of a quantitative PCR assay for the detection of *Morganella morganii*. Lett Appl Microbiol 54:292-298.

o Ferrario C, Ricci G, Fortina MG, de las Rivas B, Muñoz R, 2012, Improved study on the histidine decarboxylase of *Morganella morganii* - In: 978-975-561-423-6. - (2012 Aug), p. 597. Presented at 23rd International ICMFH Symposium, Food Micro 2012, Istanbul, Turkey, 3-7 September 2012.

o Ferrario C, Borgo F, de las Rivas B, Muñoz R, Ricci G, Fortina MG, Organization of the histamine biosynthetic locus in *Morganella morganii* and its expression under acidic condition. Submitted to J Appl Microbiol.

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Appendix 1

APPENDIX 1. COPIES OF ABSTRACTS OF PAPERS, ORAL COMMUNICATIONS AND POSTERS

1. Ferrario,C, Pegollo C, Ricci G, Borgo F, Fortina MG, 2012a, PCR detection and identification of histamine-forming bacteria in filleted tuna fish samples. J Food Sci 77:115-120.

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PCR Detection and Identification of Histamine-Forming Bacteria in Filleted Tuna Fish Samples

Chiara Ferrario, Chiara Pegollo, Giovanni Ricci, Francesca Borgo, and M. Grazia Fortina

Abstract: Total of 14 filleted yellowfin tuna fish (*Thunnus albacares*) sold in wholesale fish market and supermarkets in Milan, Italy, were purchased and tested to determine microbial count, histamine level, histamine-forming bacteria, and their ability to produce histamine in culture broth. Although histamine level was less than 10 ppm, many samples showed high total viable bacterial and enterobacterial counts that reached dangerous levels after temperature abuse for short periods of time. A PCR assay targeting a 709-bp fragment of the histidine decarboxylase gene (*hdc*) revealed that 30.5% of the 141 enteric bacteria isolated from samples were positive and potentially able to produce histamine. The *hdc* positive strains were mainly isolated from fish bought at wholesale fish market, where we observed several possible risk factors, such as handling in poor and non-refrigerated conditions during fillet preparation. These positive strains were identified as *Citrobacter koseri*/*Enterobacter* spp. and *Morganella morganii*, by 16S/23S rRNA internal transcribed spacer amplification and 16S rRNA sequence analysis. The strains showed a variable ability of histamine production, with *Morganella morganii* being the most active histamine-producing species. A direct DNA extraction from fish and a PCR targeting the *hdc* gene showed a high degree of concordance with the results obtained through microbiological and chemical analyses, and could aid in the prompt detection of potentially contaminated fish products, before histamine accumulates.

Keywords: histamine, histamine-forming bacteria, PCR detection, tuna fish

Practical Application: The use of methods for the early and rapid detection of bacteria producing biogenic amines is important for preventing accumulation of these toxic substances in food products. In this study, we used a molecular approach for the detection of histamine-forming bacteria in fish. PCR–based methods require expensive equipment and a high degree of training for the user, but are fast (< 24 h) and reliable. They now represent the best predictive methods to identify a potential risk factor in fish products during processing, storage, and marketing and can be used in the investigation of risk reduction strategies.

M: Food Microbiology & Safety

ORIGINAL ARTICLE

Species-specific DNA probe and development of a quantitative PCR assay for the detection of *Morganella morganii*

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Abstract

Aims: To develop a SYBR Green quantitative PCR assay (qPCR) for the specific detection of *Morganella morganii*, a fish pathogen responsible for the Histamine Fish Poisoning.

Methods and Results: A new primer set, amplifying a 179-bp fragment of the 16S rRNA gene, was selected for specificity, and 14 *M. morganii* strains and 32 non-*Morganella* strains were evaluated. The melting temperature of 84°C was consistently specific for the amplicon. Two standard curves were constructed: the minimum detection sensitivity was 0·563 pg of pure DNA, corresponding to DNA extracted from nine cells of *M. morganii*. The qPCR assay was evaluated in experiments with seeded fish samples, and the regression coefficient values were calculated.

Conclusions: A highly specific and rapid assay was developed for the detection of *M. morganii* in tuna fish samples.

Significance and Impact of the Study: This method represents the first study about the quantification of pathogenic *M. morganii* in fish products. This approach can be utilized to prevent the presence of this undesirable species in the food chain.

Appendix 1

Genome Sequences of *Lactococcus garvieae* TB25, Isolated from Italian Cheese, and *Lactococcus garvieae* LG9, Isolated from Italian Rainbow Trout

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Lactococcus garvieae is a fish pathogen and an emerging zoonotic opportunistic pathogen as well as a component of natural microbiota in dairy products. Here, we present the first report of a genome sequence of *L. garvieae* TB25, isolated from a dairy source, and that of *L. garvieae* LG9, isolated from rainbow trout.

Genetic investigation within *Lactococcus garvieae* revealed two genomic lineages

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Abstract

The diversity of a collection of 49 *Lactococcus garvieae* strains, including isolates of dairy, fish, meat, vegetable and cereal origin, was explored using a molecular polyphasic approach comprising PCR-ribotyping, REP and RAPD-PCR analyses and a multilocus restriction typing (MLRT) carried out on six partial genes (*atpA*, *tuf*, *dltA*, *als*, *gapC*, and *galP*). This approach allowed high-resolution cluster analysis in which two major groups were distinguishable: one group included dairy isolates, the other group meat isolates. Unexpectedly, of the 12 strains coming from fish, four grouped with dairy isolates, whereas the others with meat isolates. Likewise, strains isolated from vegetables allocated between the two main groups. These findings revealed high variability within the species at both gene and genome levels. The observed genetic heterogeneity among *L. garvieae* strains was not entirely coherent with the ecological niche of origin of the strains, but rather supports the idea of an early separation of *L. garvieae* population into two independent genomic lineages.

Genome Sequences of Two *Lactococcus garvieae* Strains Isolated from Meat

Lactococcus garvieae is an important fish pathogen and an emerging opportunistic human pathogen, as well as a component of natural microbiota in dairy and meat products. We present the first report of genome sequences of *L. garvieae* I113 and Tac2 strains isolated from a meat source

Original paper submitted to Journal of Applied Microbiology

Organization of the histamine biosynthetic locus in *Morganella morganii* and its expression under acidic condition

Ferrario C, Borgo F, de las Rivas B, Muñoz R, Ricci G, Fortina MG

The aim of this study was to improve the genetic knowledge of histamine production by *Morganella morganii*, and to study the expression of the histidine decarboxylase gene (*hdc*), for a better control of the accumulation of this toxic substance in food products. The sequence of the histidine decarboxylase chromosomal region showed the presence of two putative histidine/histamine antiporters located upstream and downstream the *hdc* gene. The second transporter is followed by a histidyl-tRNA synthetase. This genetic organization was confirmed by PCR amplifications of this region in different strains of *M. morganii* isolated from tuna samples available in our collection. Sequence comparisons with other gram negative histamine producer bacteria, showed a similar genetic organization in *Klebsiella oxytoca*. This work represents the first description and identification of the *hdc*-related genes in *M. morganii*. To study the *M. morganii* histidine decarboxylase enzyme and its expression, the corresponding *hdc* gene was cloned into *Escherichia coli* and recombinant cells grown at 37°C after 4 h of isopropyl-β-D-thiogalactopyranoside induction, were shown to over-produce histidine decarboxylase. Finally, *hdc* expression in *M. morganii* was studied by RT-PCR experiments in different growth conditions. The highest level of expression was detected under acidic conditions, with an histidine excess. This result supports the hypothesis that the histidine decarboxylation reaction in *M. morganii* may play a role in acid survival.

Appendix 1

23rd International ICMFH Symposium, Food Micro 2012, Istanbul, Turkey, 3-7 September 2012.

Improved study on the histidine decarboxylase of *Morganella morganii*

Chiara Ferrario, Giovanni Ricci, Maria Grazia Fortina, Blanca de las Rivas, Rosario Muñoz

The *hdc* gene, coding for the enzyme histidine decarboxylase (HDC), is responsible of the decarboxylation of the amine histidine to histamine, a biogenic amine that can cause a chemical intoxication called Scombroid syndrome. This gene is present in the genome of different bacterial species, both of gram negative and positive bacteria. One of these is *Morganella morganii,* a gram negative rod considered one of the most prolific histamine former in seafood, especially in tuna fish. The aim of this study was to improve the knowledge at genome level and to study the expression of the pyridoxal phosphate dependent *hdc* of *M. morganii*, for a better control of the accumulation of this toxic substance in food products.

Firstly, the nucleotidic sequence of the gene *hdc* and its flanking regions was determined by the development of a plasmid library of *M. morganii* in *E. coli* strain. After sequencing, the obtained region of 7681 bp showed the presence of two amino acids permease (*pot*E) located one before and one after the *hdc* gene, usually coded next to decarboxylases, for the histidine/histamine transport. The second permease is followed by a second histidyl - tRNA synthetase (*his*S). These results were confirmed also by PCR amplifications of all these genes in different strains of *M. morganii* isolated from tuna samples. This is the first indication and localization of these genes in *M. morganii*. A comparison with other gram negative histamine producers, showed a difference for the presence of the aminoacid permease after the *hdc*.

To study the HDC enzyme and its expression, the corresponding gene was cloned into *Escherichia coli*, grown in different cultural conditions. Proteins were extracted, the HDC specific enzyme was purified and its activity was determined by TLC methods.

Finally, *hdc* gene expression in *M. morganii* was studied by RT - PCR experiments after grow of the strain tested in different conditions of temperature, pH, presence of O_2 and adding different amount of histidine.

Variability at gene and genome levels revealed different genomic lineages in *Lactococcus garvieae*

Chiara Ferrario, Francesca Borgo, Giovanni Ricci, Maria Grazia Fortina.

Lactococcus garvieae is a pathogen that causes septicemia in fish and serious damage to fish aquaculture worldwide, but recently has been isolated from human, ruminant, water sources and several food matrices. In the present work we monitored the population structure of *L. garvieae* strains, including isolates of dairy, fish, meat, vegetable and cereal origin. This bacterial collection was explored using molecular polyphasic approach comprising PCR-ribotyping, Rep and RAPD-PCR analyses and multilocus restriction typing (MLRT) carried out on six partial genes. This approach allowed high-resolution cluster analysis in which two major groups were distinguishable: one group included dairy isolates, the other group meat isolates. Unexpectedly, some strains coming from fish grouped with dairy isolates whereas the other with meat isolates. Likewise, strains isolated from vegetables allocated between the two main groups. These findings revealed high variability within the species at both gene and genome levels. The observed genetic heterogeneity among Lactococcus garvieae strains was not entirely coherent with the ecological niche of origin of the strains, but rather supports the idea of an early separation of *L. garvieae* population into independent genomic lineages. To better define this result, a multilocus sequence typing (MLST) scheme was developed for *L. garvieae*, based on the sequence analysis of eight genes (α-acetolactate synthase *als*, α-subunit of ATP synthase *atp*A, bacterial elongation factor EF-Tu *tuf*, glyceraldehyde-3-phosphate dehydrogenase *gap*C, β-subunit DNA gyrase *gyr*B, β'-subunit RNA polymerase *rpo*C, D-alanine-D-alanyl carrier protein ligase *dlt*A, galactose permease *gal*P). On the 17 sequence types determined, to estimate intraspecific genetic diversity of data samples and to detect particular population structures several statistical analyses were carried out. The average nucleotide diversity, numbers of synonymous and nonsynonymous mutations, the measure of contribution of recombination and mutation in the creation of sample population were determined to delineate a phylogenetic framework of our collection of *L. garvieae*.

15th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 15-17 September, 2010 (Portici, Italy)

Safety and quality of fish products: a methodological polyphasic approach to study contaminating emerging pathogens

Chiara Ferrario

This PhD thesis research project aims to improve the current systems of management and control of food safety, focusing the attention on the study of two bacterial species, *Lactococcus garvieae* and *Morganella morganii* that represent valid models of contaminating emerging pathogens in fish products.

Appendix 1

16th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 21-23 September, 2011 (Lodi, Italy)

***Lactococcus garvieae* and *Morganella morganii*: two bacterial models to study quality and safety of fish products**

Chiara Ferrario

This paper reports the results obtained for two bacterial species, *Lactococcus garvieae* and *Morganella morganii,* that represent models of contaminating emerging pathogens in fish products: i) molecular characterization and genetic polymorphism of *L. garvieae* strains coming from different food niches; ii) detection of the histamine-forming *M. morganii* in tuna fish samples and its quantification through the development of a new real-time PCR assay.

17th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 19-21 September, 2012 (Cesena, Italy)

Lactococcus garvieae and *Morganella morganii* as models to study safety and quality of fish products

Chiara Ferrario

This PhD thesis reports the study of two bacterial species, *Lactococcus garvieae* and *Morganella morganii,* that represent models of contaminating emerging pathogens in fish products: i) molecular characterization and genetic polymorphism of *L. garvieae* strains coming from different food niches; ii) quantification of *M. morganii* through the development of a new real-time PCR assay and study of the gene responsible of histamine production during storage of fish belonging to the *Scombridae*'s family.

Appendix 2

APPENDIX 2. INDEX OF TABLES

Appendix 3

APPENDIX 3. INDEX OF FIGURES