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PhD Course in

Veterinary and Animal Science (Class XXX)

Doctoral Thesis

Of milk and honey: molecular tools to monitor resistance in honeybee and dairy cow diseases

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SUMMARY

Abstract

In the field situation, the potential benefit of the wide-scale application of molecular tools for healthy risk monitoring and pathogen control strategies is still under looked for a number of reasons. Among these, it should be mentioned the usually higher complexity and cost compared to most of the classical monitoring tools, and the sometimes incomplete in-field standardization of the protocols. On the other side, the molecular tools that are properly validated in terms of sensitivity, specificity and accuracy can greatly help pathogen detection especially in cases disease management programmes for reduction of morbidity and mortality relies both on prompt species-specific diagnosis and on effective profiling of species/strain virulence and host resistance. The molecular biology can support not only the identification and characterization of target pathogens, but also can help the breeding strategies aiming to increase the natural resistance of the host species.

This PhD thesis is the outcome of a range of activities and experimental results aimed to a better implementation of molecular tools in the control strategies of some honey bee, *Apis mellifera,* and dairy cow diseases. This approach was selected since the above two food-producing livestock species are representative of very different production systems and are exposed to several pathogens with complex epidemiological, pathological and control implications. In facts, the health of the hive can be at risk not only for adult honeybee diseases, mainly of viral and fungal origin, but also for also for several brood diseases, mainly of bacterial and parasitic origin. Thus, proper monitoring of hive pests and selection of colonies that are resistant to brood diseases thanks to their high hygienic behavior attitude can be of advantage for the professional beekeeper, because it implies a decreased use of drugs and longer life of the hives. Regarding dairy cows, they can be affected by several pathogens with different impact regarding animal and human health. In facts, milk can be a good source of foodborne pathogens since it carries a variety of microorganisms. Indeed, it is a direct contact with contaminated sources in the milking stall from the environment and infected animals. In particular, the bacterial species *Escherichia coli* is a widespread pathogen whose different patterns of virulence factors can lead to very severe health implications for the dairy farm. The identification and pathogenic characterization of *E. coli* strains is highly important for public health since it can reproduce in animals, humans and the environment. The routine detections

and differentiations of intestinal and extraintestinal *E. coli* in cows are usually based on a combination of biochemical tests; serotyping, phenotypic assays based on virulence characteristics and molecular detection methods. The detention of the causative agent is very important to infer the inherent sensitivity of the host animals to specific virulent strains and for the setup of an effective control strategy.

In this project, the possibility to optimize the existing methods for a reliable diagnostical evaluation and accurate risk profiling linked to severe pathogens in field conditions, as a measure of the epidemiological exposure and sensitivity of the hosts, has been evaluated. For all the proposed molecular approaches, the comparison with reference methods that are already in use have been performed as a necessary step to guarantee reliable and comparable results.

As a first topic, the research activity was focused to the development of a prognostic molecular assay targeting hygienic behavior (HB)-specific biomarkers in honeybee nurses, with the aim to test potential practical application in marker-assisted selective breeding programs. Currently, the beekeepers measure the HB score in their honeybee colonies using methods like the pin-prick test or freeze-killed brood (FKB) assays, during their in-field tests. These methods are inherently imprecise, time consuming and resource intensive for the breeders, thus limiting their suitability for widespread application. The availability of molecular biomarkers for the hygienic phenotype could fasten the recognition of this trait and the breeding process. The present work was characterized by a transversal approach: from in-field phenotypic characterization of HB with the classical methodology, to expression profiling of selected target genes in nurses, with the aim to assess differential expression of such genes between selected honeybee colonies with higher and lower HB, respectively. The expression data were obtained through qPCR analysis and the results highlighted the prominent significance of Act5 and Obp4 among the analyzed candidate markers as a distinguishable tools to infer the hygienic feature of a honeybee colony. This molecular approach has thus great potential for the use in the field as a reliable tool to measure inherent resistance of honeybee colonies to a wide range of brood pathogens.

As a second topic, a new qPCR-HRMA-based approach was setup for the monitoring of honey bee nosemosis. This is a gut disease of adult honey bee caused by the microsporidia *Nosema apis* and *N. ceranae*. These two species are morphologically similar but differ in epidemiological

pattern and virulence. Proper control strategies of the infection by beekeepers rely on correct species differentiation. The discrimination between the two species using the classical methods can be difficult since *N. apis* and *N. ceranae* spores are not easily distinguishable under microscopic examination. This may lead to misdiagnosis especially on samples from areas where both microsporidia are present. Correct species identification thus requires molecular analysis. Over the last years, several molecular methods with higher sensitivity and specificity compared to classical microscopy were designed. Nevertheless, a need for simple, rapid and cost-effective molecular protocols to discriminate between *N. ceranae* and *N. apis* still remains. High resolution melting analysis (HRMA) coupled to real-time PCR is a widely used technique to target sequence polymorphisms in different species without the need to perform DNA sequencing or to use species-specific probes. This work aimed to design a qualitative and quantitative assay for the simultaneous detection and discrimination of *N. ceranae* and *N. apis* infection in exposed honey bees, based on HRMA coupled to real time quantitative PCR (qPCR-HRMA). A fast and cost-effective qPCR-HRMA protocol to detect and differentiate simultaneously and unequivocally *N. ceranae* and *N. apis* DNA extracted from honey bees was then realized. The present method is simpler to use than most other DNA-based methods and provides comparable discrimination between the two sibling species, to be also useful as a measure of the exposition and sensitivity of the adult honeybees in defined epidemiological situation.

And as a third research activity, the presence and virulence profile of pathogenic *E. coli* in bulk-tank dairy milking compared to milking machine filters samples was evaluated on a number of samples collected in dairy farms. To achieve this purpose, the classical isolation and identification techniques have been coupled with a molecular diagnostic approach. The results underlined the different detection pattern of pathogenic *E. coli* in the bulk-tank milk with respect to milking machine filter, with special regard to EIEC strains. The implementation of a combined microbiological and biomolecular approach allowed the selective identification of *E. coli* from other bacteria on specific media and the profiling of virulence factors , leading to a rapid and sensitive identification of pathogenic strains, as a necessary step for a proper characterization of the host and human exposure, and inherent health risk. In particular, the analysis of milk filters could be an useful tool to assess the presence of a risk of contamination by *E. coli* pathotypes both at food and environmental level.

Overall, the results of the present project could help the characterization of the inherent sensitivity of the hosts to these diseases and, finally, of the inherent risk that the above pathogens may pone to the health of animals and/or humans.

Riassunto

L’utilizzo di strumenti molecolari per il monitoraggio del rischio della malattie e per le strategie di controllo degli agenti patogeni in campo è ancora sottovalutata per molti motivi. Tra queste, bisogna considerare l’elevata complessità e il costo di queste metodologie rispetto all’utilizzo della maggior parte dei metodi classici di monitoraggio e, alcune volte, l’utilizzo di protocolli standard incompleti in campo. Gli strumenti molecolari che sono stati validati per sensibilità, specificità e accuratezza, possono contribuire notevolmente al rilevamento degli agenti patogeni, soprattutto nei casi in cui i programmi di gestione delle malattie per la riduzione della patogenicità e della mortalità si basano sia su una rapida diagnosi specifica delle specie sia su un efficace profilo di virulenza di specie/ceppo e resistenza all'ospite. La biologia molecolare può supportare non solo l'identificazione e la caratterizzazione di patogeni target, ma può aiutare anche nelle strategie di allevamento per aumentare la resistenza naturale delle specie ospitanti.

Questa tesi di dottorato è la conseguenza di una serie di attività e risultati sperimentali finalizzati ad una migliore applicazione della biologia molecolari per le strategie di controllo delle malattie nelle api, in particolare *Apis mellifera* e nelle vacche da latte. Questo approccio è stato selezionato in quanto le due specie producono cibo in sistemi di produzione molto diversi e quindi sono esposti a diversi agenti patogeni con elevate implicazioni epidemiologiche, patologiche e di controllo. Infatti, la salute dell'alveare può essere messa a rischio per le malattie delle api adulte, principalmente di origine virale e fungina, e anche per le malattie di covata, principalmente di origine batterica e parassitaria. Pertanto, un adeguato monitoraggio dell'alveare e la selezione di colonie resistenti dalle malattie di covata (un elevato comportamento igienico) portano dei benefici all'apicoltore professionista, in quanto questo implica un ridotto uso di farmaci con una maggiore sopravvivenza degli alveari. Invece, le vacche da latte possono essere affette da diversi patogeni con un diverso impatto sull’animale e sulla salute umana. Il latte può essere una buona fonte di patogeni alimentari, in quanto trasporta una varietà di microrganismi. Infatti, esso è a contatto diretto con i contaminati della stalla di mungitura, di origine ambiente e da animali infetti.

In particolare, *Escherichia coli* è un patogeno batterico ampiamente diffuso, con diversi pattern di fattori di virulenza che può determinare gravi conseguenze sulla salute dell’allevamento.

L'identificazione e la caratterizzazione dei ceppi patogeni di *E. coli* è molto importante per la salute pubblica in quanto può riprodursi negli animali, nell'uomo e nell'ambiente. Le diagnosi di routine e le differenziazione di ceppi intestinali e extraintestinali di *E. coli* nelle bovine da latte si basano su una combinazione di test biochimici; sierotipizzazione, saggi fenotipici sui fattori di virulenza e sui metodi di rilevazione molecolare. Il rilevamento dell'agente patogeno è molto importante per capire la sensibilità innata dell'ospite a specifici ceppi di virulenti e per l'allestimento di una strategia di controllo efficace. In questo progetto è stata valutata la possibilità di ottimizzare i metodi esistenti per una valutazione diagnostica affidabile e per un accurato profilo del rischio legata ai patogeni, come il rilevamento epidemiologica all'esposizione e alla sensibilità dell'ospite. Per tutti gli approcci molecolari proposti è stato eseguito il confronto con i metodi standard già in uso, come step necessario per garantire affidabilità e comparabilità dei risultati.

Primo capitolo, l'attività di ricerca si è concentrata sullo sviluppo di un metodo molecolare prognostico utilizzando dei biomarkers specifici per il comportamento igienico (HB) delle api nutrici, per verificare la potenziale applicazione in programmi di allevamento selettivo. Attualmente, gli apicoltori misurano i valori dell'HB nelle colonie con il pin-prick test o con il metodo dell'azoto liquido (FKB), durante i loro test in campo. Questi metodi sono molto imprecisi, richiedono tempo e molte risorse per gli allevatori, limitando così un'applicazione diffusa. La disponibilità di biomarkers molecolari per il fenotipo igienico potrebbe velocizzare il riconoscimento di questo tratto e il processo di breeding. Questo lavoro è caratterizzato da un approccio trasversale: dalla caratterizzazione fenotipica del HB in campo con un metodo standard, al profilo dell'espressione dei geni target nelle api nutrici, per valutare la differente espressione dei geni tra le colonie selezionate per un alto e basso HB. I dati dell'espressione genica sono stati ottenuti tramite qPCR e i risultati hanno evidenziato Act5 e Obp4 tra i marcatori candidati per individuare un fenotipo igienico di una colonia. Questo approccio molecolare ha un grande potenziale per l'utilizzo nel campo come strumento affidabile per misurare la resistenza delle colonie ad differenti patogeni di covata.

Secondo capitolo, un nuovo approccio basato su qPCR-HRMA per il monitoraggio della nosemosi nelle api. Si tratta di una malattia intestinale delle api adulti causata dalla microsporidia *Nosema apis* e *N. ceranae*. Queste due specie sono morfologicamente simili ma differiscono per il pattern epidemiologico e virulenza. Le strategie di controllo dell'infezione da

parte degli apicoltori si basano su una corretta differenziazione delle specie. La discriminazione tra le due specie può essere fatta con dei metodi classici può essere difficile in quanto le spore *N. apis* e *N. ceranae* non sono facilmente distinguibili tramite l'esame al microscopico. Questo può portare a una diagnosi errata soprattutto nei campioni da aree in cui sono presenti entrambi i microsporidi. La corretta identificazione delle specie richiede quindi un'analisi molecolare. Negli ultimi anni sono stati messi a punto diversi metodi molecolari con una elevata sensibilità e specificità rispetto alla microscopia classica. Tuttavia, rimane ancora necessario l'utilizzo di protocolli molecolari semplici, rapidi ed economici per discriminare tra *N. ceranae* e *N. apis*. High resolution melting analysis (HRMA) con Real-Time PCR è una tecnica ampiamente utilizzata per mettere a punto i polimorfismi di sequenza in diverse specie senza la necessità di eseguire sequenziamenti del DNA o di utilizzare sonde specie specifiche. Questo lavoro è finalizzato per progettare un metodo qualitativo e quantitativo per la rilevazione simultanea e la discriminazione delle infezioni causate da *N. ceranae* e *N. apis*, basato su HRMA accoppiato a Real-time PCR quantitativa (qPCR-HRMA). Un protocollo qPCR-HRMA veloce ed economico per individuare e differenziare simultaneamente e inequivocabilmente il DNA di *N. ceranae* e *N. apis* da DNA estratto dalle api. Questo metodo è più semplice della maggior parte degli altri metodi basati sul DNA e fornisce una discriminazione comparabile tra le due specie, utile anche come misura dell'esposizione e della sensibilità delle api adulte in situazioni epidemiologiche definite.

Terzo capitolo, l'attività di ricerca si è basata sulla presenza e sul profilo di virulenza dei ceppi patogeni di *E. coli* nel latte di massa e nei filtri della macchina della mungitura ed è stato valutato su un certo numero di campioni raccolti nelle aziende casearie. Per raggiungere questo scopo, le tecniche di isolamento e di identificazione classiche sono state accoppiate con un approccio diagnostico molecolare. I risultati hanno sottolineato il diverso pattern di rilevazione di *E. coli* patogeni nel latte di massa rispetto al filtri della macchina della mungitura, con particolare riguardo ai ceppi enteroinvasive *E. coli* (EIEC). L'unione dei metodi microbiologico e biomolecolari permette l'identificazione di *E. coli* da altri batteri su supporti specifici e dai profili dei fattori di virulenza, portando ad un'identificazione rapida e sensibile dei ceppi patogeni, step necessario per una corretta identificazione dell'esposizione per l'ospite e per l'uomo, e valutazione del rischio di salute inerente. In particolare, l'analisi dei filtri del latte potrebbe

essere uno strumento utile per valutare la presenza del rischio di patotipi di *E. coli* sia a livello alimentare che ambientale.

In generale, i risultati del presente progetto potrebbero contribuire alla caratterizzazione della sensibilità degli ospiti per queste malattie e, infine, del rischio che questi patogeni possono provocare per la salute degli animali e/o degli esseri umani.

General Introduction

This PhD thesis is the outcome of a range of activities and experimental results aimed to a better implementation of molecular tools in the control strategies of some honey bee and cow diseases. This approach was selected since the above two food-producing livestock species are representative of very different production systems and are exposed to several pathogens with complex epidemiological, pathological and control implications.

In the field situation, the potential benefit of the wide-scale application of molecular tools for healthy risk monitoring and pathogen control strategies is still under looked for a number of reasons. Among these, it should be mentioned the usually higher complexity and cost compared to most of the classical monitoring tools, and the sometimes incomplete in-field standardization of the protocols. On the other side, the molecular tools that are properly validated in terms of sensitivity, specificity and accuracy can greatly help pathogen detection especially in cases disease management programmes for reduction of morbidity and mortality relies both on prompt species-specific diagnosis and on effective profiling of species/strain virulence and host resistance, whereas the standard practices do not provide sufficient reliability. With this aim, standardization of the molecular methods and comparison with reference methods that are already in use are always necessary to guarantee reliable and comparable results.

The possibility to optimize the existing methods for a reliable diagnosis and accurate risk profile of severe pathogens in field conditions could be considered with special regard to the widespread honeybees pests like the microsporidial organism Nosema ceranae, and to the risk for animal and human health linked to bacterial agents like specific, virulent Escherichia coli strains in dairy cow milking systems. Also, the genetic programmes against honeybee brood diseases have a strong need of rapid and efficient methods, like molecular tools could be, for selecting colonies with a high level of self-defense capabilities mainly represented by the nest-cleaning behaviour, i.e. the so-called hygienic behaviour. The present project deals with the above topics with different molecular approaches and investigation strategies. Overall, different protocols are here tested for routine veterinary diagnostics applied to in-field monitoring, and hold promises as quick methods allowing specific and sensitive detection of the targets with shorter analysis time and reduced cost, in parallel or in alternative to the classical approaches.

As for Honey

1 Caste Division in honeybee

The honeybee colony is composed by: single queen, the only fertile female; honeybee workers, thousands of sterile females and males or drones, few hundreds, that are present during spring for the reproduction (Pistoia 2010) (**Figure 1**).

The queen laid eggs of workers and drones in the cell, once the honeybee emerged from the cell that started his duties. The different behaviors of the honeybee were regulated by hormones (like juvenile hormone), genetic predisposition and in according with the colony conditions and environmental (food availability and seasonal influences) (Robinson 1992).

Morphologically the worker, the queen and the drone are easy to recognize for the specific body characterization. The worker honeybee is covered by thick hairs, the wings of a length almost equal to the body and the eyes are well separated. The drones are larger, stockier, have longer wings and huge eyes that meet each other forehead. The queens are larger and tapered, with a swollen abdomen that protrudes from wing level abundantly (Contessi 2016).

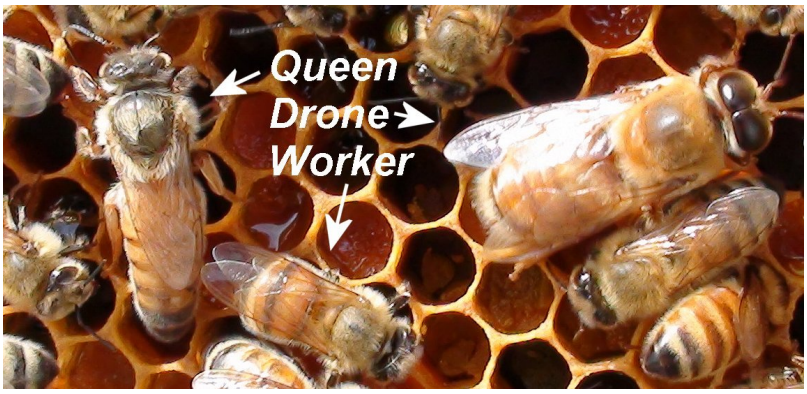


Figure 1: Caste division. From the top to the bottom: queen, drone and a worker honeybee.

The worker builds multiple combs, which consists of adjoining hexagonal cells made of wax secreted by the workers' wax glands. In the cells, they store their food (honey and pollen) in the upper part of the comb and the brood (worker and drones) in the available cells (Suwannapong et al. 2014). While, the larger queen cells are normally built at the lower edge of the comb (Seeley 1985; Otis and Hadisoesilo 1990).

- ✓ **Queen**

There is only one queen in each colony and her roles are: laid eggs (2,500 eggs a day) and produce a queen pheromone (mandibular gland pheromone), which influence the physiology and behavior of the workers and serves to maintain the colony in a "normal" state (Winston and Slessor 1992). The new queen (known as the "virgin queen") emerges 16 days after the egg was laid (Seeley 1985; Winston 1987; Winston and Slessor 1992)and she stays in the colony for a few days in order to be feed and to allow her reproductive organs to mature a little further. Following this first period, the virgin queen comes out from the colony and makes a several reconnaissance flights to prepare for the mating flight around 21 days from the emerged. The mating site usual is close to the colony and up to 30 meters from the ground. The queen flies up to the drones and she mates repeatedly with several of them until her spermatheca is full, after she comes back to the colony. The workers accommodate what she need (food, grooming and cleaning her wastes) and they control the vacant cells controlling her egg production in according to seasonal variations: increasing and decreasing the amount of stores of pollen and honey (Mackensen 1943; Anderson 1963; Woyke 1963; Winston 1987; Winston and Slessor 1992). After a few years, the mature queen reduces the number of eggs laid and less pheromones and the colony begins the replace with a new virgin queen. The workers produce a new queen, which is a new extend form more existing worker cells (at begging, there is no difference between an egg of worker or queen) to "queen cell" in the bottom or edge of the main the broodcomb. The new larva is fed by rich diet of "royal jelly" by the nurse honeybees. The rich diet fed to the developing queen larva alters the anatomy of the larva (from that of the worker). The abdomen becomes notably longer for the reproductive organs (the ovaries and spermathecal) (Mackensen 1943; Anderson 1963; Winston and Slessor 1992).

When the virgin queen is ready to emerge, she can have different fates:

1) She will be not the only virgin queen and she fight against to the others virgin queens,

2) She will became a queen of a colony, which a part of them is flight away with the old queen (a swarm),

3) She will be accepted by the colony, the workers of which will immediately start to care for her (Mackensen 1943; Anderson 1963; Winston and Slessor 1992)

This destiny happens in colonies without control of a profession beekeeper, that supervises the straight and the genetic of the colonies and directly replaces the old queen.

- ✓ **Drones**

Drones are the male honeybees and they die after workers stop feeding them because they can't feed alone for their morphology. Their body sizes are larger than workers andt hey have large compound eyes, which appear to come together at the top. Their main role is to mate with virgin queens. They arise from unfertilized eggs, indeed they are "haploid" (16 chromosomes), with respect to" workers (diploid) and queen (polyploidy) (Mackensen 1943; Anderson 1963; Winston and Slessor 1992). The queen has two distinct reproductive organs: once holding the eggs and once the sperm and she has the choice when laid a fertilized egg (worker or drone). The queen will lay drone eggs in drone cells that are larger in diameter in order to the larger body size of the drone. The drone larva is progressively fed by workers, after 24day he emerges as a young adult. After a few days, the drone will begin to carry out reconnaissance flights and they have a good ability to navigate probably for their compound eyes with respect to the workers or queen. After 2 weeks the drone will be mature enough to mate in the "mating yards" (previous describe in mating flight. During the mating, the drone's sex organs (endophallus) inflate within the queen and pumping sperm into her and a part of that is gripped in the queen's oviduct. Each drone will remove from the queen the endophallus of his predecessor before undergoing the same fate. After mating, the drone usually dies (Gary 1963; Ruttner 1966; Winston and Slessor 1992).

- ✓ **Workers**

Workers carry out all the duties that go into building and maintaining a colony (brood rearing, comb building, house cleansing, foraging, and colony defense). They have a smaller body size and they possess a sting that is a modification of ovipositor. The worker although are infertile, but they have the reproductive organs. Indeed, there is a lack of the reproductive capacity of the queen, they starts out as a normal egg laid by the queen. The worker larva is fed "brood food" which is largely a combination of nectar, pollen (protein required), sugar (royal jelly) and enzymes from the saliva of the "nurse" honeybees (Mackensen 1943; Anderson 1963; Winston and Slessor 1992). The worker diet is the same of the queen larva, however the proportion of

royal jelly in the first 3 days promotes is less respect the queen, which increase of growth hormones and decide the destiny of the larva.

There are two main labour that they make during their life in according with the age caste: young workers (nurse) and older workers (foragers), but there are flexible in base of the necessary of the colony and season (foragers season life is 6 weeks, while in winter is more than 6 months (Oster and Wilson 1978; Robinson 1992)

The emerged honeybee is still soft, and they cannot sting, and internal development continues, especially glandular development. (Winston 1987). During this period, the nurses (young honeybees) perform cell cleaning and brood capping tasks, followed by tending brood and the queen. Once the workers are about 15 days, their behavior shifts from nurse to house honeybees, and they perform tasks related to comb building, cleaning and food handling. In according with the different behavior of the worker, their glands change the development: the wax glands become more developed, while the size of the mandibular and hypopharyngeal glands diminishes gradually. After 3 weeks their start the last phase of the life cycle, the workers come out of the beehive and they begin with the first orientation flights, followed by foraging activity otherwise participate in guarding the nest and ventilating for cooling the hive or evaporating water from the honey (Crane 1990).

Plasticity or flexibility in the age related behavioral patterns of tasks performance, make that a colony can adapt very well to a constantly changing internal and external environment. During swarming for instance when many old workers establish a new colony together with the departing queen, many tasks, normally performed by young workers, such as building combs and caring for the brood, will be taken over by some of the older workers. In the original colony, where mostly young workers remain, a group of them will start foraging and defending the nest (Robinson 1992).

2. Honeybee Disease

The spread of the honeybee diseases are favored by the beekeepers that moving colonies to new area through trade and nomadism, so the parasite move from their original hosts to the new honeybee species, as *Varroa destructor*.

The infectious diseases that affected the health of honeybees can be divided into two main groups:

- **Brood diseases**: infecting the development stages before adulthood, as egg, larva and pupa. Major diseases: American and European foulbrood; chalkbrood disease and Varroosis.
- **Adult bee diseases**: infecting the honeybees after emerging from capped cells. Major disease: Nosemosis.

In terms of the regulatory aspects of honeybee pathogens, under the WTO (World Trade Organisation) agreement on the application of Sanitary and Phytosanitary Measures (SPS Agreement), the OIE (World Organisation for Animal Health) publishes sanitary standards for international trade in animals and animal products. As part of this activity, OIE develops international standards and guidelines for diagnostic tests and vaccines as well as for veterinary laboratories. Six honeybee diseases are listed by the OIE in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals to provide internationally agreed diagnostic laboratory methods ((OIE) 2008). The COLOSS BEEBOOK provides many additional protocols, which can improve pest and pathogen research and its potential impact in these times of pollinator declines. The relevant specialists in each field have been gathered to constructively list and to criticize existing research protocols. The BEEBOOK is thus an important step forward to the establishment of standardized protocols for the study of honeybee diseases.

I. American foulbrood (AFB) and European foulbrood (EFB)

AFB is a disease transmitted by gram-positive *Paenibacillus larvae*, which spores germinates in larval guts (Genersch et al. 2006; Sammataro and Avitabile 2011)). It is recognizable by irregular and sunken capping, by change in brood color which appears dark and “melted” down, and pupal tongue sticks out (**Figure 2A**) (Sammataro and Avitabile 2011). This is the most destructive of the brood diseases and it is not a rare disease. Once it occurs that causes a

considerable economic losses to beekeepers. It easily diagnosed and well controlled by the authorities, indeed it can be considered one of the major threats to honeybee health. (Ashiralieva and Genersch 2006; Genersch 2007, 2008, 2010). To control the disease, the best way is to burn all colonies infected with AFB or you can treat the infected colonies with antibiotics (Terramycin and Tylan).

EFB is disease transmitted by the *Melissococcus plutonius,* that coccus with a close phylogenetic relationship to the genus *Enterococcus* (Cai and Collins 1994). Bacteria infect younger and uncapped larvae, turning them brown (Sammataro and Avitabile 2011). The Infection is at larvae level when they ingest food contaminated with *M. plutonius*. The larvae are susceptible at any stage before cell capping, but it decreases with increasing age. Bacteria proliferate in the larval midgut assimilating much of the larval food so the larvae die after 4 or 5 days for starvation (Bailey 1983). Dead larvae are twisted around the walls or stretched out in the cell. Some of infected larva may survive and pupate after discharging bacterially contaminated faeces, which are deposited on the walls of the brood cells (Bailey and Ball 1991). Surviving infected larvae produce pupae and adults of subnormal weight (Bailey 1960). EFB has two saprophytic bacteria (*Paenibacillus alvei* and *Enterococcus faecalis*) as secondary invaders, that decomposed the larvae infected (**Figure 2 B**).

It is commonly present in weak or stressed colonies and during the spring season, slowing the growth of the colony, but may disappear with the onset of a good honey flow (Forsgren 2010; Sammataro and Avitabile 2011). The treatment in-field is three dustings of terramycin, 5 to 7 days apart. If the symptoms return, repeat the steps above to clear up the bacteria. Tylosin is another broad spectrum antibiotic used to treat gram positive organisms. It is also detectable using a PCR techniques specific for *M. plutonius* (Govan et al. 1998; Forsgren et al. 2005; Roetschi et al. 2008).

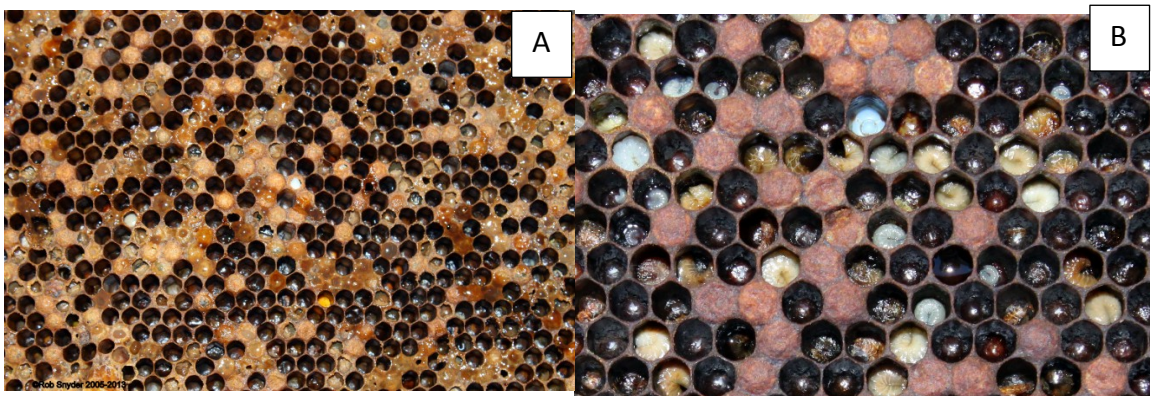


Figure 2. A, American Foolbrood disease; **B**, European Foolbrood disease.

II. Chalkbrood disease

It is caused by the fungus *Ascosphaera apis*, and *Aspergillus* spp., also known as stone brood disease, origin in Japan (Morse and Flottum 1997; Qin et al. 2006; Aronstein and Murray 2010). It look like as non-homogeneous brood and the appearance of white, mummified larvae, first in the cells and then on the bottom board (**Figure 3**) (Sammataro and Avitabile 2011; Contessi 2016). The spore infection are formed during sex reproduction of fungi and it can be transmitted by food or the body surface. It is observed in larvae that is three to four days. The dead larvae swell to the size of the cell, covered with the whitish fungi of the fungus, subsequently, the dead larvae mummify, harden, shrink and appear chalklike. The color of the dead larvae varies with the stage of growth of the fungi: first white, then grey and finally, when the fruiting bodies are formed, black. In the laboratory the fungus can be identified by its morphology ((OIE) 2008). The brood diseases can be controlled keeping strong colonies and kept under control applying beekeeping procedures as re-queening the colony.

Figure 3: Larvae infested by chalkbrood disease

(http://www.ars.usda.gov/is/graphics/photos/aug98/k8144-3.htm)

III. Varroosis

One of the major parasite that affect the health and the survival of the Western honeybee, it is the ectoparasitic mite *Varroa destructor* (Anderson and Trueman 2000). Originating in South-East Asia, where it parasites *Apis cerana*, but in the last decades *V. destructor* made a host switch to *A. mellifera* and spread around the world (Rosenkranz et al. 2010).

Life cycle of *V. destructor* has two distinct phases: a phoretic phase on adult bees (**Figure 4 B**) and a reproductive phase within the sealed drone and worker brood cells (**Figure 4 A**).

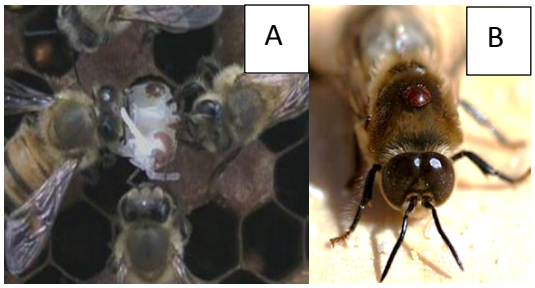


Figure 4. **A**, *V. destructor* inside the brood during the reproductive phase. **B**, phoretic phase of *V. destructor* on adult bee

The Infection began from the female mites, which were transported within the colony by adult bees during the phoretic stage. The reproductive stage of the mite began when the female mite enter brood cells, before the cell is capped (**Figure 5**). From pupation to emerge of the honeybee, foundress mites must produce at least a mature male and mature female mite within the natal cell in order to produce viable offspring (Donzé et al. 1996). The mother mite and her offspring regularly feed on the host pupa sucking haemolymph and eventually transmitting a wide range of viruses to the bee larvae, *e.g.* Deformed wing virus; Israeli acute paralysis virus; Kashmir bee virus; Acute bee paralysis virus (Di Prisco et al. 2011; Martin et al. 2012). When the newly-emerged honeybee from the cell, adult female mites and any fully developed offspring are released into the colony, meanwhile the male and any immature females die. Adult female mites can also disperse from one colony to another when transported on the bodies of drifting, robbing or swarming worker honeybees (Rosenkranz et al. 2010). Indeed, this efficient way of transmission and the foraging behaviour allow rapid spreading of the mite, and facilitates viral transmission to bees, beside inflicting nutritional

stress and immune suppression (Chen et al. 2006; Chen and Siede 2007; de Miranda and Genersch 2010; Rosenkranz et al. 2010; Dainat et al. 2012)

Figure 5: *V. destructor* life cycle.

The main strategy to fight *Varroa* infestation is the use of chemical and/or organic pharmaceutical products. Besides, the pharmacological control of *V. destructor* can lead to some negative outcomes, *e.g*. loss of efficacy after prolonged use due to the development of mite resistance, propagation of susceptible colonies by beekeepers, increased beekeeping costs, and the occurrence of acaricide residues in honeybee products like honey and wax (Milani 1999; Oldroyd 1999; Bogdanov 2006). Such approach, it is not sufficient to eradicate the mite.

IV. **Nosemosis**

Microsporidia are single-cell organisms and are possibly the smallest organisms with a true nucleus (eukaryotes). Mature spores measure 1.5 - 10 µm in length, depending on the species.

Many pathogens of insects, as some bacteria and fungi, can germinate and reproduce in the environment at least during certain life stages, but microsporidia are obligate pathogens, and can only multiply inside the cells of the host. The only phase that can survive for long period out of environment is the infective mature spore, which is essentially a dormant stage and must be ingested by a susceptible host and invade the tissue cells of the alimentary tract to reproduce once again. Most microsporidian species reproduce only in the cytoplasm of the host cells, but a few species have been known to utilize the cell nucleus.

Nosemosis is a highly prevalent and serious disease affecting honey bee, *A. mellifera*, caused by two microsporidia, *Nosema apis* and *N. ceranae*. These two species are morphologically similar but differ in epidemiological pattern and virulence. Classical type A nosemosis was detected for the first time by Zander in 1907, it is caused by *N. apis* and afflicts honey bees with reduced honey yield, increased winter mortality and poor spring build-up (Fries 1993). In 1996 *N. ceranae* has been reported infecting *A. ceranae* (Fries et al. 1996). In the following years, several studies showed the widespread expansion of *N. ceranae* infection host range to *A. mellifera,* and now this microsporidian parasite is considered a worldwide emerging pathogen with a high prevalence in honey bee colonies (Higes et al. 2006, 2010; Paxton et al. 2007).

The Nosema microrganism passes the active reproductive phase of its life cycle within the digestive cells lining the mid-gut of the adult honey bee. On entering one of these cells, the animal grows and multiplies rapidly, utilizing the cell contents for its food supply. Reproduction ceases after several days with the formation of a large number of spores. The cell then reptures, shedding the spores into the mid-gut lumen. The spores pass down the gut into the small intestine and accumulate in the rectum from which they are voided at intervals in the bee's faeces. Within the spore, the parasite enters its passive, resting stage until swallowed by another bee. When this happens, the spore "germinates" as it enters the ventriculus from the crop. It enters an epithelial cell and begins to grow.

Nosema spores contain simple but important and interesting organelles. These structures, which are only visible using transmission electron microscopy at a magnification of more than 5,000X, are nearly all related to invasion of host cells and reproduction. **Figure 6** reports a longitudinal section of a mature spore containing a double nucleus (all true *Nosema* species have double nuclei called diplokarya; other groups may have a single nucleus), layers of membranes at the apical end of the spore named polaroplast, a vacuole at the distal end of the spore and, most spectacularly, a polar filament that is attached at the apical end of the spore and winds like a spring around the inside spore wall. The mature spore is covered by an outer exospore formed of protein and an endospore that is composed of a protein-chitin matrix. This thick, tough spore wall protects the spore once it leaves the host cell and is exposed to environmental conditions.

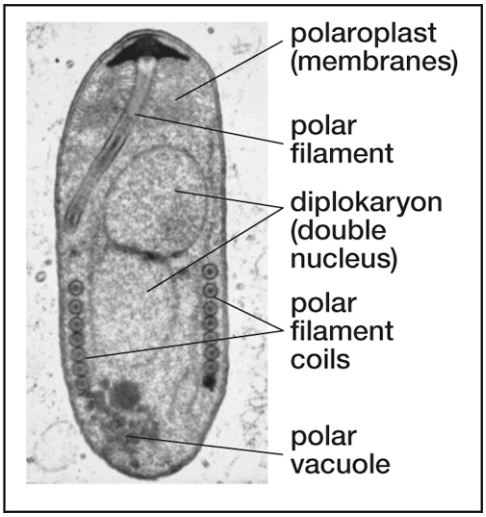


Figure 6: Major organelles inside an infective microsporidian spore. Courtesy photo of the Society for Invertebrate Pathology.

Nosema spp. invade the intestinal cells coating the mid-intestine of the honey bee, then they multiply fastly and within a few days (3-7 days) the cells are full of spores, the resting phase of the parasite. When the bee intestine cell ruptures, it sheds the spores into the gut where they accumulate in masses, to be later excreted by the bees. If spores from the excreta are picked up and swallowed by another bee, they can germinate and once more become active, starting another round of infection and multiplication (**Figure 7** and **Figure 8**).

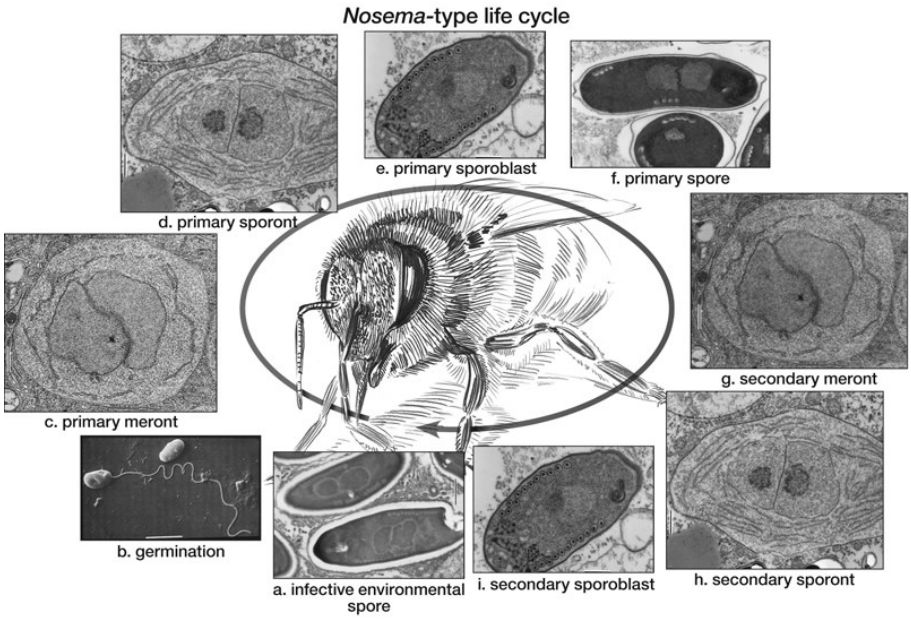


Figure 7: life cycle of Nosema (Scanning electron micrograph of germinating spore courtesy the Society for Invertebrate Pathology; transmission electron micrographs of other stages (courtesy of Wiley Publishing Co.)

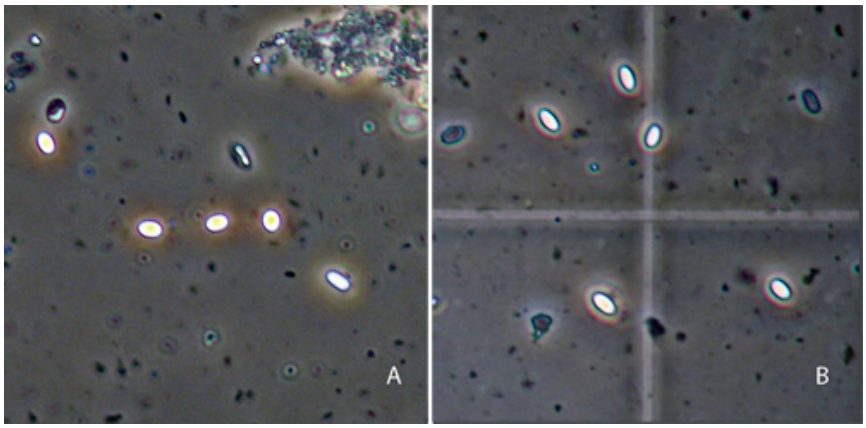


Figure 8: Mature infective spores of *N. apis* (a) and *N. ceranae* (b), pathogens of the honey bee. Courtesy photos by Huang.

Nosema infection has been associated with impact on pheromone production, immune response, flight behavior, energetic stress, behavioral fever and hunger mediated conduct (the bees are less inclined to share their food with other bees), effects on brood care, thermoregulation, defense and foraging. All these factors may have an impact not only at the individual but also at the social level and have detrimental effects on colony homeostasis (Botías et al. 2012b).

N. apis is more prevalent in cooler climates and affects honeybees primarily in the spring and early summer. it has been referred to as “Spring dwindling”, with dysentery and fouling of the outside of hives with faeces and it is known to age bees more rapidly in that they take on the duties of older bees and subsequently die sooner than non-infected bees (Fries 2010). These precocious foragers have been shown to be less effective and resilient than normal foragers. Infection with *N. apis* has also been shown to raise Juvenile Hormone III (JHIII) levels in bee hemolymph which is associated with behavioural changes. There is also evidence that colonies infected with *N. apis* collect significantly less pollen than uninfected colonies due to the reduction in their adult bee population. Although feeding pollen supplements in the laboratory increases the lifespan of bees infected with *N. apis,* feeding field colonies with supplemental pollen has been reported not to counter the reduction in worker lifespan, presumably as it was used primarily for brood rearing. It was observed that *N. apis* colonizes the entire gut in approximately 2 weeks after infection and an average of 3-5 x 10^6 spores are present at the end

of this period, although there was no apparent relationship between bee longevity and spore dose (Fries et al. 2013).

N. ceranae is more adaptable and less host specific than *N. apis* (Botías et al. 2012b). There is evidence that *N. ceranae* is far more virulent than *N. apis*, causing colony deaths in warmer drier climates, is more stable at warmer temperatures and that it has a greater effect on longevity. The stress may be an important factor contributing to the increased virulence of *N. ceranae* observed. In a recent study, feeding experiments on caged bees showed that both mortality and sugar syrup consumption were higher in *N. ceranae* infected bees than in N. apis-infected and control bees (Martín-Hernández et al. 2011). The mortality and sugar syrup consumption were also higher in *N. apis*-infected bees than in controls, but less than in N. ceranae-infected bees. With both microsporidia, mortality and sugar syrup consumption increased in function of the increasing spore counts administered for infection, from 10^3 to 10^5 spores/bee. The differences in energetic requirements between Nosema spp. confirms that their metabolic patterns are not the same, which may depend critically on host– parasite interactions and, ultimately, on host pathology. The repercussions of this increased energetic stress may even explain the changes in host behavior due to starvation, lack of thermoregulatory capacity, or higher rates of trophallaxis. *N. c*eranae appears to be more prevalent in the summer than *N. apis* but seasonal infection peaks appear to differ between geographical zones (Martín-Hernández et al. 2011; Chen et al. 2012).

It was reported that there were no differences in virulence of strains isolated from Spain and France and it was considered that the response of the strain of honeybee to infection may be more critical, e.g. susceptibility of *A. mellifera iberiensis* (Dussaubat et al. 2013).

The biological cycle of Nosema spp. in honeybees has been reported to depend on temperature. When expressed as total spore counts per day after infection (2 weeks old bees infected with 10,000 spores), the biotic potentials of *N. apis* and *N. ceranae* at 33°C were similar, but a higher proportion of immature stages of *N. ceranae* than of *N. apis* were seen. At 25 and 37°C, the biotic potential of *N. ceranae* was higher than that of *N. apis* (**Figure 9**). The better adaptation of *N. ceranae* to complete its endogenous cycle at different temperatures clearly supports the observation of the different epidemiological patterns and supports the increased incidence of problems associated with *N. ceranae* infections in warmer climates,

whereas the viability of *N. ceranae* spores is significantly reduced following one week in a deep freezer, which is not the case for *N. apis* (Martin-Hernandez et al. 2007).

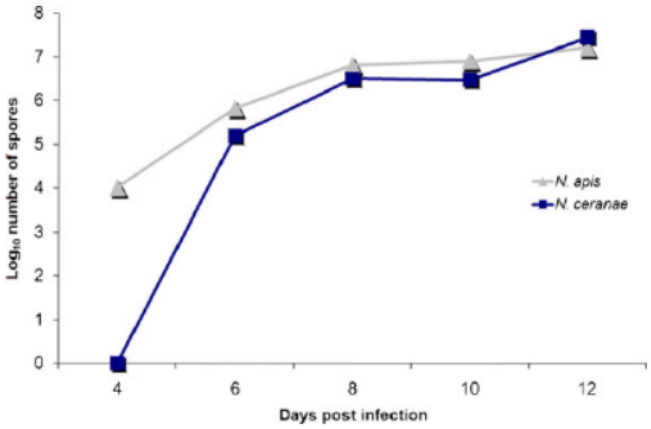


Figure 9: Biotic Potential of N. apis and N. ceranae (Fries et al. 2013)

N. ceranae has been reported in a number of European countries, including Spain, France, Germany, Switzerland, Denmark, Finland, Greece, Hungary, Holland, United Kingdom, Italy, Serbia, Poland, Slovenia, Bosnia Herzegovina and Sweden (Higes et al. 2010). A multiplex PCR assay was used to detect coinfections by the two species of Nosema in a screening approach in Europe. Screening of bee samples from Spain, Switzerland, France, and Germany using the PCR technique revealed a greater presence of *N. ceranae* than of *N. apis* in Europe, although both species are widely distributed. From the year 2000 onward, statistically significant differences have been found in the proportions of *Nosema* spp. spore-positive samples collected between and within years. In the first period examined (1999 to 2002), the smallest number of samples diagnosed as Nosema positive was found during the summer months, showing clear seasonality in the diagnosis, which is characteristic of *N. apis*. From 2003 onward a change in the tendency resulted in an increase in Nosema-positive samples in all months until 2005, when a total absence of seasonality was detected (Martin-Hernandez et al. 2007).

The disease caused by *N. ceranae* in honey bees, has been named as type C nosemosis, and has a more severe impact on colony health compared to infections by *N. apis* (Fries 2010; Higes et al. 2010). Nosemosis caused by *N. ceranae* is now considered to be an internationally emergent

disease (Higes et al. 2010). Thus, correct species differentiation is needed in order to establish proper control strategies of the infection by beekeepers.

Microscopical examination is the mainly utilized method for the diagnosis of honey bee nosemosis, but the spores of the two microsporidia are not easily distinguishable. Consequently, a molecular analysis is usually required for species identification (OIE) 2008; Botías et al. 2012; Fries et al. 2013). In the last years, several molecular protocols have been published for the identification of *N. ceranae* and *N. apis*, including species-specific PCR assays, or simultaneous detection of the *Nosema* species using multiplex PCR (Higes et al. 2006; Martin-Hernandez et al. 2007; Chen et al. 2008; Carletto et al. 2013). Until now, published real time PCR-based assays capable of simultaneous identification and quantification of both species in the same reaction use separate primers pairs and species-specific probes (Chen et al. 2009; Bourgeois et al. 2012).

High Resolution Melting Analysis (HRMA) is a molecular technique allowing single-nucleotide discrimination that is increasingly used in diagnostic microbiology and parasitology for species identification and genotyping. This technique offers a low-cost, closed-tube approach to amplicon analysis with the capacity for single-nucleotide discrimination and easy integration with real time PCR using a single pair of (Reed et al. 2007). Several real-time PCR-HRMA based assays have been published in the recent years for parasite diagnosis (Areekit et al. 2009; Wongkamchai et al. 2013; Albonico et al. 2014). At the present time, no real time PCR-HRMA based methods have been validated for simultaneous detection and discrimination of the two closely related *Nosema* species affecting honey bees.

3. Hygienic behavior in honeybees

The honeybees are selected for traits that permits a more sustainable apiculture (as gentleness, low swarming tendency, high wintering ability) and for productivity (as beeswax, propolis and pollen import). Another traits of selection in honeybees are brood production, disease and tolerance disease (Sammataro and Avitabile 2011; Contessi 2016)

Since the 1930s, the hygienic behavior (HB) is known as a behavioral response of nurse honeybee to stop the spreading of infections inside the colony, conferring resistance against diseases that affect, *inter alia*, the honeybee brood (Park 1937; Wilson-Rich et al. 2009).

Hygienic bee are able to detect the presence of an infected larva or pupa and respond by 'uncapping' the wax cover of a brood cell, if the cell was sealed, and by removing the diseased individual. HB evolved as a general mechanism of resistance to brood pathogens including *P. larvae*, *A. apis* and *V. destructor* (Gilliam et al. 1983; Spivak and Reuter 1998a, b, 2001b; Harbo and Harris 1999; Invernizzi 2001).

In recent years, there has been substantial progress in developing the HB in honeybees, using a documented for selective breeding realize in Minnesota and Russia (Ibrahim and Spivak 2006; Rinderer et al. 2010; Büchler et al. 2010).

General HB also confers some resistance toward *Varroa* mite and may contribute to the resistance of *Varroa*-surviving colonies in France (Spivak and Reuter 2001a). A large proportion of *Varroa* mites in resistant colonies enter cells and either die, produce no progeny and produce male or immature progeny (Harbo and Harris 1999). In 2006, Ibrahim & Spivak found that non-reproduction of mites was related to HB and the term *Varroa* Sensitive Hygiene (VSH) was coined.

Specific HB toward *Varroa* is the basis of resistance in the phenotype *Varroa* Sensitive Hygienic (VSH) (Dietemann et al. 2012). These honeybees preferentially remove mite-infested pupae that are between 3 to 5 days post-capping (Harris 2007). *Varroa* mite reproduction is heavily synchronized with the reproductive timing of its bee host; therefore, mites removed from cells by HB may be forced to reinvade new cells in order to complete reproduction (Steiner et al. 1994; Harris 2007). A break in synchrony between the reproductive cycle of the re-invading mite and the development of its host has previously been shown to negatively influence on mite reproduction (Kirrane et al. 2011; Frey et al. 2013). Thus, the removal behavior by VSH bees significantly lowers the population of *Varroa* mites in these colonies and suppresses their reproduction.

Despite the hygienic phenotype is deeply investigated by the scientific community, HB and VSH are still poorly characterized from a molecular perspective. Recent studies have determined HB to be a quantitative trait and up to seven loci may be responsible for its expression (Lapidge et al. 2002; Gramacho and Spivak 2003; Oxley et al. 2010; Danka et al. 2011). Quantitative trait loci (QTL) mapping of genotypes of individual VSH stock identified candidate genes associated with vision, olfaction, memory and dopamine reception (Tsuruda et al. 2012). Differential up-

regulation and down-regulation of genes related to neuronal wiring, olfactory and visual signaling has been shown in VSH and non-VSH stocks (Le Conte et al. 2011).

In solitary insects, cellular or humoral-based defenses provide the only known system for immunity, but honeybees' genome reveals that bees retain these systems for immunity too, but the number of immunity genes is lower than those of solitary insects such as flies, moths and mosquitoes. As an apparent compensation for this, social insects have evolved collective systems of behavior that provide defenses against disease and parasitism (Evans et al. 2006).

- Evaluation of HB in-field

Since HB is considered to be heritable as a quantitative trait, many honeybee breeders try to select colonies with the highest HB. During selection process and in-field evaluation, HB is phenotypically measured using the pin-prick method or freeze-killed brood (FKB) assay.

The pin-killing method kills or damages the brood by perforating and killing the pupa within the brood cell, which can be easily performed in the field or in the laboratory. This process produces a small hole in the capped cell and the treated brood exudes body fluid. Sometimes this fluid is visible on the cell cap.

The freeze killed brood (FKB) test consist in a cylinder of thin metal, 6–8 cm in diameter, was twisted into a comb portion containing sealed brood until it reached the mid-rib. Approximately 300 ml of liquid nitrogen were poured slowly into the cylinder, in order to kill the brood in the selected area (**Figure 10 A**). Once thawed, the cylinder was extracted and the frame was marked to indicate its location within the brood nest. Photographs of each patch were taken, and the frame was replaced in its hive. From 24 hours later, the frame was removed, and the comb was photographed again (**Figure 10 C**). The photographs were then used to determine the percentage of capped brood cells that had been removed by each colony HB was assessed as the number of cells with capped brood that had been cleaned out (cell uncapped and dead brood removed) by honeybee nurses.

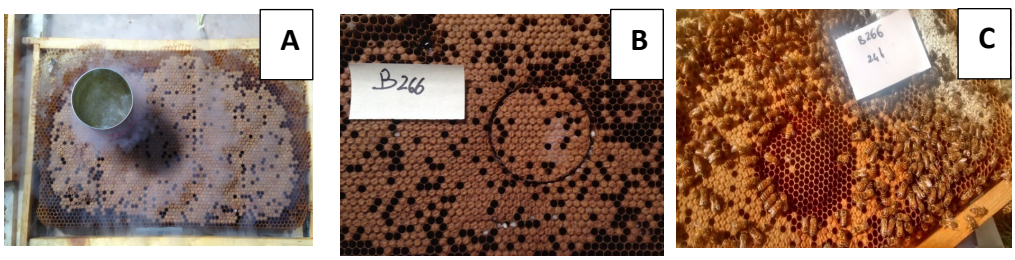


Figure 10. Principal steps of FKB test: **A**: nitrogen liquid in the cylinder on the brood comb; **B** after nitrogen liquid treatment; **C**, after 24 hour in the test colony**.**

Since all these methods are time consuming and resource intensive for the breeder, limiting their suitability for widespread application, the availability of molecular biomarkers of the hygienic phenotype could fasten the recognition of this trait and also the breeding processes. However, the removal of killed brood is not always correlated with the removal of disease or parasitized brood (Palacio et al. 2010).

4. Gene marker for honeybee evaluation

The effective defense of honeybees against such diseases is one of the most essential achievements of the honeybee colony.

In modern, the main strategy to fight brood disease relies on the use of chemical and/or organic pharmaceutical products, often coupled with specific beekeeping practices to minimize the load of parasites in the colony. Such approach, unfortunately, is not sufficient to eradicate the disease like varroosis, nosemosis and foulbrood diseases. Besides, the pharmacological control of *V. destructor* can lead to some negative outcomes, e.g. loss of efficacy after prolonged use due to the development of mite resistance, propagation of susceptible colonies by beekeepers, increased beekeeping costs, and the occurrence of pharmacological residues in honeybee products like honey and wax (Milani 1999; Oldroyd 1999; Bogdanov 2006).

The effective defense against disease is one of the most essential achievements of the honeybee colony. The individual honeybee's immune system functions in a similar way to that of vertebrate animals, although the most effective defense mechanism that can lead to self-healing of the honeybee colony is the social behavior of removing as many pathogen agents or parasites as possible from the honeybee colony. This behavioral defense (entrance reduction and/or stinging) prevents parasites from penetrating the honeybee colonies, or their killing or removal. If the dead organism is too large to remove, as with mice, the honeybees completely cover it with propolis. The most important defense against disease, however, is the honeybees' HB. In latest years, there has been substantial progress in developing the HB in honeybees

In honeybees, the gene expression studies mostly performed were on brain tissue, which have shown that division of labor and several other eusocial species, is associated with task-specific RNAm transcriptional profiles (Whitfield et al. 2003; Adams et al. 2008; Oxley et al. 2010; Liu et al. 2011; Daugherty et al. 2011)

In 2014, was performed a high-throughput sequencing identification of genes involved with *V. destructor* resistance in the western honeybee, *A. ceranae* (Ji et al. 2014). In Varroa-resistant *A. ceranae* colonies was found three members of the odorant binding protein (Obp) colony (obp4, obp17 and obp18) and two transcription factors were significantly upregulated like cAMP-responsive element-binding protein-like-2-like and mushroom bodies like – 1-like, (Mblk-1) (Ji et al. 2014).

Many studies have proposed that honeybees with the Higher olfactory sensitivity in general initiate HB as they can detect and discriminate abnormal brood at low stimulus level (Swanson et al. 2009). Such differences in olfaction could be related to honeybee Varroa tolerance and can also account to increased grooming and HB, which are important traits known to be implicated with *V. Destructor* resistance (Navajas et al. 2008). The recognition and discrimination of thousands of odorous compounds is mediated by olfactory sensory neurons (Ji et al. 2014). In insects, chemosensory neurons are surrounded by an aqueous environment that acts like a barrier to volatile and hydrophobic molecules. Many airborne molecules, such lipophilic odorants and pheromones, must be recognized by a specialized class of proteins that facilitate their delivery to the olfactory receptors. This function is provided by Obp in insects (Deyu and Leal 2002). Thus upregulation of Obps found by Ji et al. (2014)could be related to the higher olfactory sensitivity of hygienic colonies. Recently in the article of Boutin et al. (2015), the Obp4 is the only Obp colony over expressed in the brain of Non-High HB honeybee. On the other hand, as reported in the paper of Foret & Maleszka (2006) Obp4 is expressed exclusively in the antennae of adult honeybees, with chemotactic functions.

Another interesting gene, Mblk-1 transcription factor, is expressed in the mushroom bodies and therefore it may function in the mushroom body neuronal circuits (Takeuchi et al. 2001; Park et al. 2003). Mushrooms bodies are thought to be involved in sensory integration, learning, and memory in insects (Groh et al. 2006). Compared to those in other insects, mushrooms bodies in honeybees are well-developed, and could be connected to their advanced social behaviors

(Blenau and Erber 1998; McQuillan et al. 2012). Actin 5c (Act5C), a cytoskeletal gene, is upregulated during memory formation (Lamprecht and LeDoux 2004). This gene, in addition to the memory formation, induces synaptic and structural plasticity and long-term potentiation in the honeybee brain (Hotulainen and Hoogenraad 2010; Ojelade et al. 2013). The housekeeping gene Ribosomial protein L32 (Rpl32) is considered a suitable reference gene for gene expression studies because it is stably expressed during development of honeybee larvae and pupae (Reim et al. 2013). Also, its expression is significantly stronger in the brains honeybee for all life (Lourenço et al. 2008; Reim et al. 2013).

5. Aim

The selection of colonies that are resistant to diseases can be of advantage for the professional beekeeper, because it implies a decreased use of drugs and longer life of colonies. The monitoring and the prevention of honeybee diseases (brood and adult honeybees) can also promote the health of beehive through improved diagnostic assays and control strategies, but for some needs the currently used in-fields methods are not always accurate and precise. At this regard, molecular biology can support and/or replace classical methodologies, to assist in particular the selection of colonies with higher HB and the monitoring of adult bee pathologies. Thus, the availability of properly validated molecular targets and assays can be very useful in supporting the management processes aimed to increase honeybee health. This is particularly true when considering some of the most dangerous pathogens affecting brood, like bacterial pests and Varroa destructor, and bee adults, like *Nosema ceranae*.

With the above goal, research activities of this thesis were focused to the development of a) a prognostic assay for already known HB-specific biomarkers, opening the way to potential practical application in marker-assisted selective breeding programs, and b) the validation of a molecular method to monitor *N. ceranae* infection in adult bees.

Regarding the aim in a), the research should involve the in-field phenotypic assessment of brood disease hygienic behaviors with one of the classical methodologies already in use, followed by the sampling of honeybee nurses that can be in charge of the removal of diseased larvae. It can be hypothesed that the differences in a phenotype such as a behavior could be driven by expression levels of relevant genes in cognitive organs. Thus, brains of the sampled nurses would be the most representative targets for such analysis. The identification of potential diagnostic targets whose expression levels correlates with HB intensity is the basis for the development of rapid molecular assays that can improve the currently slow and resource-intensive process of selective colony breeding. The validation of HB molecular expression assays should also take into consideration the potential variability linked to seasonal and environmental conditions. Regarding the aim in b), the research should focus on the setup of a suitable method to monitor nosemosis in adult bees ensuring increased speed and cost-effectiveness compared to the existing molecular methods. Proper validation of their proposed assays should thus imply the comparison with the performances of reference methods like

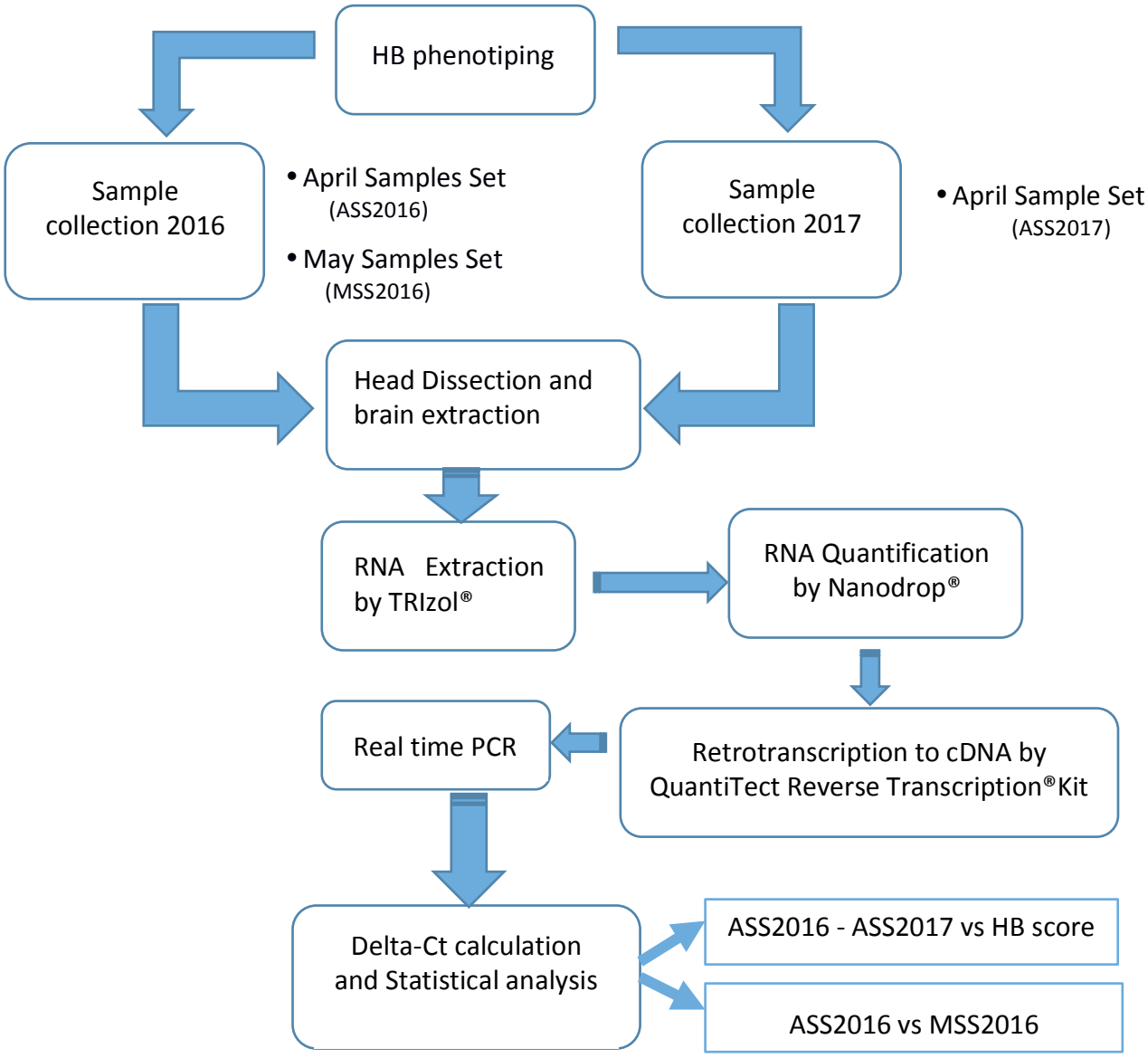
microscopy by OIE, and molecular detection and quantitation by Martin-Hernandez et al. (2007), respectively

Chapter 1: Obp4 and Act5C gene expression is related to Hygienic Behavior in honeybee colonies

1.1 Materials and Methods

1.1.1 Overview of the experimental design

The experimental design involved several steps from the in-field evaluation of colony (HB score) to the statistical analysis of the candidate target genes in nurse brains, as follows.



1.1.2 Sample collection and storage

The experimental project started in 2016 and two samples sets were collected in two consecutive months: April and May, respectively. In 2017, one samples set was collected in April. The colonies represented a mixture of European races (predominantly *Apis mellifera ligustica*).

The collection of colonies sampled in 2016: one comb with sealed and nascent brood was extracted from each hive and placed outside for a few minutes to let foragers to fly away. The nurses were collected using a test tube, keeping the comb in vertical position and dragging the tube up and down on the surface of the comb (**Figure 1.2 A)**.

Two samples sets collected in April and May respectively were analyzed. The April Samples Set (ASS2016) included:

- 5 colonies with Higher HB scores;
- 8 colonies with Lower HB scores.

The May Samples Set (MSS2016) included:

- 1 colonies with Higher HB score;
- 10 colonies with Lower HB scores.

The colonies analyzed were hosted in the experimental apiary of the Centro Zootecnico Didattico Sperimentale in Lodi and in the apiary of professional beekeeper Lorenzo Sesso in Varese, Northern Italy.

The collection of colonies sampled in 2017: the sampling procedure was improved as follows. Fifty newly-emerged honeybees were marked (**Figure 1.2 B)** with a different color for each colony. After 15-days, 21 marked honeybees were sampled from each colony. The marked honeybees were immediately put in nitrogen liquid to avoid the degradation of mRNA, and then stored individually in 1.5 ml Eppendorf tube (**Figure 1.2 C and D**).

During 2017 in April, the colonies analyzed were from the experimental apiary of the Centro Zootecnico Didattico Sperimentale in Lodi.

The April Samples Set (ASS2017) included:

- 5 colonies with the Higher HB score;

- 8 colonies with Lower HB score.

Figure 1.2: A: The tube was dragging up and down on the surface of the comb**; B:** marking of newly-emerged honeybees; **B:** recovering of 15-days old marked honeybees with a pair of tweezers; **C**: immediate freezing in liquid nitrogen.

During transport from in-field to the laboratory, samples were kept under refrigerated temperature in a polystyrene container. Once in laboratory, samples were put at -80°C, until further processing.

1.1.3 HB phenotyping

The phenotypic characterization of HB of the candidate colonies was performed through FKB test, as explained in introduction, Section 3. The ASS2016, MSS2016 and ASS2017 sample sets were phenotypically assessed for Higher and Lower HB score through FKB test with a range between 0 and 1. In particular, for each sample set the five colonies showing a higher HB score compared to the remaining colonies were classified into the “higher HB score” experimental group, whereas the remaining colonies were classified into the “lower HB score” experimental group. Consequently, the colonies of 2017 sample set had a “higher HB” threshold over/equal to 0.84, while the colonies of April 2016 sample set had a “higher HB” threshold over/equal to 0.75. A notable exception was the sample set collected in May 2016, when only a colony showed a reasonably high HB score and all the remaining colonies (HB less than 0.75) were considered as “lower HB score”.

1.1.4 Head dissection and brain extraction

Honeybee heads were dissected and brains were extracted working on dried ice cold Petri dishes, under a stereo microscope (M50, Leika). First, the head was separated from the body, which has been discarded. Afterwards, the antennae were removed, while the compound eyes together with optic lobes and mouth part were cut away with a scalpel. Then the brain was extracted simply by lifting front and back cuticles using a pair of tweezers. Pools of 7 brains (approximately 40 mg) from each colony were placed in 1,5 ml Eppendorf tubes and 1 ml of TRIzol® Reagent (Invitrogen) was immediately added to each pool. Samples were then stored at -20°C until further process.

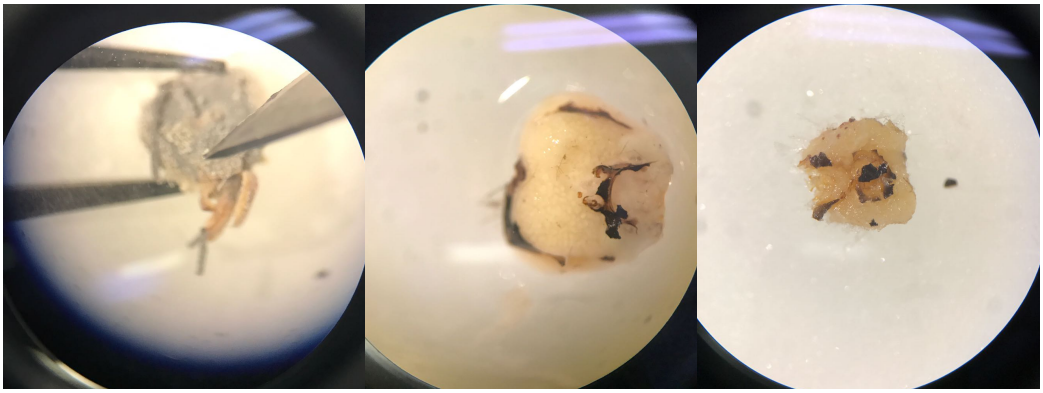


Figure 1.3: Head dissection on dried ice cold Petri dish

1.1.5 RNA extraction

Samples were thawed, and immediately lysed by manual homogenisation using a pestle, until all brains were dissolved. RNA extraction was performed following the manufacturer's instructions of TRIzol®.

The protocol as follows:

I. PHASE SEPARATION: the homogenised samples were incubated for 5 minutes at room temperature to allow the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added and tubes was shaken vigorously by hand for 15 seconds and incubated for 3 minutes at room temperature. The samples were centrifuged 12.000 x g for 15 minutes at 4 °C. Following centrifugation, the mixture was separated into a lower red, phenol-chloroform phase, an inter phase, and a colourless upper aqueous phase. RNA remained exclusively in the aqueous phase.

II. RNA PRECIPITATION: The aqueous phase was transferred to a fresh tube. The RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropyl alcohol. The samples were incubated at room temperature for 10 minutes and centrifuged at 12.000 x g for 10 minutes at 4°C. The RNA precipitate formed a gel-like pellet on the side and bottom of the tube.

III. RNA WASH: the supernatant was discarded and RNA pellet was washed once with 75% ethanol by vortexing and centrifuged at 7.500 x g for 5 minutes at 4°C.

IV. REDISSOLVING THE RNA: after discarding ethanol, RNA pellets were air dried for 5-10 minutes. Finally, RNA was dissolved in 0.1 ml Rnase-free water by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55°C.

1.1.6 RNA quantification

The concentration and purity of extracted RNAs was assessed using a spectrophotometer ND-100 (NanoDrop® Technologies Inc., Wilmington, DE, USA). The NanoDrop® measures the concentration in terms of nanograms/microliter (ng/µl) and the purity as the ratio between absorbance at 260 nm and absorbance at 280 nm (A260/A280 ratio); ideally, the ratio should be between 1.9 and 2.2.

1.1.7 Target gene expression analysis

For expression analysis of the target genes, all RNA samples were diluted to reach a final concentration of about 600 ng/µl before undergoing reverse transcription.

a. Reverse transcription

RNA reverse transcription was performed using QuantiTect® Reverse Transcription kit (Quiagen) following the manufacturer's instruction. The reaction is two steps procedure: first, elimination of genomic DNA and second, reverse transcription.

- Elimination of genomic DNA

The genomic DNA elimination reaction was prepared for every sample on ice using a mix containing: 1 µl RNA template, 2 µl gDNAWipeout Buffer and 11 µl Rnase-free water. Then, every tube was incubated for 2 minutes at 42°C, then placed immediately on ice.

- Reverse transcription

In each reaction tube from the previous genomic elimination reaction the following reagents were added: 1 µl Quantiscript Reverse Transcriptase, 1 µl RT Primer Mix and 4 µl 5x

Quantiscript RT Buffer. Every reaction tube was incubated for 15 minutes at 42°C, followed by incubation for 3 minutes at 95°C to inactivate Quantiscript Reverse Transcriptase.

b. Conventional PCR

Conventional PCR runs were performed on T100™ Thermal Cycler-Bio-Rad. The reactions were carried out in a total volume of 10µl containing 1µl of cDNA, 1X Taq buffer (containing 1.5mM MgCl2), 0.2mM dNTPs, 0,2 µM each primer, 1.25 U TaqPromega; H_20 was added to reach a total volume of 10µl. The thermal protocol for the PCR was: denaturation at 95° C for 4min, 40 cycles annealing at a T °C depending on each primer pair, and final elongation at 72°C for 10 min. Primers sequences, amplicons size and annealing temperatures for each primer pair are reported in **Table 1.1**.

Table 1.1. Primers sequences, amplicon size and annealing temperature.

Primer	Sequence	Amplicon size	Annealing	Reference
Obp4 F	5' TGCGCTGGTTCACGCAGACA 3'	288 bp	63°C	(Oldroyd and Thompson 2006)
Obp4 R	5'ATGCATTCGTCTTCGTCTGCA 3'			
Obp3 F	5' ATGATGGTTCGTTGTGACGA 3'	161 bp	55°	(Forêt 2007)
Obp3 R	5' GCACTTATCCTCGTTCTTAGCA 3'			
Obp16 F	5' TTACAGCCAATTTGCGTAGGC 3'	285 bp	55°	(Forêt 2007)
Obp16 R	5' CTGCTTGCACAATATCTCTGGTA 3'			
Obp18 F	5' TCGGGCTACGTGCTGTGGTGC 3'	187 bp	60°	(Forêt 2007)
Obp18 R	5' TCCACCATCGTCATAGCCA 3'			
Act5C F	5'TTCCCATCTATCGTCGGAAG 3'	96 bp	63°C	(Cristino et al. 2014)
Act5C R	5' CTCTCTTTGATTGGGCTTCG 3'			
Mblk-1 F	5'TGCCAAGCACTACACCAAAA 3'	120 bp	59°C	(Takeuchi et al. 2001);
Mblk-1 R	5' GGGGAATCTTAAAGGCGAAG 3'			
Rpl32 F	5'AGTAAATTAAAGAGAAACTGGCGTAA 3'	129 bp	60°C	(Reim et al. 2013)
Rpl32 R	5'TAAAACTTCCAGTTCCTTGACATTAT 3'			

The amplification products were run on 2% agarose gels and visualized under a trans illuminator.

c. Real Time PCR

All Real Time PCRs were run in duplicate using a IQ5™ Cycle Bio-Rad. The same primer pairs

used for conventional PCR were used for Real Time PCR. The Real-Time PCR was carried out in a total volume of 20 µl: each reaction was made of 2µl of cDNA, 10µl Supermix SsoFast EvaGreen, 0,2 µM each primer, and H_2O to reach a total volume of 20 µl. The thermal protocol was 95 °C for 3 min, 40 cycles at 95 °C for 10sec, and variable annealing temperature for each primer pair (Table 1.1). The melting protocol was 55 °C for 1min, then temperature raise to 95 °C through 81 steps of 0.5 °C for 10 sec each. The threshold line of the qPCR run was set up on 200. Threshold cycles (Ct) were assessed through the interpolation of each amplification curve with the threshold line.

d. Reference curves

The reference curves were designed using a mix of 5 cDNA undiluted samples (TQ); from this mix three serial dilutions were made: 1:5, 1:25, 1:125. For each target gene, serial dilutions were analysed through Real Time PCR as previously described, and amplification efficiency value was calculated by the instrument software. Ideally, amplification efficiency is a measure of the reliability of the amplification: the closer the value is to 100%, the more reliable is the amplification.

e. ΔCt analysis

The cDNAs threshold cycle (Ct) values, obtained by qRT-PCR, were used to calculate the ΔCt of each gene pair as follows:

$$\Delta Ct = Ct \text{ (target gene 1)} - Ct \text{ (target gene 2) or (EC gene)}$$

where Ct (target gene 1) or (target gene 2) indicates the value of the threshold cycle for the gene of interest, Ct(EC gene) indicates the value of the threshold cycle for the gene used as normalizer (rpl32).

1.1.8 Statistical analysis

The statistical test used for data analysis were:

- The Shapiro-Wilk test: this was used to test if the data were normally distributed. (http://scistatcalc.blogspot.it/2013/10/shapiro-wilk-test-calculator.html)
- The Mann-Whitney U test, that is a nonparametric test: this test allows two groups or conditions or treatments to be compared without making the assumption that values

are normally distributed (http://www.socscistatistics.com/tests/mannwhitney/Default.aspx)

- The Median test, that is a nonparametric test: which the null hypothesis that the medians of the populations from two or more samples are identical. The data in each sample are assigned to two groups, one consisting of data whose values are higher than the median value in the two groups combined, and the other consisting of data whose values are at the median or below.

For all tests, the significance level (p) was set at ≤0.05

1.2RESULTS

1.2.1 Hygienic behavior phenotyping

The results are detailed below as ASS2016 and ASS2017 and a comparison of ASS and MSS2016

The evaluation of HB score for the colonies collected in April2016, May2016 and April2017 were reported in **Table 1.2**.

Table 1.2: FKB assay score of the samples analyzed during April2016, May2016 and April 2017. The colonies with Higher HB score are typed in bold.

April 2016	HB Score	May2016	HB score	April 2017	HB Score
14	**1**	**25**	**0.91**	**8B**	**0.98**
LS10	**0.95**	26	0.73	**3B**	**0.90**
13	**0.87**	24	0.64	**6B**	**0.86**
LS12	**0.86**	22	0.58	**5B**	**0.85**
LS11	**0.75**	17	0.57	**11B**	**0.84**
LS8	0.58	19	0.47	1B	0.79
LS13	0.33	27	0.33	2B	0.77
11	0.28	20	0.3	9B	0.71
12	0.28	18	0.22	13B	0.69
10	0.25	23	0.12	10B	0.64
9	0.19			7B	0.63
8	0.18			14B	0.33
7	0.08			4B	0.63

After the evaluation of the HB score, the samples were analyzed for the selected single gene target, and the performance of the corresponding reference curves.

1.2.2 Reference curves

In order to obtain reference curves for each gene target, a mixed solution of the extracted cDNA was prepared and analysed through Real Time PCR as described in the M&M section. For each target, the corresponding reference curve and amplification efficiency is shown in **Figure 1.4.**

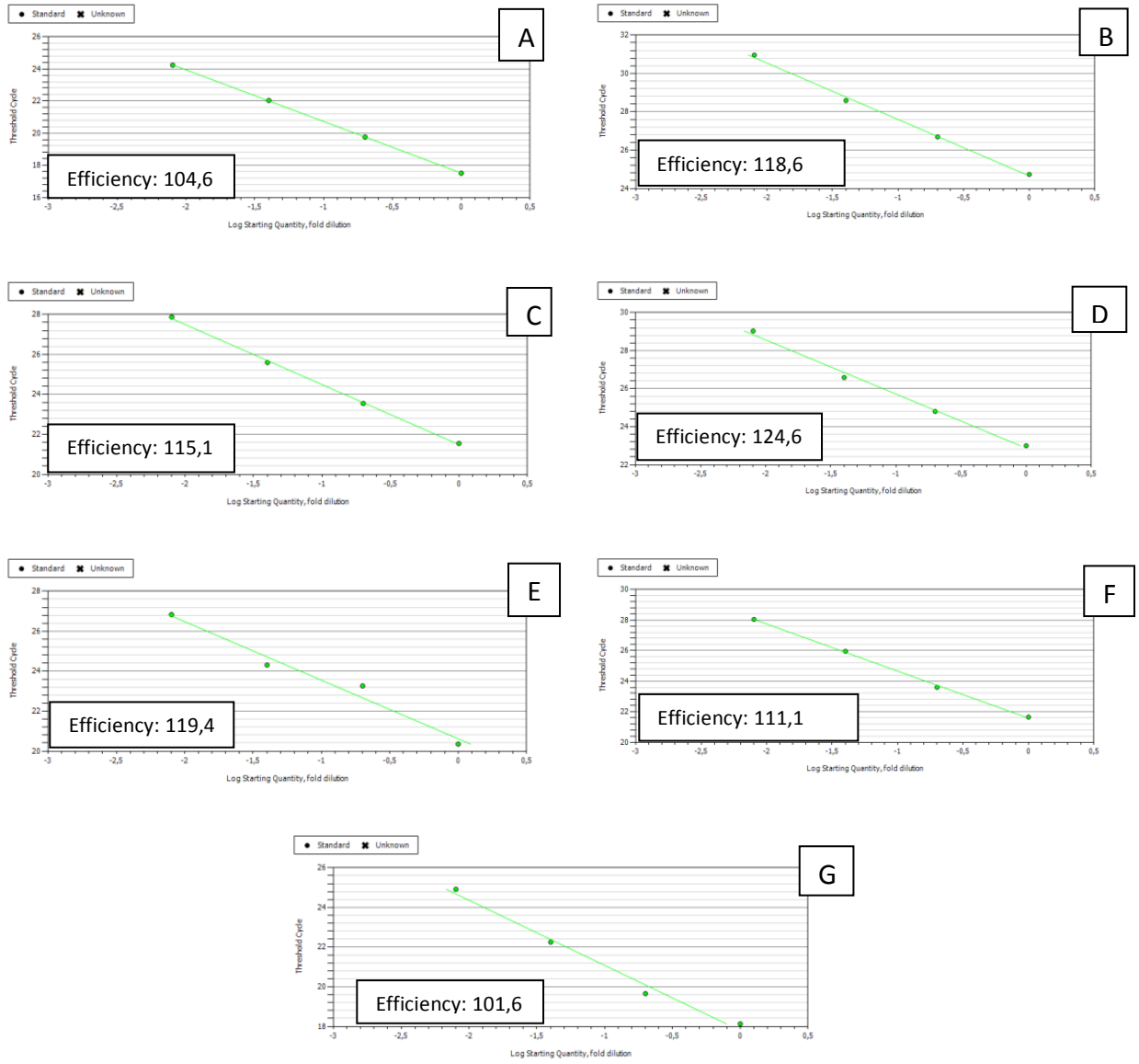


Figure 1.4: Reference curve and amplification efficiency corresponding to the following genes: **A**, Act5C; **B**, Mblk-1; **C**, Obp4; **D**, Obp3; **E**, Obp16; **F**, Obp18; **G**, Rpl32.

1.2.3 Real time PCR ASS2016, MSS2016 and ASS2017

The ASS2016 and ASS2017 cDNA samples were amplified through Real Time PCR, as shown in **Figure 1.5** for each coding gene targets**.** The Ct obtained from each amplification run are reported in **Table 1.3**, **Table 1.4** and **Table 1.5.**

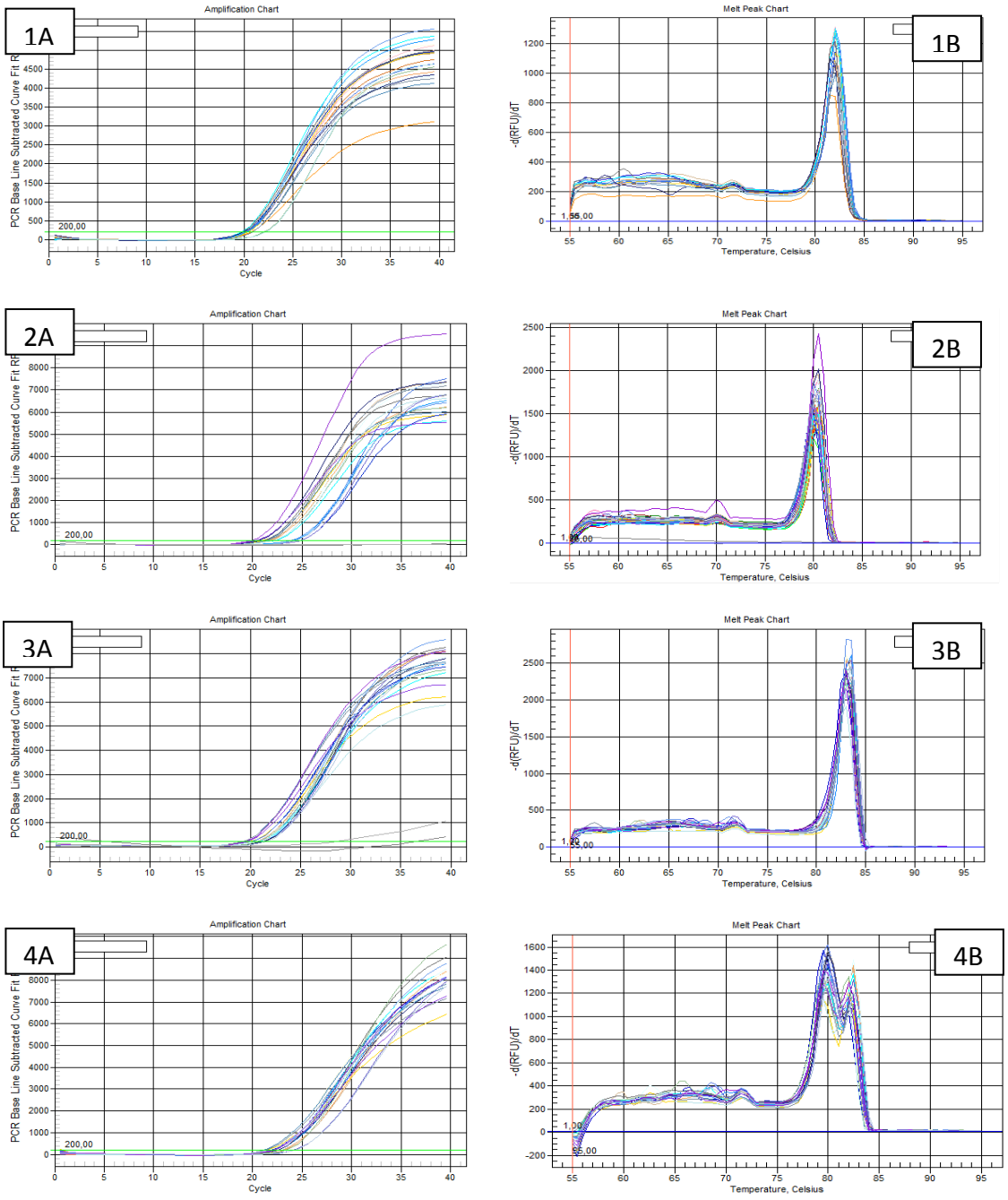


Figure 1.5: Amplification (A) and melting (B) curves of the analyzed gene targets: **1,** Act5C; **2**, Mblk-1; **3,** Obp4; **4**, Obp3; **5**, Obp16; **6,** Obp18; **7,** Rpl32.

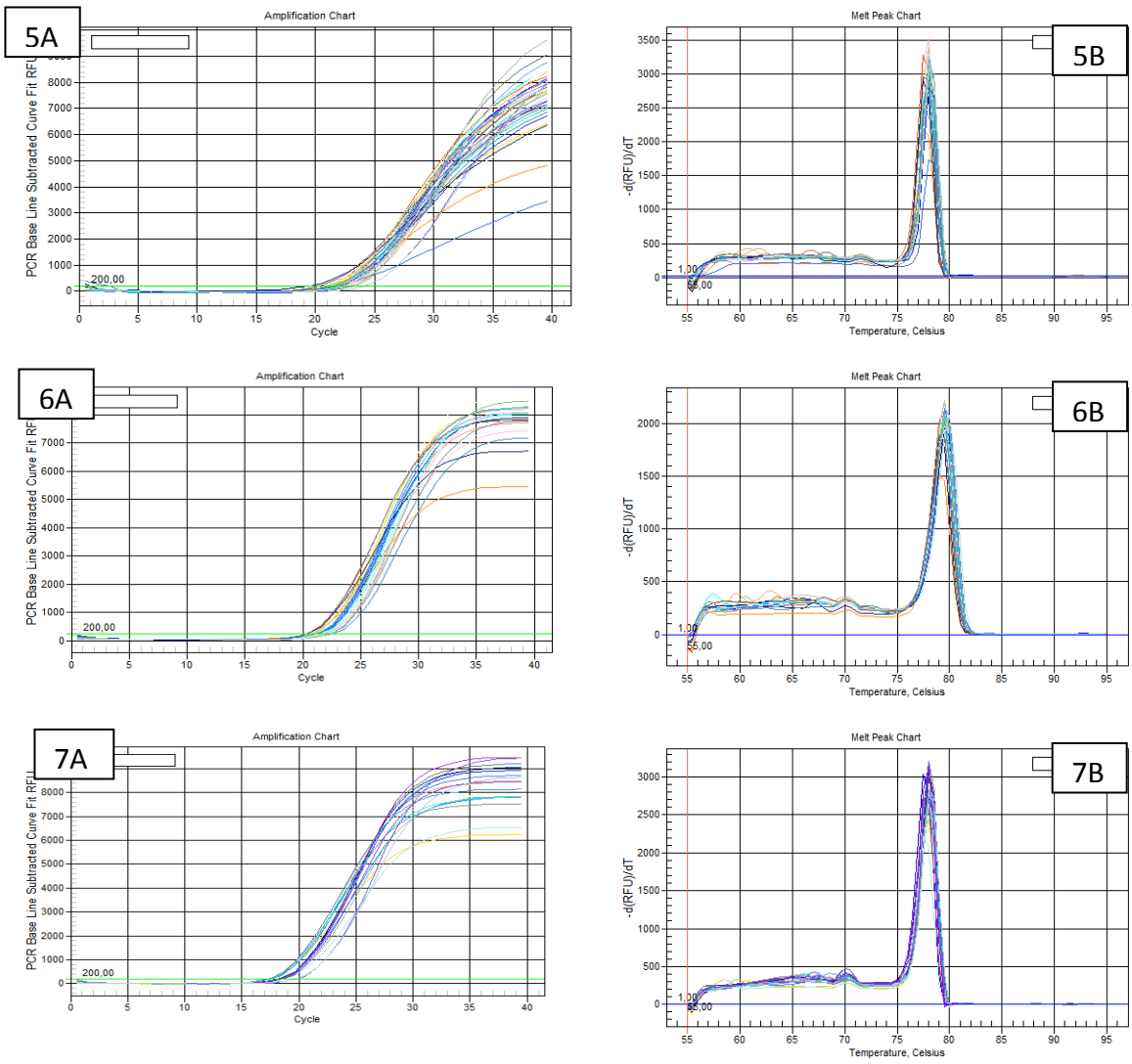


Figure 1.5: (follow)

Table 1.3. Ct values of ASS2016. The colonies with Higher HB score are typed in bold

ASS2016	HB score	Obp4	Act5C	Mblk-1	rpl32	Obp4	Act5C
14	**1**	**24.02**	**18.96**	**23.5**	**20.37**	**24.02**	**18.96**
LS10	**0.95**	**24.02**	**20.6**	**24.7**	**22.99**	**24.02**	**20.6**
13	**0.87**	**23.94**	**19.67**	**24.01**	**20.33**	**23.94**	**19.67**
LS12	**0.86**	**29.13**	**24.58**	**28.56**	**22.81**	**29.13**	**24.58**
LS11	**0.75**	**27.28**	**23.62**	**28.53**	**22.63**	**27.28**	**23.62**
LS8	0.58	26.73	25.19	27.41	22.67	26.73	25.19
LS13	0.33	29.09	27.56	30.86	24.67	29.09	27.56
11	0.28	26.13	24.74	27.35	23.01	26.13	24.74
12	0.28	27.42	24.86	28.86	21.5	27.42	24.86
10	0.25	28.07	27.27	30.75	22.57	28.07	27.27
9	0.19	26.05	23.75	26.86	23.48	26.05	23.75
8	0.18	27.61	25.38	28.29	23.44	27.61	25.38
7	0.08	27.4	26.66	30.42	22.25	27.4	26.66

Table 1.4: Ct values of MSS2016. The colonies with higher HB score are typed in bold.

MSS2016	HB score	Obp4	Act5C	Mblk-1	Rpl32
25	**0.91**	**28.93**	**26.36**	**26.12**	**22.76**
26	0.73	26.65	25.83	27.01	21.99
24	0.64	32.07	29.07	27.97	24.48
22	0.58	26.58	25.99	26.46	22.7
17	0.57	30.18	29.42	28.68	23.21
19	0.47	30.1	28.02	28.92	24.56
27	0.33	28.73	26.84	29.84	20.94
20	0.3	28.8	29.61	28.92	23.57
18	0.22	28.85	28.07	28.4	24.2
23	0.12	28.8	27.37	28.1	24.77

Table 1.5. Ct value of ASS2017. The colonies with Higher HB score are typed in bold.

ASS2017	HB score	Act5C	Mblk-1	RPL32	OBP3	OBP4	OBP16	OBP18
8B	**0.98**	**19.82**	**24.83**	**18.73**	**22.59**	**20.86**	**19.98**	**20.36**
3B	**0.90**	**19.89**	**24.79**	**18.00**	**21.75**	**20.23**	**21.00**	**20.77**
6B	**0.86**	**20.11**	**26.06**	**18.27**	**22.53**	**21.51**	**21.54**	**21.17**
5B	**0.85**	**20.11**	**24.94**	**17.55**	**21.85**	**20.93**	**21.73**	**21.37**
11B	**0.84**	**21.50**	**23.61**	**20.24**	**21.56**	**21.29**	**23.16**	**21.81**
1B	**0.79**	**20.36**	**24.88**	**17.61**	**23.00**	**20.77**	**22.28**	**21.97**
2B	0.77	20.45	24.13	17.55	21.21	20.02	21.13	20.51
9B	0.71	20.79	25.23	18.60	21.94	19.72	21.68	22.51
13B	0.69	21.58	29.02	19.52	26.17	22.09	24.96	20.81
10B	0.64	22.10	28.31	19.86	24.15	21.72	22.36	22.07
7B	0.63	19.77	25.08	17.37	21.03	19.33	21.56	21.66
4B	0.63	20.30	25.82	18.40	22.64	21.21	21.06	20.88
14B	0.33	20.64	24.41	19.62	21.45	21.26	21.75	20.53

1.2.4 Gene expression analysis: ASS2016 and ASS2017 vs HB score

The Ct values obtained for each target genes after qRT-PCR, were used to calculate the ΔCt of each gene pair. For each sample, the ΔCt is the difference between the Ct of one target gene and the Ct of another target gene, as reported in **Table 1.6** for ASS2016 and **Table 1.7** for ASS2017.

Table 1.6: ΔCt values of ASS2016. The colonies with Higher HB score are typed in bold.

ASS2016	ΔCt Obp4-Act5C	ΔCt Obp4-Mblk-1	ΔCt Obp4-Rpl32	ΔCt Act5c-Mblk1	ΔCt Act5c-Rpl32	ΔCt Mblk1-Rpl32
14	**5.06**	**0.52**	**3.65**	**-4.54**	**-1.41**	**3.13**
LS10	**3.42**	**-0.68**	**1.03**	**-4.1**	**-2.39**	**1.71**
13	**4.27**	**-0.07**	**3.61**	**-4.34**	**-0.66**	**3.68**
LS12	**4.55**	**0.57**	**6.32**	**-3.98**	**1.77**	**5.75**
LS11	**3.66**	**-1.25**	**4.65**	**-4.91**	**0.99**	**5.9**
LS8	1.54	-0.68	4.06	-2.22	2.52	4.74
LS13	1.53	-1.77	4.42	-3.3	2.89	6.19
11	1.39	-1.22	3.12	-2.61	1.73	4.34
12	2.56	-1.44	5.92	-4	3.36	7.36
10	0.8	-2.68	5.5	-3.48	4.7	8.18
9	2.3	-0.81	2.57	-3.11	0.27	3.38
8	2.23	-0.68	4.17	-2.91	1.94	4.85
7	0.74	-3.02	5.15	-3.76	4.41	8.17

Table 1.7: ΔCt values of ASS samples of 2017. The colonies with Higher HB score are typed in bold.

ASS2017	ΔCt Act5c-Mblk1	ΔCt Act5c-Rpl32	ΔCt Act5c-Obp3	ΔCt Act5c-Obp18	ΔCt Act5c-Obp16	ΔCt Mblk1-Rpl32	ΔCt Mblk1-Obp3	ΔCt Mblk1-Obp4	ΔCt Mblk1-Obp16	ΔCt Mblk1-Obp18	ΔCt Rpl32-Obp3	ΔCt Rpl32-Obp4	ΔCt Rpl32-Obp16	ΔCt Rpl32-Obp18	ΔCt Obp3-Obp4	ΔCt Obp3-Obp16	ΔCt Obp4-Act5c-	ΔCt Obp3-Obp18	ΔCt Obp4-Obp16	ΔCt Obp4-Obp18	ΔCt Obp16-Obp18
8B	**-5.01**	**1.09**	**-2.77**	**-0.54**	**-0.16**	**6.1**	**2.24**	**3.97**	**4.85**	**4.47**	**-3.86**	**-2.13**	**-1.25**	**-1.63**	**1.73**	**2.61**	**1.04**	**2.23**	**0.88**	**0.5**	**-0.38**
3B	**-4.9**	**1.89**	**-1.86**	**-0.88**	**-1.11**	**6.79**	**3.04**	**4.56**	**3.79**	**4.02**	**-3.75**	**-2.23**	**-3**	**-2.77**	**1.52**	**0.75**	**0.34**	**0.98**	**-0.77**	**-0.54**	**0.23**
6B	**-5.95**	**1.84**	**-2.42**	**-1.06**	**-1.43**	**7.79**	**3.53**	**4.55**	**4.52**	**4.89**	**-4.26**	**-3.24**	**-3.27**	**-2.9**	**1.02**	**0.99**	**1.40**	**1.36**	**-0.03**	**0.34**	**0.37**
5B	**-4.83**	**2.56**	**-1.74**	**-1.26**	**-1.62**	**7.39**	**3.09**	**4.01**	**3.21**	**3.57**	**-4.3**	**-3.38**	**-4.18**	**-3.82**	**0.92**	**0.12**	**0.82**	**0.48**	**-0.8**	**-0.44**	**0.36**
11B	**-2.11**	**1.26**	**-0.06**	**-0.31**	**-1.66**	**3.37**	**2.05**	**2.32**	**0.45**	**1.8**	**-1.32**	**-1.05**	**-2.92**	**-1.57**	**0.27**	**-1.6**	**-0.21**	**-0.25**	**-1.87**	**-0.52**	**1.35**
1B	-4.52	2.75	-2.64	-1.61	-1.92	7.27	1.88	4.11	2.6	2.91	-5.39	-3.16	-4.67	-4.36	2.23	0.72	0.41	1.03	-1.51	-1.2	0.31
2B	-3.68	2.9	-0.76	-0.06	-0.68	6.58	2.92	4.11	3	3.62	-3.66	-2.47	-3.58	-2.96	1.19	0.08	-0.43	0.7	-1.11	-0.49	0.62
9B	-4.44	2.19	-1.15	-1.72	-0.89	6.63	3.29	5.51	3.55	2.72	-3.34	-1.12	-3.08	-3.91	2.22	0.26	-1.07	-0.57	-1.96	-2.79	-0.83
13B	-7.44	2.06	-4.59	0.77	-3.38	9.5	2.85	6.93	4.06	8.21	-6.65	-2.57	-5.44	-1.29	4.08	1.21	0.51	5.36	-2.87	1.28	4.15
10B	-6.21	2.24	-2.05	0.03	-0.26	8.45	4.16	6.59	5.95	6.24	-4.29	-1.86	-2.5	-2.21	2.43	1.79	-0.38	2.08	-0.64	-0.35	0.29
7B	-5.31	2.4	-1.26	-1.89	-1.79	7.71	4.05	5.75	3.52	3.42	-3.66	-1.96	-4.19	-4.29	1.7	-0.53	-0.44	-0.63	-2.23	-2.33	-0.1
14B	-3.77	1.02	-0.81	0.11	-1.11	4.79	2.96	3.15	2.66	3.88	-1.83	-1.64	-2.13	-0.91	0.19	-0.3	0.62	0.92	-0.49	0.73	1.22
4B	-5.52	1.9	-2.34	-0.58	-0.76	7.42	3.18	4.61	4.76	4.94	-4.24	-2.81	-2.66	-2.48	1.43	1.58	0.91	1.76	0.15	0.33	0.18

In particular, the analysis of ASS2016 highlights the correlation of ΔCt between with respect to HB scores (R^2 = 0.74) and p value < 0.05 (**Figure 1.6**).

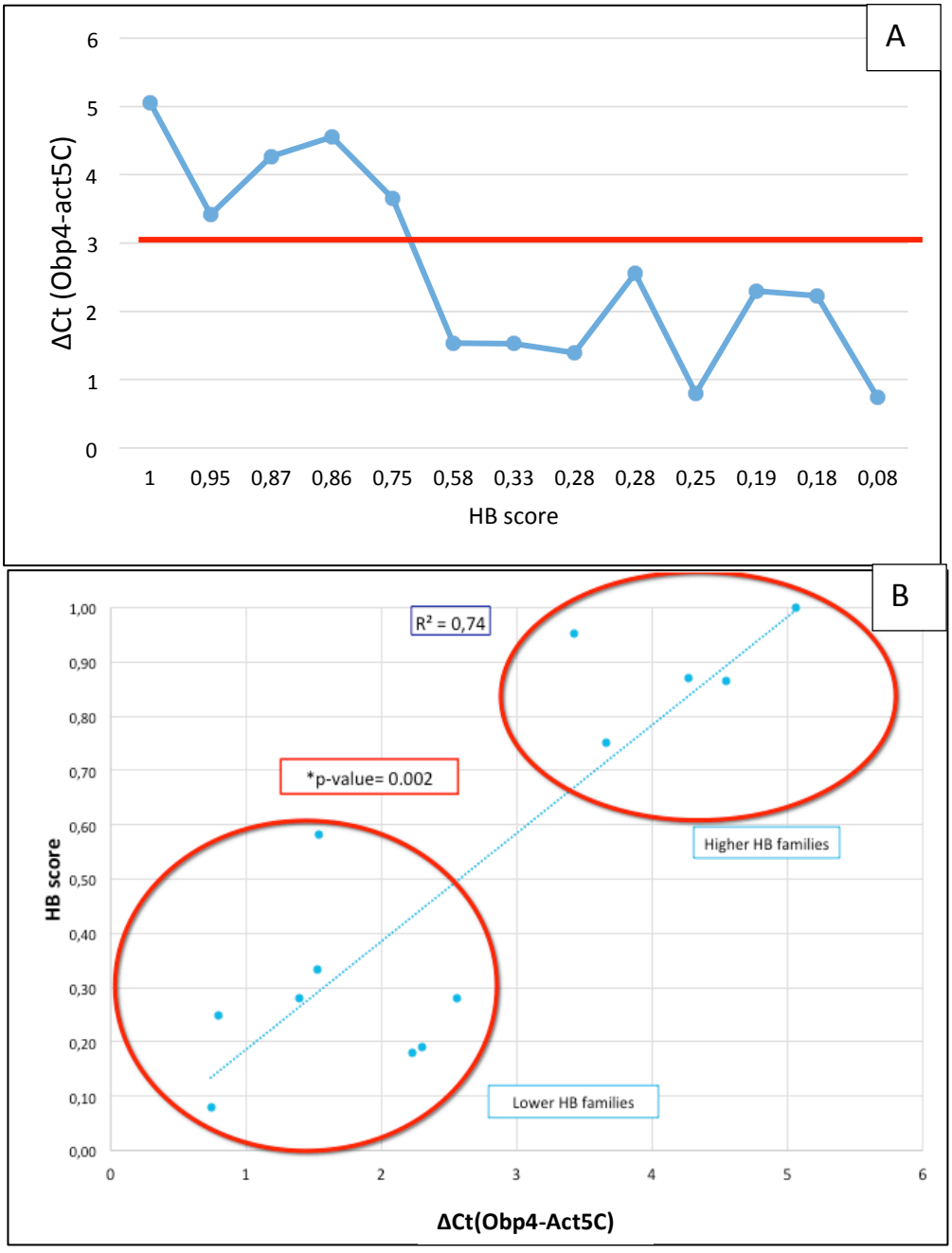


Figure 1.6: Analysis of ΔCt(Obp4-Act5C) versus HB score in ASS2016. Panel A: descriptiv correlation analysis.

The ΔCt(Obp4-Act5C) of ASS2017 was the only expression parameter whose nearly significant after statistical analysis (p-value= 0,06).

Then, the HB score and ΔCt(Obp4-Act5C) data obtained from ASS2016 and ASS2017 were combined into a single data set (**Table 1.8**). The threshold for Higher HB score was then set over/equal to 0,85.

Table 1.8: Combined ASS2016 and ASS2017 data set. The colonies with Higher HB score are typed in bold.

Samples ID	HB score	ΔCt OBP4-Act5C
14	**1**	**5.06**
8B	**0.98**	**1.04**
LS10	**0.95**	**3.42**
3B	**0.90**	**0.34**
13	**0.87**	**4.27**
LS12	**0.86**	**4.55**
6B	**0.86**	**1.4**
5B	**0.85**	**0.82**
11B	0.84	-0.21
1B	0.79	0.41
2B	0.77	-0.43
LS11	0.75	3.66
9B	0.71	-1.07
13B	0.69	0.51
10B	0.64	-0.38
7B	0.63	-0.44
4B	0.63	0.91
LS8	0.58	1.54
LS13	0.33	1.53
14B	0.33	0.62
11	0.28	1.39
12	0.28	2.56
10	0.25	0.8
9	0.19	2.3
8	0.18	2.23
7	0.08	0.74

The combined ASS2016 and ASS2017 dataset was analyzed using the median test. The result showed that the ΔCt(Obp4-Act5C) median value of the colonies with the Higher-HB score was indeed higher (2,41) compared to the colonies with the Lower HB score (0.77) and to the whole set of colonies (0,97) (**Figure 1.7**). The statistical analysis was performed using Mann-Whitney U test, that confirmed the statistically significant difference (p value = 0.04) between the two sets of colonies with Higher and Lower HB score as for ΔCt(Obp4-Act5C).

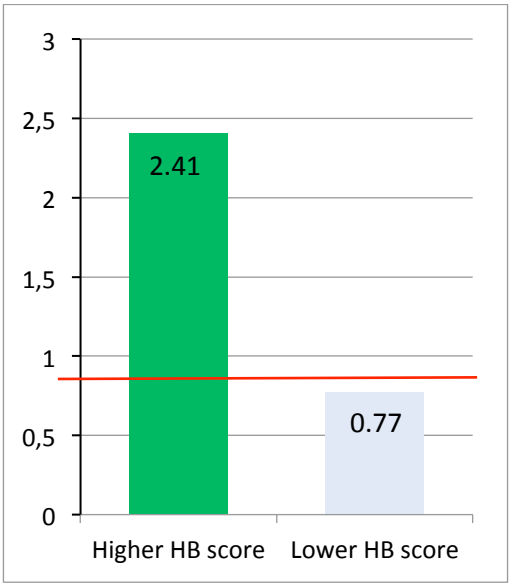


Figure Errore. Lo stile non è definito.**7:** Median values of ΔCt(Obp4-Act5C) for Higher HB and Lower HB score colonies.

1.2.5 Comparison of ASS2016 and MSS2016

1.2.5.1 Gene expression analysis of MSS2016 vs ASS2016

As already detailed, the Ct values obtained for each target genes after qRT-PCR, were used to calculate the ΔCt of each gene pair. For each sample, the ΔCt is the difference between the Ct of one target gene and the Ct of another target gene, as reported in **Table 1.9**

Table 1.9: ΔCt values calculated for MSS2016. The colony with higher HB score is typed in bold.

MSS2016	ΔCt Obp4-Act5C	ΔCt Obp4-Mblk-1	ΔCt Obp4-Rpl32	ΔCt Act5c-Mblk1	ΔCt Act5c-Rpl32	ΔCt Mblk1-Rpl32
25	**2.57**	**2.81**	**6.17**	**0.24**	**3.6**	**3.36**
26	0.82	-0.36	4.66	-1.18	3.84	5.02
24	3	4.1	7.59	1.1	4.59	3.49
22	0.59	0.12	3.88	-0.47	3.29	3.76
17	0.76	1.5	6.97	0.74	6.21	5.47
19	2.08	1.18	5.54	-0.9	3.46	4.36
27	1.89	-1.11	7.79	-3	5.9	8.9
20	-0.81	-0.12	5.23	0.69	6.04	5.35
18	0.78	0.45	4.65	-0.33	3.87	4.2
23	1.43	0.7	4.03	-0.73	2.6	3.33

The results of MSS2016 highlights the loss of correlation between ΔCt(Obp4-Act5C) and HB scores (**Figure 1.8**).

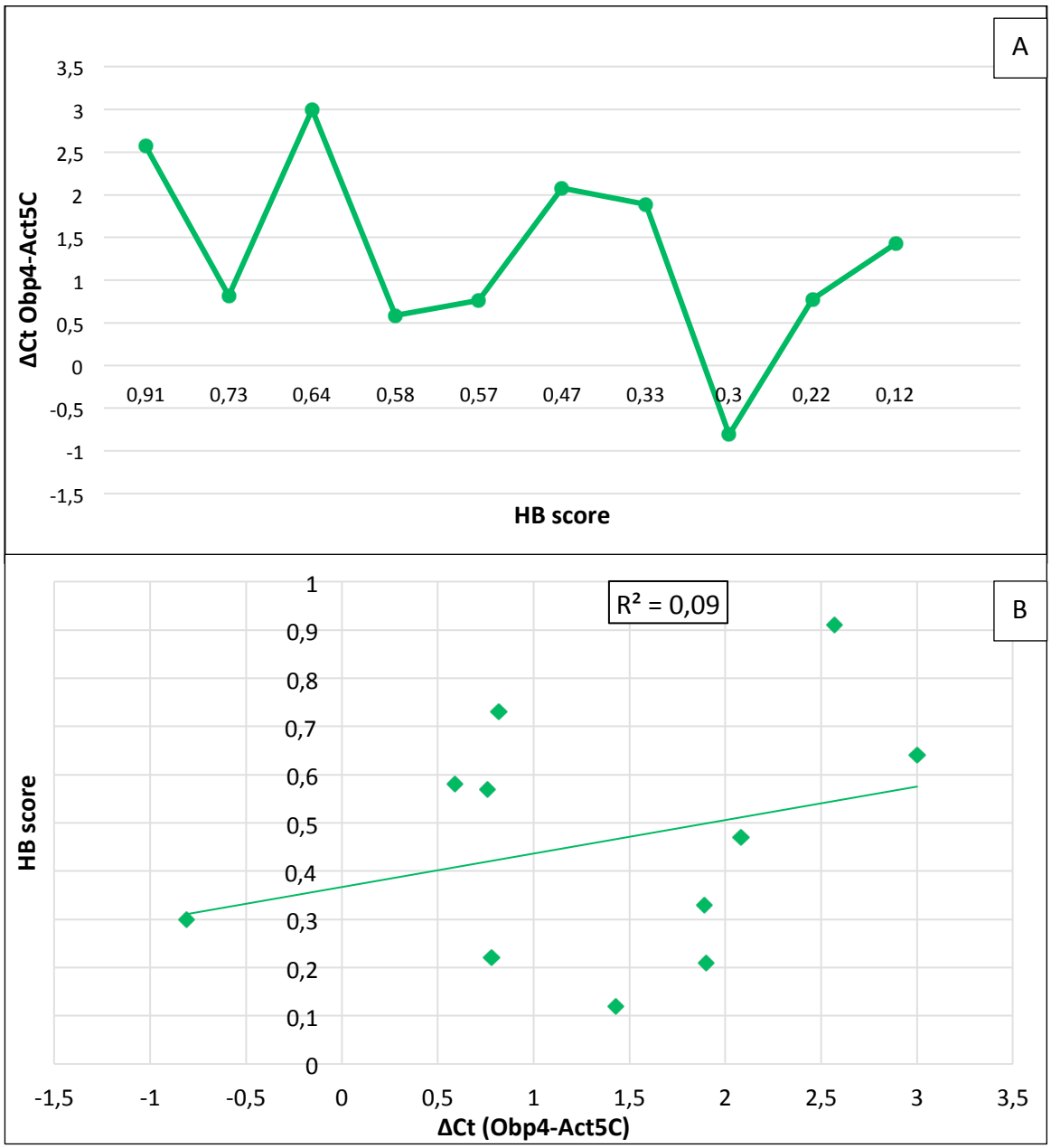


Figure 1.8: Analysis of ΔCt(Obp4-Act5C) versus HB score in MSS2016. Panel A: descriptive graph. Panel B: correlation analysis.

Then, the HB score and ΔCt(Obp4-Act5C) data obtained from ASS2016 and MSS2016 were combined into a single data set (**Table 1.10**). The threshold for Higher HB score was then set over/equal to 0,75. In this dataset, a low correlation was found between HB score and ΔCt(Obp4-Act5C) (**Figure 1.9)**.

Table 1.10: Combined ASS2016 and MSS2016 data set. The colonies with Higher HB score are typed in bold.

Samples ID	HB score	ΔCt Obp4-Act5C
14	**1**	**5.06**
LS10	**0.95**	**3.42**
25	**0.91**	**2.57**
13	**0.87**	**4.27**
LS12	**0.86**	**4.55**
LS11	**0.75**	**3.66**
26	0.73	0.82
24	0.64	3
LS8	0.58	1.54
22	0.58	0.59
17	0.57	0.76
19	0.47	2.08
LS13	0.33	1.53
27	0.33	1.89
20	0.3	-0.81
11	0.28	1.39
12	0.28	2.56
10	0.25	0.8
18	0.22	0.78
9	0.19	2.3
8	0.18	2.23
23	0.12	1.43
7	0.08	0.74

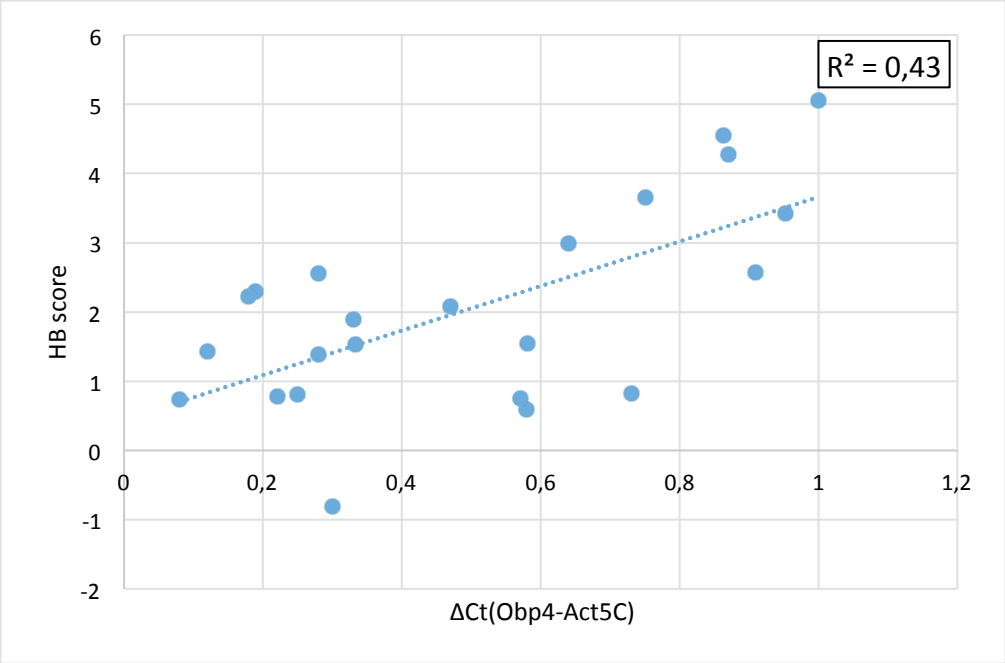


Figure 1.9: Correlation analysis between HB score and ΔCt(Obp4-Act5C) data from combined ASS2016 and MSS2016 dataset.

1.3 CONCLUSION

In the *Apis mellifera,* HB is a heritable phenotype that confers colony level resistance against brood diseases, including the damaging parasitic mite by *Varroa destructor*. Currently, the beekeepers measure the HB score in their honeybee colonies using methods like the pin-prick test or freeze-killed brood (FKB) assays, during their in-field tests. These methods are inherently imprecise, time consuming and resource intensive for the breeders, thus limiting their suitability for widespread application. The availability of molecular biomarkers for the hygienic phenotype could fasten the recognition of this trait and the breeding process. Currently, the genetic and biochemical elements driving the manifestation of this behavior are largely unknown. Once identified, such genes can be potentially used as biomarkers to aid identification and selection of colonies resistant to disease. More generally, gene expression analysis can provide a valid tool to identify coding genes that are known controlling behavioral processes.

The present work was characterized by a transversal approach: from in-field phenotypic characterization of HB, to expression profiling of selected cerebral coding (RNAm) genes, with the aim to assess differential expression of such genes between selected honeybee colonies with the Higher and the Lower HB, respectively. The gene targets of this work have been selected from previous literature evidences as a subset of honeybee nurse brain coding genes that may be involved in HB intensity: Act5C, a cytoskeletal gene involved in memory formation; Obp4, as a brain-specific olfactory gene and also expressed in antennae; Obp3, Obp16 and Obp18 as olfactory genes being expressed in a wide range of organs and tissues with different patterns; Mblk-1, as a transcription factor specifically expressed in mushroom body. Beyond, Rpl32 was targeted as a housekeeping gene with stable expression in honeybees as reported in previous studies.

Le Conte et al., (2010) reported the analysis of brain transcriptome of highly Varroa-hygienic bees and the identification of a set of genes involved in social immunity including some Obps. The function of these candidate genes did not seem to support higher olfactory sensitivity in hygienic bees. Thus the correlation between Obps and HB could be linked to other, still unknown functional mechanisms behind the binding of olfactory molecules. The results of this thesis highlight the prominent significance of Act5 and Obp4 among the analyzed candidate

markers as a distinguishable tools to infer the hygienic feature of a honeybee colony. In particular, the evaluation of a candidate gene panel in nurse brains allowed to clarify a significant relationship between Obp4 and Act5C vs HB. More in details, ΔCt of Obp4 and Act5C was a discriminating parameter for the colonies with higher HB score with respect to the colonies with lower HB. The results showed repeatability in two different sampling years on a total 26 colonies.

As reported in literature, HB is influenced by external environmental factors, as the abundance of nectar and other unknown factors, and it is not constant in time in each hive (Xonis et al., 2015). Noteworthy, when comparing years 2016 and 2017, the Obp4 and Act5C gene pair showed somewhat different expression levels. The variability of the relationship between Obp4 and HB score could be correlated to the different nectar flow in April 2017 compared to April 2016 in the apiary site (Lodi) and related honeybee colony functional activity (during April 2016, the nectar flow in Lodi was indeed markedly higher compared to April 2017). The different abundance of potential OBPs ligands (mainly, small hydrophobic volatile molecules of 10-20 carbon atoms, as reported by Zhou, 2010) could affect the expression of OBPs with functional involvement in metabolic pathways not linked to brood cleaning. In particular, this could be likely especially for Obp4, since its expression in brain can be associated with odorous stimuli attracting honeybees looking for pollen and nectar. Besides, a different balance between younger (nurses less than three weeks of age) and older honeybees is also appreciable during strong nectar flows, mainly due both to impaired egg deposition by the queen (due to scarcity of empty cells) and to an increased need for foragers. Consequently, HB should be considered a dynamic, rather than a static, phenotype being heavily affected by a variety of environmental stressors. The appreciable effect of seasonal conditions on the level of HB phenotype could be a reason for the inconstant correlation with OBP4 and/or Act5 that has been observed in the present thesis. Notwithstanding this, the overall results obtained from the pooled 2016-2017 sample set confirmed the suitability of ΔCt(Obp4-Act5C) as a marker to infer HB condition.

From the perspective point of view, the identification of colonies with high HB trait using molecular markers should be based on repeated measurements during the brood season. Evaluation of more honeybee colonies for large-scale in-field validation of marker-assisted HB-selection is also necessary. As a further objective for the future, technical improvements such

as the setup of a new duplex protocol allowing measurement of both candidate genes (Obp4 and AC5C) through a single analysis would be highly advisable.

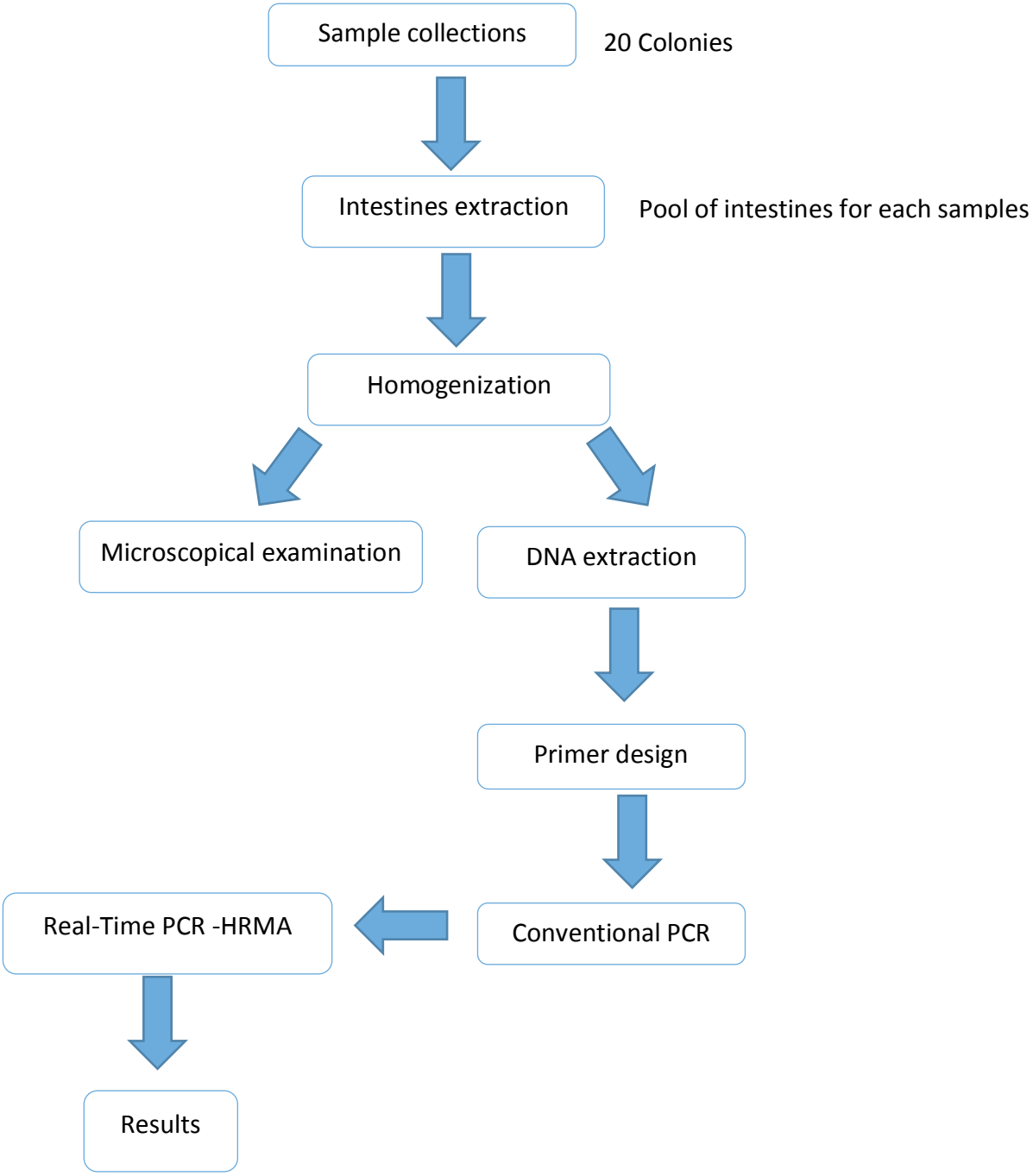
The present study was reported as a manuscript to be submitted for publication:
(**Dell'Orco F**., Facchini E., Rizzi R., Mortarino M. The expression ratio between Act5 and Obp-4 is a marker of hygienic behavior in honeybees. Manuscript to be submitted)

Chapter 2: Detection and quantitation of *Nosema ceranae* in honey bees by real time PCR coupled to High Resolution Melting Analysis

2.1. Materials and methods

2.1.1. Overview of the experimental design

The experimental design involved several steps from the collection of the samples to biological analysis, as follows:



2.1.2. Honey bee sample collection

For the setup of the qPCR-HRMA assay for *Nosema spp* (named as " HRMNosem"), 20 samples were collected in-field during summer months from honeybee colonies already known as infected by *Nosema spp*. From each colony, 25 foraging bees were collected at the hive entrance.

2.1.3. Honey bee sample processing and DNA extraction

Honeybee samples were processed for microscopical spore counting and genomic DNA (gDNA) extraction following a published protocol with some modifications (Bourgeois et al. 2010). Briefly, from each colony sample the intestines of the honey bee were collected and pooled. Then the intestine pool samples were omogenized in 5 ml of double-distilled water and filtered using a metal tea strainer. After two centrifugations at 800 g for 6 minutes, the pellets were resuspended in 1 ml of double-distilled water. At this step, the spore load for each sample was assessed using the method described in the OIE reference manual ((OIE) 2013). Then a 500 µl aliquot was sonicated for three cycles of 10 seconds each (output=2) using a Sonifier 250 instrumentation , at setting output=2 (Branson Ultrasonics, Danbury, CT, U.S.A). Then 1 ml of TNNT lysis buffer (0,5% SDS, 0.5% Tween 20, 0.5% Nonidet P-40, 10mM NaOH, 10mM Tris [pH 7.2]) and 8 µl of 20 mg/ml proteinase K were added to each sample. After overnight incubation at 56 °C, the proteinase K was heat-inactivated at 95 °C for 10 min. After this step, DNA was isolated using the standard phenol-chloroform-isoamyl alcohol extraction followed by ethanol precipitation (J. Sambrook, Fritsch, Maniatis, Molecular Cloning: A Laboratory Manual 2nd Edition, Vol. 3, pages E3 - E4; Cold Spring Harbor Laboratory Press 1989). Then the DNA pellet was resuspended in 50 µl of PCR-grade water and then stored at −20 ◦C before analysis by real-time PCR. For each sample, total DNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and adjusted to 20 ngr/µl using PCR-grade water. Positive control samples of *N. ceranae* and *N. apis* were provided from the archival of Istituto Zooprofilattico Sperimentale of Lazio and Tuscany, Rome. Honeybee samples previously known as free from *Nosema* spp infection were also included as negative controls. All used chemicals and reagents were from Sigma (Sigma-Aldrich S.r.l., Milano, Italy).

2.1.4. Primers design

Nosema spp ribosomal gene 16S was chosen as gene target. The nucleotide sequences of 16S ribosomal RNA gene from *N. ceranae* (FJ789797.1, FJ789789.1, FJ789785.1, FJ789799.1, FJ789786.1, FJ789788.1, FJ789791.1, DQ374656.2, DQ673615.1, FJ789795.1, EU045844.1, U26533.1) and *N. apis* (FJ789796.1, FJ789790.1) were obtained from GenBank (available at http://www.ncbi.nlm.nih.gov/genbank/) and aligned using the online version of Clustal Omega software (available at http://www.ebi.ac.uk/Tools/msa/clustalo/). The following primer pair: HRMNosem forward (HRMNosemF): 5' –AGGGGCGAAACTTGACCTAT- 3' and reverse (HRMNosemR): 5' – GCATCAATCATCATACACACTC- 3', was designed on conserved regions of the alignement, and substantially avoiding polymorphic regions that have been reported in *N. ceranae* 16S rRNA gene sequence (Sagastume et al. 2014) (**Figure 2.1**). The expected amplicon size was 162 bp for *N. ceranae* and 160 bp for *N. apis*. The specificity of primer sequences for *Nosema* species was confirmed using the Basic Local Alignment Search Tool program (available at http://blast.ncbi.nlm.nih.gov/).

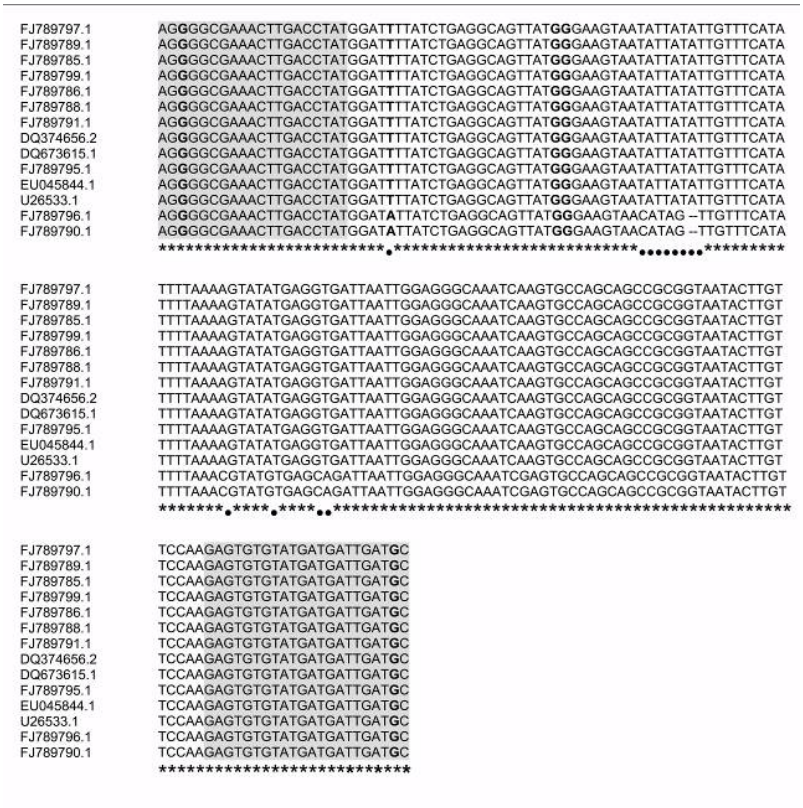


Figure 2.1. Alignment of partial *Nosema* ribosomal 16S gene sequences of *N. ceranae* (FJ789797.1, FJ789789.1, FJ789785.1, FJ789799.1, FJ789786.1, FJ789788.1, FJ789791.1, DQ374656.2, DQ673615.1, FJ789795.1, EU045844.1, U26533.1) and *N. apis* (FJ789796.1, FJ789790.1). Asterisks indicate identity; dots indicate sequence variations; gray areas indicate primer sequences (HRMNosemF and HRMNosemR) targeting conserved regions with no intra-species variability and with maximized interspecies variability design.

2.1.5. Conventional PCR

The designed primer pair (HRMNosemF and HRMNosemR) was first tested by conventional PCR as follows: 20 ng of extracted gDNA, 1U GoTaq® G2 DNA Polymerase (Promega Corporation, Madison, WI, USA), 1X Green GoTaq® Reaction Buffer (Promega Corporation), 0.2 mM PCR Nucleotide Mix (Promega Corporation), 0.3 mM of each primer pairs and PCR-grade water to a final volume of 20 µl. The thermal protocol was set as follows: 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 sec and elongation at 72°C for 30 sec. The amplification ended with a final elongation step at 72°C for 7 min. The amplification was performed on iCycler thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). Amplification products were run on 2% agarose gel and UV-visualized.

2.1.6. Real-time PCR - High Resolution Melting Analysis (HRMA)

Amplification product melting analysis is not a novel concept and exploits a fundamental property of DNA, called melting: the separation of the two strands of DNA with heat. PCR product melting analysis in combination with real-time PCR that was first introduced with the LightCyclers and the stability of DNA duplexes was detected using intercalating dye, like SYBR Green I. This is able to detect primer-dimers or other non-specific products. This procedure, called Low Resolution Melting has been performed for over a decade. A Low Resolution Melt curve is produced when the temperature increases, normally in steps of 0.5 °C increment per second, thereby gradually denaturing a previously amplified DNA target. Since SYBR Green I is only fluorescent when bound to dsDNA, fluorescence decreases as the double strand of the DNA amplicon is denatured. The melting profile depends on the length, GC content, sequence and heterozygosity of the amplified target. The highest rate of fluorescence decrease is normally at the melting temperature of the DNA sample (Tm). The Tm is defined as the temperature at which 50% of the DNA sample is doublestranded and 50% is single-stranded. The Tm is typically higher for DNA fragments that are longer and/or have a high GC content.

The fluorescence data from low resolution melting curves can easily be used to derive the Tm by plotting the derivative of fluorescence vs temperature (-dF/dT against T) as reported in **Figure 2.2** (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

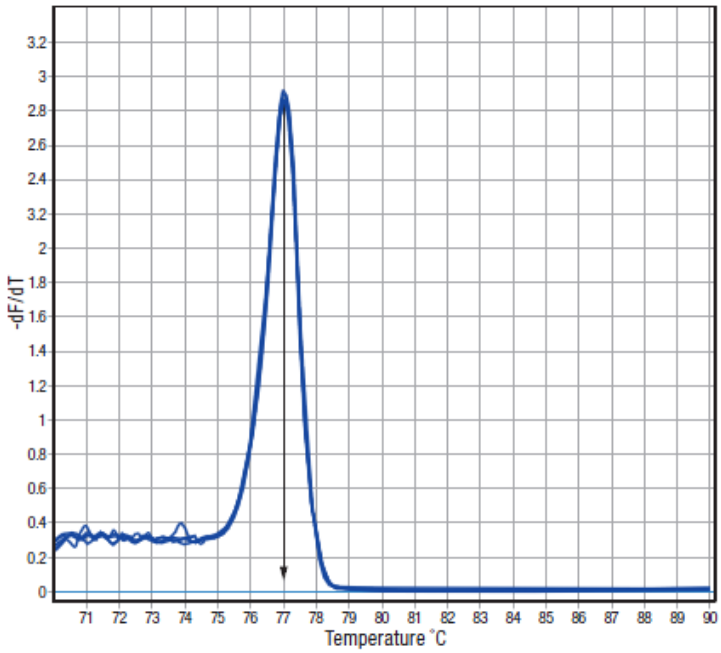


Figure 2.2: Low Resolution Melt profile derivative plot (-dF/dT against T). The steepest slope is easily visualized as a melt peak. In this example the Tm of the amplicon is 77 °C (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

The principle of High Resolution Melting Analysis (HRMA) is the same as a Low Resolution Melt, except that the temperature difference between each fluorescence reading is reduced. During a Low Resolution Melt curve analysis, the temperature increases are typically in 0.5 °C steps, but for HRMA this is reduced to 0.008 - 0.2 °C increments, depend on the property of the instrument.

Briefly, the first step of the HRM protocol consists in the amplification of the region of interest (usually, through a real-time PCR protocol), in the presence of a specialized double-stranded DNA (dsDNA) binding dye. This particular dye is highly fluorescent when bound to dsDNA and inadequately fluorescent in the unbound state. This change allows to monitor the DNA amplification during PCR (as in quantitative PCR). After completion of the PCR step, the amplified target is gradually denatured by increasing the temperature in small increments, in order to produce a characteristic melting profile; this is termed melting analysis. The DNA

sequence is gradually heat-denatured, the dye is released from the amplicon and therefore resulting in a decrease of fluorescence. If tuned in the correct way, this process allows a much more detailed analysis of the melting behavior. HRMA is thus able to detect a single change in the nucleotide sequence. HRMA uses low-cost dyes and requires less optimization than similar systems based on TaqMan chemistry and fluorescence resonance energy transfer (FRET) probes. Besides, HRMA is a simpler and more cost-effective way to characterize multiple samples (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com). HRMA sensitivity and reliability has been improved with the use of a variety of new dsDNA intercalating dyes and the availability of new instruments and more sophisticated analysis software.

There are a variety of dsDNA intercalating dyes, which have particular different characteristics. The conditions of the dyes used for HRMA are different from dyes typically used for standard quantitative PCR (qPCR) assays. Factors critical in qPCR, such as the signal-to-noise ratio and amplification efficiency, are not essential requirements for HRMA. Instead, the dye must provide detailed information on the melting behavior of an amplified target. Ideally the dye should not bind preferentially to pyrimidines or purines, change the Tm of the amplicon, or inhibit DNA amplification (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com). The three main classes of dsDNA binding dyes currently available are:

- **Non-saturating dyes.** SYBR® Green I is the most common non-saturating dsDNA intercalating dye. This type of dye is not very suitable to be used in HRMA. SYBR® Green I stabilizes the dsDNA when used at high concentrations, but it inhibits DNA polymerase. In contrast, low concentrations are not distributed in an appropriate way in the strand of DNA that results in poor discrimination-based difference (**Figure 2.3**). For a good performance the dye must be used at the right concentration.

- **Saturating dyes.** These dyes, unlike the previous ones do not inhibit the DNA polymerase and do not alter the Tm of the fragment. These dyes in contrast to other can be added in higher amounts so that the entire sequence is full of dye. Unfortunately this dye is not able to redistribute during melting because the dsDNA is saturated. More accurate examination

of the melting performance is therefore possible, as reported in **Figure 2.3**. Dyes such as SYTO9® and LCGreen® can be used at the saturating concentrations required for HRMA.

- **Release-on-demand dyes.** The "release-on-demand" class of dyes, which include EvaGreen®, can be added at non-saturating concentrations. This characteristic depends on the new method of fluorescence emission. In fact, the dye signal turns off when the fluorescent is free in solution. After the dye is tied in with the filament dsDNA, when the temperature drops the dye starts to emit the signal. This allows non-saturating concentrations of the dye to be used, ensuring that there is no PCR inhibition, whilst the unique dye chemistry provides highly sensitive HRMA analysis.

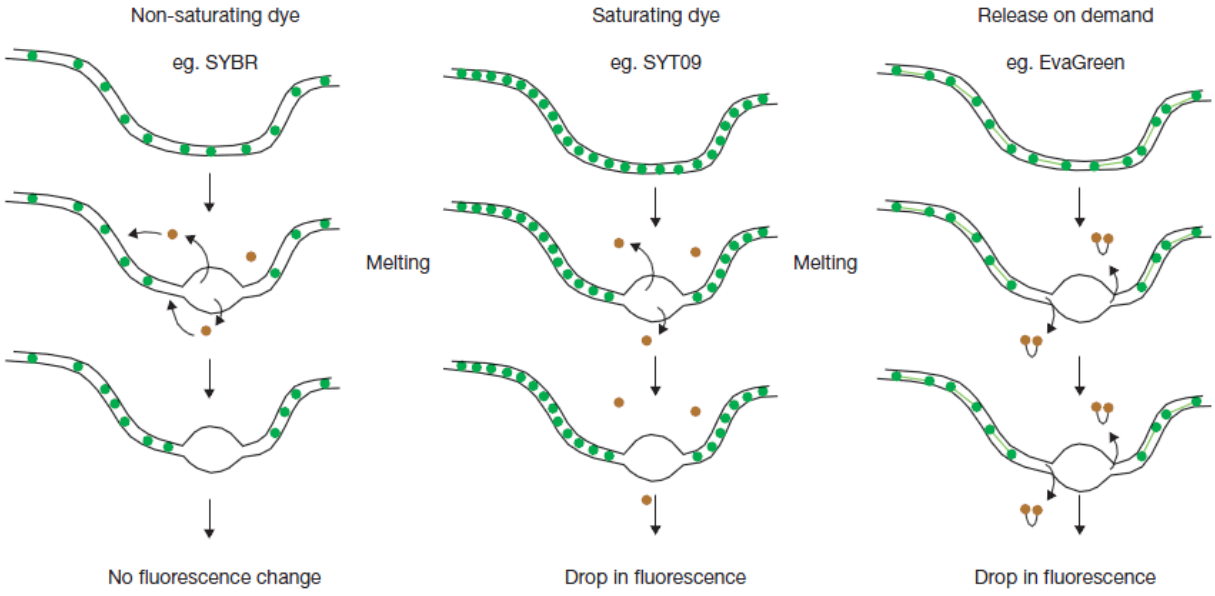


Figure 2.3: Non-saturating, saturating and 'release-on-demand' dsDNA intercalating dyes (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com). Melting of the duplex as the temperature increases releases the intercalated dyes. At non-saturating concentrations the dye rapidly rebinds to regions that remain double stranded; consequently there is no drop in fluorescence. Saturating and 'releaseon-demand' dyes do not redistribute from the melted regions of single-stranded DNA back to dsDNA, resulting in a reduction of fluorescence. This difference gives dyes such as EvaGreenR the high sensitivity required for HRM analysis.

The HRMA is a very sensitive technique and for this reason the development of the method is crucial. There are several factors that influence the behavior of melting, as genomic DNA (gDNA) quality, the background pattern, the concentration of $MgCl_2$ and polymerase inhibitors. Small differences in the initial configuration can significantly impact the final results. Achieving specific amplification is critical to the success of an assay, since any non-specific amplification

will greatly impair the melt analysis. The following most important aspects are listed (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com):

- **DNA quality and quantity:** One of the main factors that can affect the quality of the results is the relationship between DNA samples and PCR preparation. Unsuitable purification techniques can distort the signal. The presence of salts can change the melting behavior of the PCR product, and may result in poor reproducibility, low sensitivity and incorrect genotype calls.

Buffer carryover from the template DNA will not only modify the Tm and melting behavior during the HRM, but can encourage also nonspecific amplification during PCR.

To avoid possible bias in the results, some tricks may be used, as for example: a) to extract the DNA to be analyzed using always the same kit, b) to check the concentration of salts in the kit because any excess can distort the signal, and c) to check that the DNA concentration is equal for all samples. **Table 2.1** shows the recommended concentration of DNA for the different types of targets. At low concentrations of the target, there is a greater possibility of incorporating a mutation early in the PCR that will affect the melting behavior. By contrast, high concentrations of DNA can result in high fluorescence due to an increase in dsDNA intercalation of dye. The positive control(s) for all genotypes should be included if possible, preferably at the same concentration of their corresponding samples. Control DNA should also be eluted and/or diluted in the same buffer of the samples.

Type of DNA	Recommended amount for HRM analisys/reaction
Genomic DNA (gDNA)	10 ng - 100 pg
Plasmid DNA (1-10 kb)	1 ng - 10 fg
Amplicon DNA	10 pg - 1 fg

Table 2.1: Recommended concentration of DNA for different types of template (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com)

- **Primer design:** the primers for HRM are not easy to draw, since they should adhere to several requirements. In fact, it should be noted that the HRM analysisis is preceded by a qPCR, then amplicons can not be very long to ensure high sensitivity. Potential primer sets and full-length amplicons can then be tested for specificity using a BLAST search. Factors such as the position of the primer, the need for a GC region on the 3 'end, the presence of any secondary structures in the primers or target, and the specificity of the predicted amplification should be

carefully considered. Software as Primer3 or Primer3Plus can be very useful for drawing of primer HRMA, because the user can specify the location, the length and any other characteristics that are needed.

After the primers have been designed, the following factors must be checked:

- ✓ The presence of a GC 'clamp' on the 3' end. This is not essential, but usually improves amplification efficiency.
- ✓ The specificity of the primers and the presence of known sequence variations. A BLAST search with both primer sets and the expected amplification product should be performed. If possible, sequence variations should also be checked.
- ✓ The secondary structure of the primers and the expected amplification product. This should be examined using appropriate softwares, for example, m-fold, http://mfold.rna.albany.edu/?q=mfold/dna-folding-form, or operon, http://www.operon.com/tools/oligo-analysis-tool.aspx. Amplification of any secondary structure within the product may result in unusual melting profiles. Primers may need to be redesigned to avoid areas of secondary structure.
- ✓ Amplicon size. This must be optimal for the specific application, typically between 100 - 300 bp.

- **Primer concentration and purification:** the concentration of primer does not significantly affect the overall result in HRMA, however the recommended primer concentration to avoid nonspecific amplification is between 0.05 and 0.5 µM final. For normal purposes, it can be used the final concentration of 0.3 µM as recommended by the Evagreen's manufacturers.

- **Effect of $MgCl_2$ on HRM analysis:** different concentrations of $MgCl_2$ influence the efficiency of amplification, as is the case with conventional PCR. The amount of $MgCl_2$ leads to reduced non-specific amplification, and allows a clearer distinction of sequence variations. It is important to realize that the optimum $MgCl_2$ concentration for amplification efficiency Is not necessarily optimal sensitivity for HRM. The effect of $MgCl_2$ is shown in **Figure 2.4**. As the concentration of $MgCl_2$ is increased, the HRM difference graphs change distinctively.

- **Amplicon length:** in HRMA it is difficult to specify the optimal length of the amplicon, since there are many factors to be considered. For example, one factor is the number of

nucleotide changes in the sequence, i.e. the less variations there are, the shorter should be the amplicon. Normally the amplicon length can vary from 50 to 300 bp. Clearer discrimination is usually seen with shorter amplicons. However, small amplicons can result in lower fluorescence values, presumably because of decreased incorporation dye. By contrast, very long amplicons are allowed to create complex structures resulting in melting behaviours that can be misinterpreted in the end. When screening for unknown sequence differences, longer amplicons (typically 200-500 bp) can be used. This is useful in gene scanning or determining the variation within at population (e.g., viral) and reduces the number of required primer sets. Furthermore, too short amplicons are difficult to sequence.

- **Effect of PCR enhancers on HRM analysis:** PCR enhancers are commonly added to end-point PCR in order to help reducing non-specific amplification and the number of reaction cycles, and increasing yield. These enhancers function by assisting the melting and annealing of primers and templates, and can affect HRMA performance. For GC-rich amplicons, the addition of DMSO appears not to affect the HRMA significantly, and is recommended if amplification is difficult. Overall, the addition of PCR enhancers is not recommended as they usually detract from HRM performance.

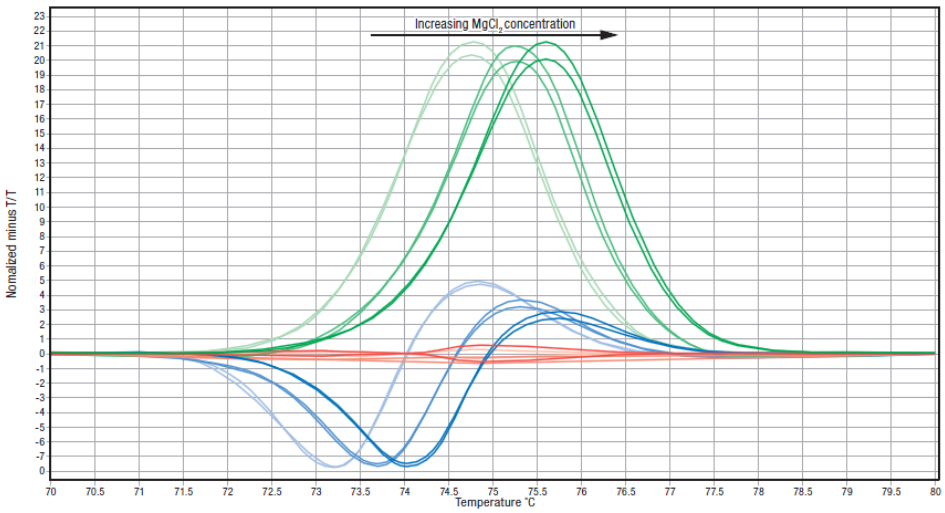


Figure 2.4. Effect of increasing $MgCl_2$ on melting behavior. Three Difference Graphs are overlayed to demonstrate the dependence of amplicon melting on $MgCl_2$ concentration. The effect of $MgCl_2$ is most pronounced in the heterozygote samples (blue), a result of the magnesium stabilizing the annealing of mispairs. The $MgCl_2$ concentrations are 1.5 mM, 2.5 mM and 3.5 mM final. G/G = green, G/A = blue, and A/A = red (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

The capacity of HRM analysis depends upon the sensitivity of the instrument and the nature of the PCR reagents used. Certain instruments have been designed specifically for HRM analysis. These can be summarized into two distinct classes:

1) **Block based instruments** – Samples are placed in a block for cycling and a scanning head or stationary camera is used for detection.
2) **Rotary** – Samples reside in a single chamber and spin past an optical detector.

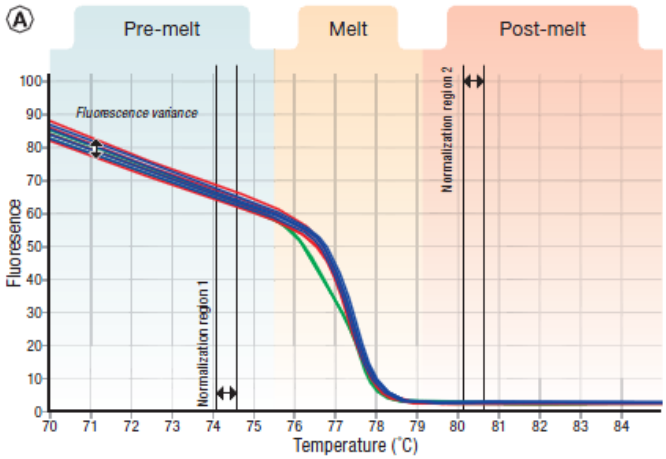
There are advantages and disadvantages with each class, and the users should choose the type of instrument that best suits their needs. As a general rule, for high sensitivity and reproducibility a rotary-based instrument should be used, whilst for high-throughput and ease-of-handling, block-type instruments are optimal. The HRM run can vary considerably between instruments; some take considerably longer than others. Every instrument should be calibrated regularly (as recommended by the manufacturer, typically every 6 months), kept in a clean area on a secure and stable benchtop, and checked daily for dust buildup around the optics. The computer hardware and software must be capable of handling the large quantities of data usually generated during a HRM experiment. Analysis of the data is relatively easy with the appropriate software. HRM specific software uses various algorithms to analyze the data and display the results in user-friendly formats to help discriminate between sequence variants. Additional software packages, designed for more detailed analysis, are available from some instrument manufacturers (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

Data analysis is typically straightforward, with the correct software, allowing multiple samples to be analyzed at the same time. Low resolution melt plots are often viewed as a derivative plot (−dF/dT against T); however, HRM raw melting curves must be analyzed differently. It is important to know what to look for when interrogating results; unforeseen errors made during setup can be identified at this point.

Amplification plots. For HRMA, the reproducibility of amplification is more important than efficiency amplification, compared with qPCR. As an example, the HRM analysis can still be performed if the product amplifies later than expected (for example due to limitations in the design of primers), provided that all the samples are constants, the no template control (NTC) does not amplify and the product is specific.

Raw data melt curve. The data collected during the analysis HRM has a variety of (pre-melt) initial values of fluorescence. These variations make it difficult to interpret the results, even if different genotype groups can be visible. **Figure 2.5** shows how to analyze the HRM results and a 124 bp product covering a Type IV SNP is used as an example (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com). The panel A of this figure illustrates how the selection of pre-and post-melt regions is used to align data. It is important to adjust these bars so that the melt region is not selected. If the preor post-melt regions are not clearly defined, it is possible to repeat the HRM run only (without repeating the amplification step), and adjust the temperature range as required. Pre- and post-melt regions must be selected for each primer set, by positioning the parallel double-bars as shown.

Normalization data. If the data is normalized correctly, it will appear as shown in the panel B of the **Figure 2.5**. This is termed 'Normalization Data'. Here the fluorescence variance seen in the Figure has been eliminated, and only the temperature range between the outer bars of the pre- and post-melt regions is shown. The genotypes are now more distinct, but the two homozygote samples (in red and blue) are still difficult to distinguish.



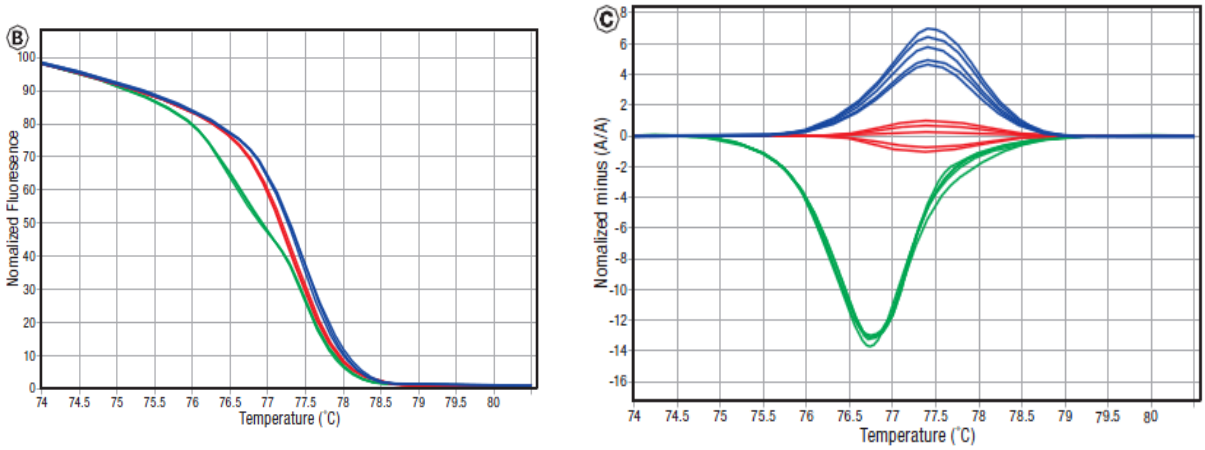


Figure 2.5. Analysis of HRM data from a type 4 SNP (A/T). Different genotypes are highlighted in different colors. A/A = red, A/T = green, and T/T = blue. **A.** Raw data showing the pre-melt, melt and post-melt regions. Notice the fluorescence variance and the positioning of the pre- and post-melt identification bars. **B.** Normalization Data derived from the raw data plots in A. Positioning of the pre-melt, and post-melt bars provides a more detailed view of the melt region. **C.** Difference Graph derived from the Normalization Data (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

Difference graph. Some HRM software applications allow calculation of the difference plot. This is achieved by subtracting the normalized fluorescence data of a user-defined genotype from that of each of the other samples in the HRM analysis. In panel C of **Figure 2.5**, the A/A genotype has been selected as a baseline (any genotype can be selected, but usually one of the homozygotes is used). The position of each sample relative to the baseline is plotted against the temperature. This output of HRM analysis is an aid for visualizing the normalization data. Automatic calling of genotypes of sequence variants can usually be performed with most instrumentation softwares; sometimes confidence ratings are given. However, checking the difference to confirm the genotype is recommended.

The gDNA extracted was tested by real-time PCR using an EcoTM Real-Time PCR System (Illumina, Inc., San Diego, CA, USA). The reaction mix was prepared as follows: 20 ng of extracted gDNA, 7.5 µl Supermix SsoFast EvaGreen mix (Bio-Rad Laboratories Inc.), 0.3 µM of each primer and PCR-grade water to a total volume of 15 µl. PCR-grade water was used as blank sample in each experiment. Each sample was tested in duplicate. The thermal protocol for the real time PCR was 95°C for 600 s, 45 cycles of 95°C for 15 s and 60°C for 30s. The High Resolution Melting protocol was 95°C for 15 s, 55°C for 15 s, ramping to 95°C with continuous

fluorescence data acquisition at each 0.1°C increment and a final step at 95°C for 15 s. The Eco_v4.0 software was used to analyze the HRM curves. Raw melting-curve data were normalized by setting the pre-melt (initial fluorescence) and post-melt (final fluorescence) signals of all samples to relative values of 100%, and 0% respectively.

2.1.7. Amplicon cloning and sequencing

The performance of the qPCR-HRMA method for the identification and quantification of *Nosema ceranae* was analyzed by building reference curves obtained from ten-fold serial dilutions of the corresponding cloned amplicon. Briefly, conventional PCR amplification products from positive control *N. cerana*e and *N. apis* DNA were run on 2% ethidium bromide agarose gel electrophoresis and purified using QiaquickTM Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The concentration of purified amplicons was spectrophotometrically measured using using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). Each quantitated amplicon was ligated to the plasmid pGEM T-easy at a concentration of 50 ng/µl (Promega Corporation) as reported in manufacturer's instructions. Then, DH5α competent cells were transformed with the corresponding plasmid at 3:1 concentration. After an overnight incubation at 37° C, the obtained colonies were screened for presence of the amplicon-ligated plasmidby conventional PCR using T7-SP6 plasmid-specific primers (pGEM® Vectors). One positive colony for each target was grown and the corresponding plasmid clone was purified using Qiaprep™ Spin Miniprep kit (Qiagen) and spectrophotometrically quantitated. The plasmid clones were then sequenced using T7 and SP6 primes and ABI technology in order to confirm the identity of each ligated target. The performance of the amplification was evaluated through the analysis of ten-fold serial dilutions of the corresponding plasmid from 10^6 to 10^1 copies/well. Mixed reconstructed samples containing different concentrations of *N. ceranae* and *N. apis* cloned DNA samples (10:1, 1:1, and 1:10 respectively) were also prepared in order to test the capability of the assay to distinguish mixed infection with different parasite load ratios.

The performance of the qPCR-HRMA method was assessed by comparing the results obtained from the same samples with the reference PCR method that is currently utilized in conventional and real time PCR for the identification and differentiation of *N. ceranae* and *N. apis* (Martin-

Hernandez et al., 2007; Fries et al., 2013). This method is based on the primer pairs 218MITOC and 321APIS for the amplification of *N. ceranae* and *N. apis* respectively.

2.1.8. Statistics analysis

Pearson's correlation coefficient was calculated as a measure of the strength of the linear relationship between two variables using Social Science Statistics online tool (http://www.socscistatistics.com/tests/pearson/Default.aspx).

2.2. Results

The new qPCR-HRMA method was tested both in conventional and real-time PCR, demonstrating high specificity and sensitivity for the target sequences. All samples showed a reliable and reproducible amplification signal. The amplicons were visualized on agarose gel electrophoresis at the expected size: 162 bp for *N. ceranae*, 160 bp for *N. apis* and 160/2 bp for the mixed samples, with undetectable non-specific amplication or primer dimers. No amplification was obtained from *Nosema*-negative sample, confirming the specificity of the protocol (**Figure 2.6**). The DNA sequencing confirmed the identity of the corresponding *Nosema* amplicons (data not shown).

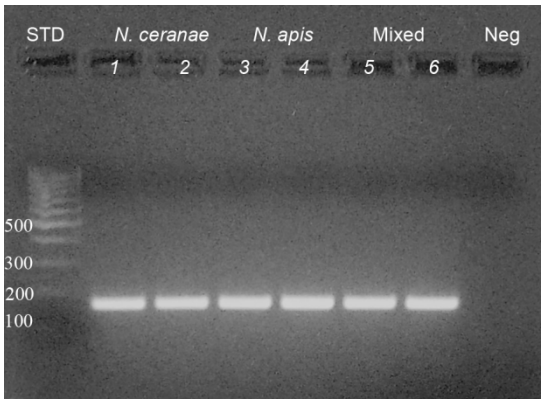


Figure 2.6 Agarose gel electrophoresis performed after conventional PCR. STD = 100 bp molecular weight marker; lines 1, 2 = *N. ceranae* positive control samples, lines 3, 4 = *N. apis* positive control samples, lines 5, 6 = 1:1 mixed samples; Neg = negative control.

The HRMA that was performed after real time PCR allowed the clear discrimination of the two species based on the different melting temperature of the two amplicons. In the normalized plot, the *N. ceranae* pure sample was clearly distinguishable from the *N. apis* pure sample and the mixed samples respectively . In addition, the differential plot allowed sample classification by subtracting the corresponding curves from a reference curve, i.e., the mixed 1:1 sample *N. ceranae/N. apis* (**Figure 2.7**).

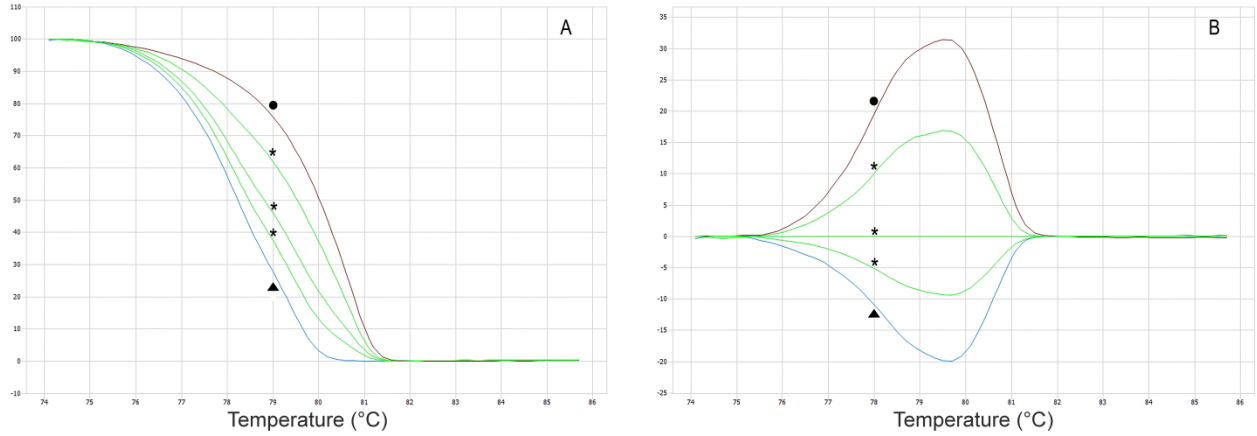


Figure 2.7. Quantitative PCR-HRMA of *Nosema* positive samples. **A**, Normalized and temperature shifted melting curves; **B**, differential plot of the same curves. From bottom to top, three different types of samples are distinguishable in both panels: the sample with pure *N. ceranae* infection (indicated by triangles), the samples with mixed *N. ceranae*/*N .apis* infection (indicated by asterisks) and the sample with pure *N. apis* infection (indicated by dots). The mixed samples correspond to a *N. ceranae*/*N. apis* copy number ratio of 10:1, 1:1 and 1:10 from bottom to top, respectively. In panel B, the mixed sample corresponding to a 1:1 *N. ceranae*/*N. apis* copy number ratio was used as a reference curve.

The real time PCR amplification of 10-fold serial dilutions of *N. ceranae* target clones allowed the set up of the reference curve, and a comparable amplification efficiency between HRMNosem and 218MITOC assay respectively was shown (**Figure 2.8**) The reference curve for HRMNosem and 218MITOC methods were also used to quantitate the *N. ceranae* target DNA in the 20 honeybee samples that were analyzed with both methods. The corresponding values obtained with the two methods for the same positive samples underwent correlation analysis. The resulting plot showed a very good correlation (R=0.9) between the *N. ceranae* load calculated with the new HRMA-based method and the reference method based on 218MITOC primers pair (**Figure 2.9**).

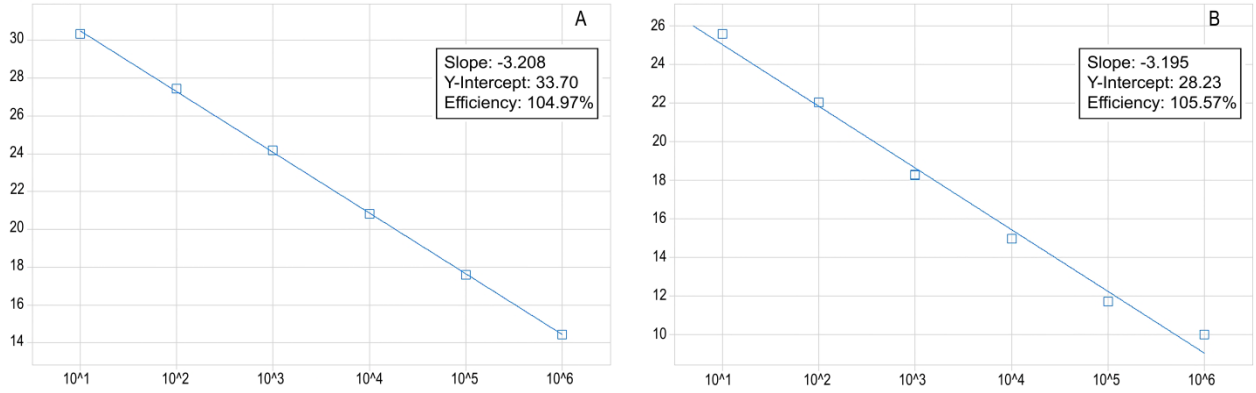


Figure 2.8. Reference curves for *N. ceranae* using HRMANosem method (panel A) and 218MITOC method (panel B) on a target plasmid serial diluition ranging from 10^6 to 10^1 copies/well.

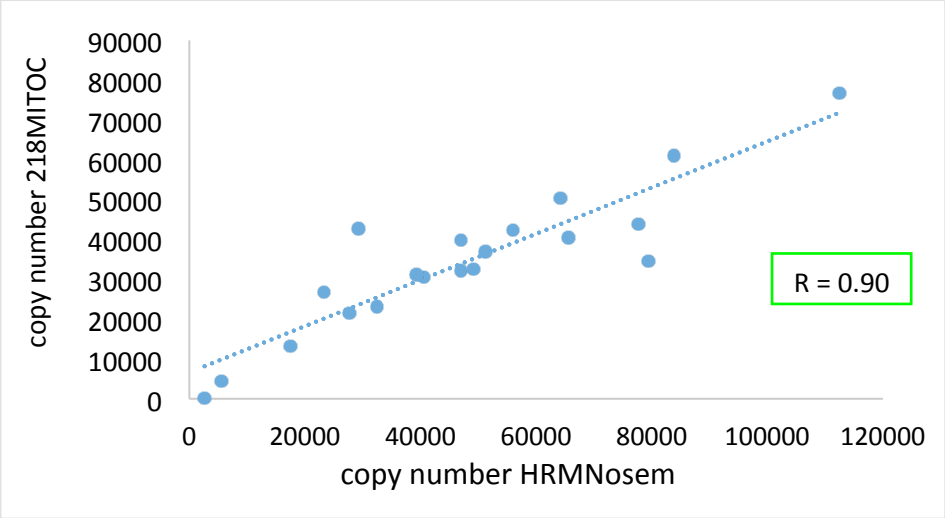


Figure 2.9 Correlation analysis of the *N. ceranae* load evaluated with HRMNosem and 218MITOC methods.

The tested samples ranged from 49166.67 to 1800000.00 mean spores/honeybee as determined by microscopical counting. The comparison between the mean number of spores per honeybee and the DNA copy number showed a moderate (R=0.64) correlation between the values obtained by the HRMNosem assay and by the microscopical method from the same samples (**Figure 2.10**).

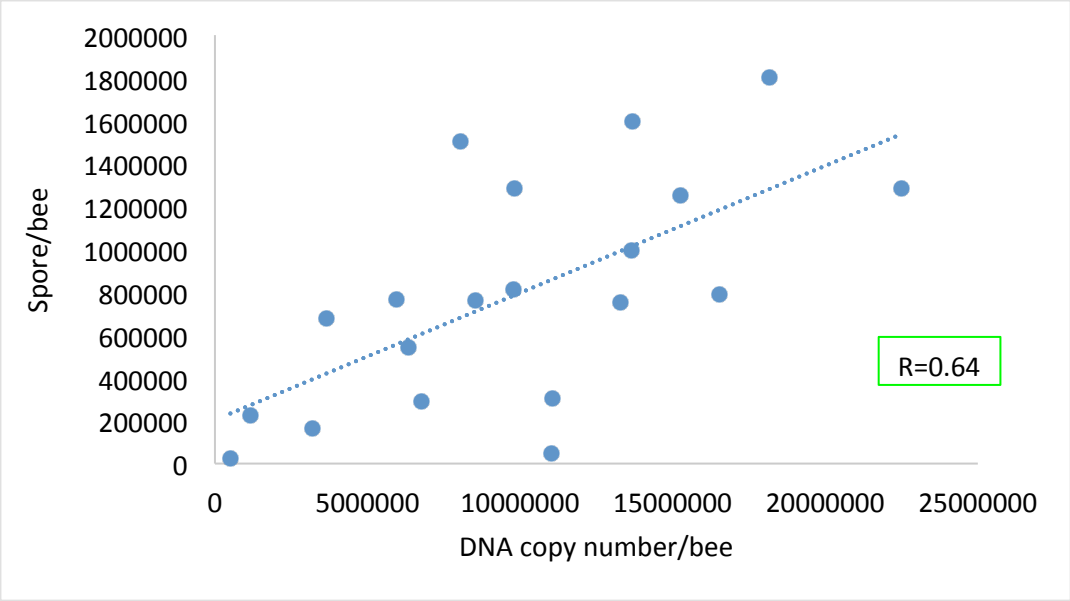


Figure 2.10. Correlation analysis between molecular (by HRMNosem method) and microscopic assessment of *N. ceranae* load for each sample

2.3. Discussion

N. ceranae and *N. apis* are honeybee parasites widely circulating inside the apiaries around the world. The infestation of *A. mellifera* by *N. apis* represents a real danger to the health of the beehive. The type A nosemiasis caused by this parasite is a notifiable disease under the provisions of the Veterinary Police Regulations (Botías et al. 2013). Only recently, *N. ceranae* has been widely admitted as a pathogen of the honey bee. At present the dynamics of the infection and the pathological consequences it may have on the host are not well defined and give rise to controversial opinions among experts in the sector. The rapid diffusion of the parasite caused emerging concern and for this reason careful monitoring of this phenomenon is required (Martin-Hernandez et al. 2007). To take precautionary measures that are efficient and durable it is first necessary to make an accurate assessment of the pathogen at the apiary level. To do this it is important to develop methods of certain identification of the parasite that are able to distinguish the two species of *Nosema* correctly and to provide information on the degree of infestation of honeybees. Positive identification of *Nosema* spp. may be established by standard light microscopical detection of the typical spores in the honey bee ventriculus or faeces. Microscopical examination can be routinely performed on individual or pooled honey bee samples. Due to age-related infection pattern, the highest proportions of infected honey bees are usually found in foragers, thus older workers are generally sampled from the hive entrance (Meana et al. 2010). Due to the high variability in spore load among workers, a relevant (at least 25-60 honey bees) sample size is usually recommended. After grinding the honey bees or their abdomen or ventriculus in a reliable liquid medium, spores are usually detected in the macerate at 400x magnification. Giemsa's staining or phase contrast light microscopy can be used to improve differential diagnosis with yeast cells, and spores and cysts from similar microorganisms (Fries et al. 2013). Spore count has been suggested as a marker of the severity of the infection especially regarding *N. ceranae*, even if the relationship between spore count and the health of the colony has been questioned (Meana et al. 2010). A compound microscope using 400 X magnification is sufficient for observing *Nosema* spp. spores in macerated bee preparations. During microscopical analysis, it is possible to perform spore counting by using a standard haemacytometer. The proportion of infected honey bees into the colony can show a good correlation with the average spore counts in pooled samples (Fries et

al. 2013). Nevertheless, under standard microscopical examination the spores of the two microsporidia, even if slighty different in size, are not easily distinguishable, especially whem mixed infections occur. Electronic microscopy can rely on the different number of polar filament coins to distinguish between the two species, but this technique is clearly not reliable for routine in-field diagnosis.

Molecular analysis can be suitable tool for highly sensitive and cost-effective pathogen species identification both for laboratory and field purposes. In the last years, several molecular protocols have been published for the identification of *N. ceranae* and *N. apis*, including conventional species-specific PCR allowing separate identification of the two pathogens, and PCR-RFLP and multiplex PCR allowing amplification for the two species simultaneously (Martin-Hernandez et al. 2007; Paxton et al. 2007; Chen et al. 2008; Carletto et al. 2013). Most published real time PCR (qRT-PCR)-based assays are capable of simultaneous identification and quantification of both species in the same reaction, using separate primers pairs and species-specific probes (Chen et al. 2009; Bourgeois et al. 2012). The OIE recommend a qualitative multiplex PCR method as the reference protocol for molecular diagnosis of *Nosema* (Martin-Hernandez et al. 2007; (OIE) 2008). This method was also adapted for duplex-real time PCR with melting curve analysis, thus improving the sensitivity, specificity and speed of the original assay (Burgher-MacLellan et al. 2010).

The present study validated a new qPCR-HRMA protocol assay for infection assessment of the two *Nosema* species affecting honey bees by comparison with the results obtained with two largely used reference methods. Taken together, the results of the present study support qPCR-HRMA as an alternative method for parasite identification and load estimation. This assay is promising for extensive routine use in in-field diagnosis as a quick and sensitive single step protocol to identify both Nosema species using an unique primer pair and saturating dye and avoiding the use of expensive sequence-specific probes. Thus, this assay has some advantages compared to already existing reference methods, not requiring multiplex methods or time-consuming post amplification techniques like agarose gel amplification and/or DNA sequencing. Specific features of this assay are a shorter analysis time, a reduced cost, and a comparable amplification efficiency for both targets, especially useful in case of simultaneous infection with the two *Nosema* species.

The present study was reported as a manuscript currently submitted for publication (**Dell'Orco F.**, Loiacono M., Albonico F., Zanzani S., Cersini A., Formato G., Colombo M., Mortarino M.. Detection and quantitation of *Nosema ceranae* in honey bees by real time PCR coupled to High Resolution Melting Analysis**.** Parasitology Research, submitted manuscript)

As for Milk

Chapter 3: Identification of pathogenic *Escherichia coli* in bulk-tank milk and filter milking machine through molecular profiling of virulence genes

3.1 Introduction

3.1.1 Escherichia coli

Domain	Bacteria
Phylum	Proteobacteria
Class	Gamma-Proteobacteria
Order	Enterobacteriales
Family	*Enterobacteriaceae*
Genus	*Escherichia*
Species	*E. coli*

Escherichia coli is a member of the genus *Escherichia* within the family *Enterobacteriaceae*. It is a gram-negative , rod-shaped, mostly motile and facultative anaerobic bacterial species (Edwards & Ewing 1962). *E. coli* can live in two different habitat during his life cycle: The first one is host-associated in the lower intestine of warm-blooded animal/human, in which it establishes commensal associations (Macfarlane & Gibson 1997); the second one is non-host-associated (Savageau 1983). Indeed, *E. coli* is released in the environment through faecal deposition, in which it can survive for long periods and potentially replicate on algae in water and soils in tropical, subtropical and temperate climates (Ishii & Sadowsky 2008).

Beside these habitats, certain strains have the potential to cause a wide spectrum of intestinal and extra-intestinal diseases such as urinary tract infection, septicaemia, meningitis, and pneumonia in humans and animals (Michael S. Donnenberg 2002). *E. coli* is frequently used as a faecal indicator bacterium (FIB) for assessing water quality, and the survival and growth of *E. coli* in the environment raises concerns regarding the use of this bacterium for indicating faecal contamination. In addition, since some *E. coli* strains and serotypes can cause human diseases, understanding the ecology of this bacterium is important to prevent infection and spread of this pathogen to food, soil, and water (Jang et al. 2017).

The first information about the genome sequence of *E. coli* was in 1997, the last available analysis allowing the division into seven major phylogenetic groups: A, B1, B2, C, D, E, and F, with the remaining unclassified subtypes placed in an eighth group, *Escherichia* cryptic clade *I*

(Herzer et al. 1990; Clermont et al. 2013). These groups encompass saprophytic (A) and pathogenic (in particular B2, D) types and are often considered to be the result of long evolution time.

The phenotypes of the different *E. coli* organisms types are related to genomic differences and the patterns of gene expression, while the genomic islands define the different behavioral types (Touchon et al. 2009). In immunocompromised hosts or when gastrointestinal barriers are disrupted, even nonpathogenic strains of *E. coli* can cause infection (Cullor & Smith 1996)

- **Morphological structure**

The cell wall of *E. coli* is composed of a thin peptidoglycan layer and an outer membrane. The external cell membrane contains phospholipids, membrane proteins and lipopolysaccharide (LPS). LPS comprises lipid-A, the lipopolysaccharide core and repeated polysaccharide units called O-antigens and the toxic effects are caused by lipid-A (Cullor & Smith 1996). The polysaccharide layer called capsule can be divided into on three types of somatic (O), capsular (K) and flagellar (H) antigens (Cullor & Smith 1996). Different *E. coli* serotypes are defined by combination of O and H antigens, among which more than 700 are identified (Kaper et al. 2004; Ishii & Sadowsky 2008). *E. coli* strains also diverge in other phenotypic characteristics, such as carbon utilization patterns, antibiotic resistance profiles, flagellar motility, ability to form biofilms, and the ability to cause diseases. The *E. coli* adaptability to specific hosts would lose fitness in other environments, that phenotypic variation could explain the ecological adaptation(Franz & van Bruggen 2008).

Major groups of *E. coli* virulence factors include adhesins, toxins, polysaccharide capsules and O-antigens, proteins secreted into host cells and other mechanisms to resist killing by complement or to bind iron ions. Bacteria do not produce virulence factors continually but only when intercepting particular signals from the host or environment (China & Goffaux 1999).

The horizontal gene transfer plays an important role in the acquisition of new genes in *E. coli* (Doi et al. 2012), while genetic mutations can also contribute to the *E. coli* phenotypic diversity such as carbon metabolism (Cooper & Lenski 2000). High genotypic diversity has been identified in *E. coli* based on the repetitive extragenic palindromic PCR (rep-PCR) DNA fingerprinting (Jang et al. 2015; Byappanahalli et al. 2012) and pulsed field gel electrophoresis

(Johnson et al. 2013). Such genotypic diversity appears to be common among environmental strains (Ishii & Sadowsky 2008).

3.1.2 Pathotypes of *E. coli*

The virulence factors can be present in the *E. coli* genome or on plasmids (Harel & Martin 1999).

The environmental conditions can influence *E. coli* genomic traits of the bacterium (Tenaillon et al. 2010). In the host-animal intestine, *E. coli* population structure is determined by the conditions of the gastrointestinal tract, which in turn, is influenced by host physiology and diet. Similarly, the genetic structures of *E. coli* populations in the natural environment are shaped by abiotic and biotic environmental factors (van Elsas et al. 2011).

Pathogenic *E. coli* can be divided into two groups: intestinal and extraintestinal pathogens.

The major intestinal pathogens include:

- enteroaggregative *E. coli* (EAEC),
- enteroinvasive *E. coli* (EIEC),
- enterotoxigenic *E. coli* (ETEC).

The major extraintestinal pathogens include:

- neonatal meningitis-associated *E. coli* and sepsis-causing *E. coli* (NMEC)(Dale & Woodford 2015).
- uropathogenic *E. coli* (UPEC)
- urinary tract infections (UTIs)

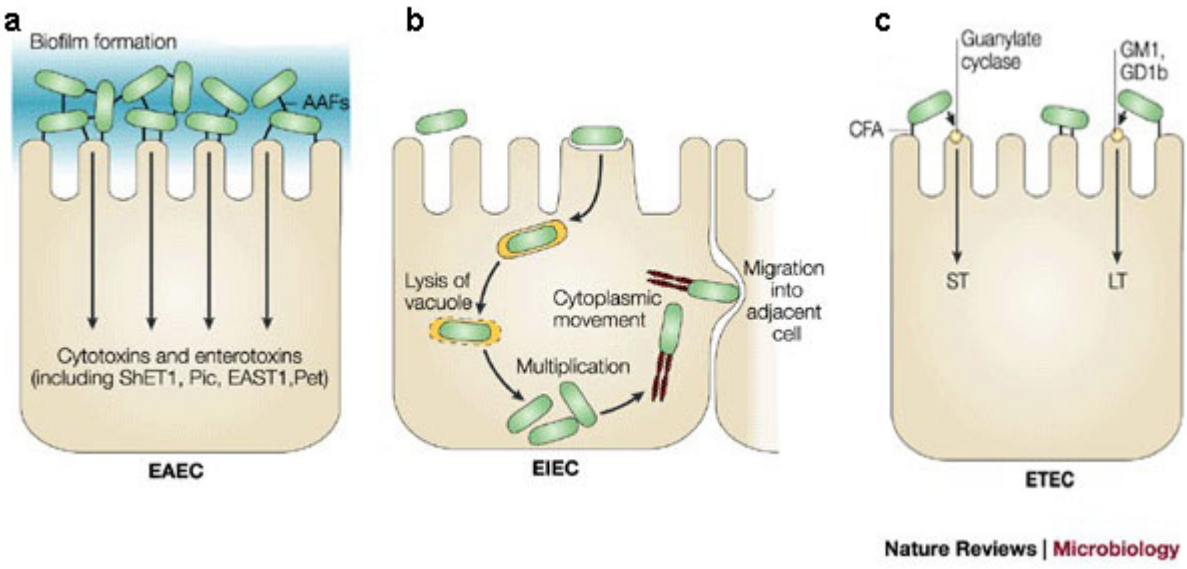


Figure 3.1: Pathogenic schema of intestinal E. coli. a) EAEC adheres to small and large bowel epithelia in a thick biofilm and elaborates secretory enterotoxins and cytotoxins. b) EIEC invades the colonic epithelial cell, lyses the phagosome and moves through the cell by nucleating actin microfilaments. The bacteria might move laterally through the epithelium by direct cell-to-cell spread or might exit and re-enter the baso-lateral plasma membrane. c) Similarly, ETEC adheres to small bowel enterocytes and induces watery diarrhoea by the secretion of heat-labile (LT) and/or heat-stable (ST) enterotoxins (Kaper, 2004).

- **Enteroaggregative *E. coli* (EAEC)**

The first identification of EAEC was in 1987, being defined by its aggregative patterns of adherence to cultured cells (Nataro et al. 1987). EAEC strains are important causative agents of traveler's watery diarrhea with mucus accompanied by fever, vomiting, and abdominal pain (Okhuysen and Dupont 2010).

The invasion process can be divided into three steps. First, the aggregative adherence fimbriae of EAEC allows the colonization of the intestinal mucosa and induces IL-8 release. Second, it produces a mucus-mediated biofilm on the enterocyte surface. Third, EAEC releases the toxins, causing the inflammatory response, intestinal secretion and mucosal toxicity (Pereira et al. 2008). In particular, the cytotoxins can stimulate neutrophil transmigration across the epithelium, which can itself lead to tissue disruption and fluid secretion (Kaper et al. 2004) (**Figure 3.1**).

EAEC has a pattern known as autoaggregative, in which bacteria adhere to each other in a 'stacked-brick' configuration (Nataro & Kaper 1998), indeed EAEC produces plasmid-encodeds aggregative adherence virulence factors as:

- ✓ aggregative adherence fimbriae I (AAF/I; aggA),
- ✓ aggregative adherence fimbria II (AAF/II; aafA),
- ✓ aggregative adherence fimbria III (AAF/III; agg3A),
- ✓ aggregative adherence fimbria IV (AAF/IV; agg4A),
- ✓ aggregative adherence fimbria V (AAF/V; agg5A),
- ✓ plasmid-encoded toxin (Pet), that can lead to cytoskeletal changes and epithelial-cell;
- ✓ enteroaggregative heat stable toxin 1 (EAST1; astA) which causes the watery diarrhoea
- ✓ dispersin (aap), a protein which increases the spread of infection across the mucosal surface or the penetration of the mucous layer (Sheikh et al. 2002),
- ✓ transcriptional activator AggR (aggR) regulon, that increases faecal concentrations of IL-8 and IL-1 (Jiang et al. 2002).
- ✓ enterotoxin ShET2
- ✓ *Shigella* enterotoxin 1 (ShET1), it seems contributing to the secretory diarrhea (Kaper, Nataro, and Mobley 2004; Aslani et al. 2011; Chaudhuri et al. 2010; Jønsson et al. 2015)

The typical symptoms caused by EAEC are typical of strains carrying AggR and AggR-regulated genes, indeed the term 'atypical EAEC' is used for strains lacking the AggR regulon (Kaper et al. 2004).

The infection treatments are oral rehydration therapy and antimicrobial therapy, but the antibiotic resistance is increasing worldwide (Aslani et al. 2011). The detection of EAEC is on the detection of aggregative adherence (AA). The assays utilized are the cell culture (HEp-2 assay) (Cravioto et al. 1979) and DNA probe (Nataro et al. 1985). A PCR assay with primers derived from the AA probe sequence shows similar sensitivity and specificity (Nataro & Kaper 1998).

- **Enteroinvasive *E. coli* (EIEC)**

EIEC strains are biochemically, genetically, and pathogenetically closely related to *Shigella* spp.. Several studies have shown that *Shigella* and *E. coli* are taxonomically indistinguishable at the species level (Wei et al. 2003). EIEC can be distinguished from *Shigella* by a few minor biochemical tests, but these pathotypes share essential virulence factors. EIEC strains are generally lysine decarboxylase negative, non- motile, and lactose negative (Brenner et al. 1973).

EIEC causes an invasive inflammatory colitis, and occasionally dysentery, but in most cases watery diarrhea. EIEC is invasive, but dissemination of the organism past the submucosa is rare.

The infection starts with epithelial cell penetration, followed by lysis of the endocytic vacuole, intracellular multiplication, directional movement through the cytoplasm and extension into adjacent epithelial cells. Movement within the cytoplasm is mediated by nucleation of cellular actin into a 'tail' that extends from one pole of the bacterium (**Figure 3.1**) (Kaper et al. 2004).

Much of EIEC pathogenesis seems to be the result of the multiple effects of plasmid-borne type III secretion system (Sansonetti et al. 2000). It secretes different protein:

- ✓ IpaA, involved in binding the vinculin and induces actin depolymerization, thereby helping to organize the extensions that are induced by IpaC into a structure that enables bacterial entry;
- ✓ IpaB, involved in binding the signaling protein CD44 and the macrophage caspase 1, causing apoptosis and release of IL-1 from macrophages;
- ✓ IpaC, involved in actin polymerization, which leads to the formation of cell extensions by activating the GTPases Cdc42 and Rac;
- ✓ IpgD, involved in the reorganization of host-cell morphology by uncoupling the cellular plasma membrane from the actin cytoskeleton, which leads to membrane blebbing (Kaper et al. 2004).

The type III secretion system apparatus, which is encoded by mxi and spa genes, enables the insertion of a pore containing IpaB and IpaC proteins into host cell membranes.

Although the extensively characterized type III secretion system is essential for the invasiveness characteristic of EIEC and Shigella species, this process is also contributed by the plasmid-encoded serine protease SepA, the chromosomally encoded aerobactin iron-acquisition system and other secreted proteases that are encoded by genes present on pathogenicity islands.

The detection of EIEC is on the ability of the strain to invade epithelial cells and to spread from cell to cell to distinguish from *Shigella* spp (Sereny test) (Kopecko 1994), enzyme-linked immunosorbent assay (ELISA) to detect the ipaC gene (Inv) (Pal et al. 1997) and DNA probes (Gomes et al. 1987; Wood et al. 1986). A PCR and multiple PCR assay with primers derived from ial gene (Frankel et al. 1990; Frankel et al. 1989).

- **Enterotoxigenic *E. coli* (ETEC)**

ETEC strains were first recognized as causes of diarrheal disease in piglets, with lethal infection in newborn animals (Alexander 1994). Studies of ETEC in piglets first elucidated the mechanisms of disease, including the existence of two plasmid- encoded enterotoxins (ST and LT). The descriptions of ETEC in humans reported that certain *E. coli* isolates, first in children, following in adult with diarrhea, elicited fluid secretion by the intestine (DuPont et al. 1971). ETEC are also an important cause of diarrhoeal disease in animals and these animal strains express fimbrial intestinal colonization factors, which are not found in human ETEC strains.

The pathogenesis begins when the ETEC colonizes the surface of the small bowel mucosa using fibrillar colonization (CF) factors and elaborates enterotoxins, which give rise to intestinal secretion.

ETEC enterotoxins can be divided in two groups: the heat-labile enterotoxins (LTs) and the heat-stable enterotoxins (STs). ETEC strains can express LT and ST alone, or both. LT is a potent mucosal adjuvant, independently of its toxic activity (Pizza et al. 2001) and has been incorporated into numerous vaccine candidates containing a variety of antigens, thus resulting in increased antibody responses to these antigens. STs are small, single-peptide toxins that include two unrelated classes — STa and STb — which differ in both structure and mechanism of action. Indeed, STa toxin can affect humans (Nataro & Kaper 1998), while STb toxin can affect animals (Dubreuil 1997) ETEC is largely a pathogen of developing countries, and it is well known that these countries typically have a low rate of colon cancer. So the high prevalence of ETEC in developing countries might have a protective effect against this important disease, and indicates that infectious diseases might exist in a complex evolutionary balance with their human populations (Kaper et al. 2004).

The detection of ETEC is related on the expression and analyses enterotoxins LT and ST. The assays developed are the immunoassays (Honda et al. 1981), including a radioimmunoassay for ST (Giannella et al. 1981), ELISA (Cryant 1990; Yolken et al. 1977) and latex agglutination (Ito et al. 1983). The molecular techniques developed are DNA probes to detect LT- and ST-encoding genes in stool and environmental samples (Nataro & Kaper 1998). Also, the "multiplex" PCR assay (Lang et al. 1994; Stacy-Phipps et al. 1995) in which several PCR primers are combined to detect one of several different diarrheagenic *E. coli* pathotypes in a single reaction.

- **Extraintestinal pathogenic *E. coli* (EXPEC)**

The ExPEC strains predominantly cluster in the B2 and D phylogenetic groups while APEC strains have also expanded into C and F groups (Johnson et al. 2001; Nicolas-Chanoine et al. 2007; Totsika et al. 2011). ExPECs colonize and infect a wide range of host species, using different virulence factors (VFs) that are not restricted to the ExPEC pathotype. ExPECs respond to environmental stimuli using several signaling networks; the best characterized of these signaling systems are two-component systems (Breland et al. 2017). The common VFs for ExPEC are pili assembled by the chaperone-usher pathway (CUP), protein adhesins, toxins, iron acquisition systems, transport systems, and other non-essential factors (Russo & Johnson 2000).

ExPEC strains colonizes different tissues: UPEC, urethra, bladder and kidneys; NMEC, brain and nervous tissues; UTIs, urinary tract. Despite the existence of these classes of ExPEC, there is great overlap in the variety of VFs expressed by each class, making these designations rather artificial (Russo and Johnson, 2000).

ExPEC infections in all afflicted populations are typically treated with antibiotics, although this practice can cause multi-drug resistance, thus affecting the healthcare setting and making more difficult eradication of ExPEC strains (Breland et al. 2017).

The detection of the ExPEC is based on VFs of the different strains(Russo & Johnson 2000).

3.2 Aim

The identification of *E. coli* strains is important for public health since it can reproduce in animals, humans and the environment. The routine detections and differentiations of intestinal and extraintestinal *E. coli* are usually based on a combination of biochemical tests; serotyping, phenotypic assays based on virulence characteristics and molecular detection methods. The detention of the causative agent is very important to infer the inherent sensitivity of the host animals to specific virulent strains and for the setup of an effective control strategy.

The aim of this work was to evaluate the presence and virulence profile of pathogenic *E. coli* in bulk-tank milk compared to milking machine filters samples on a number of samples collected in dairy farms.

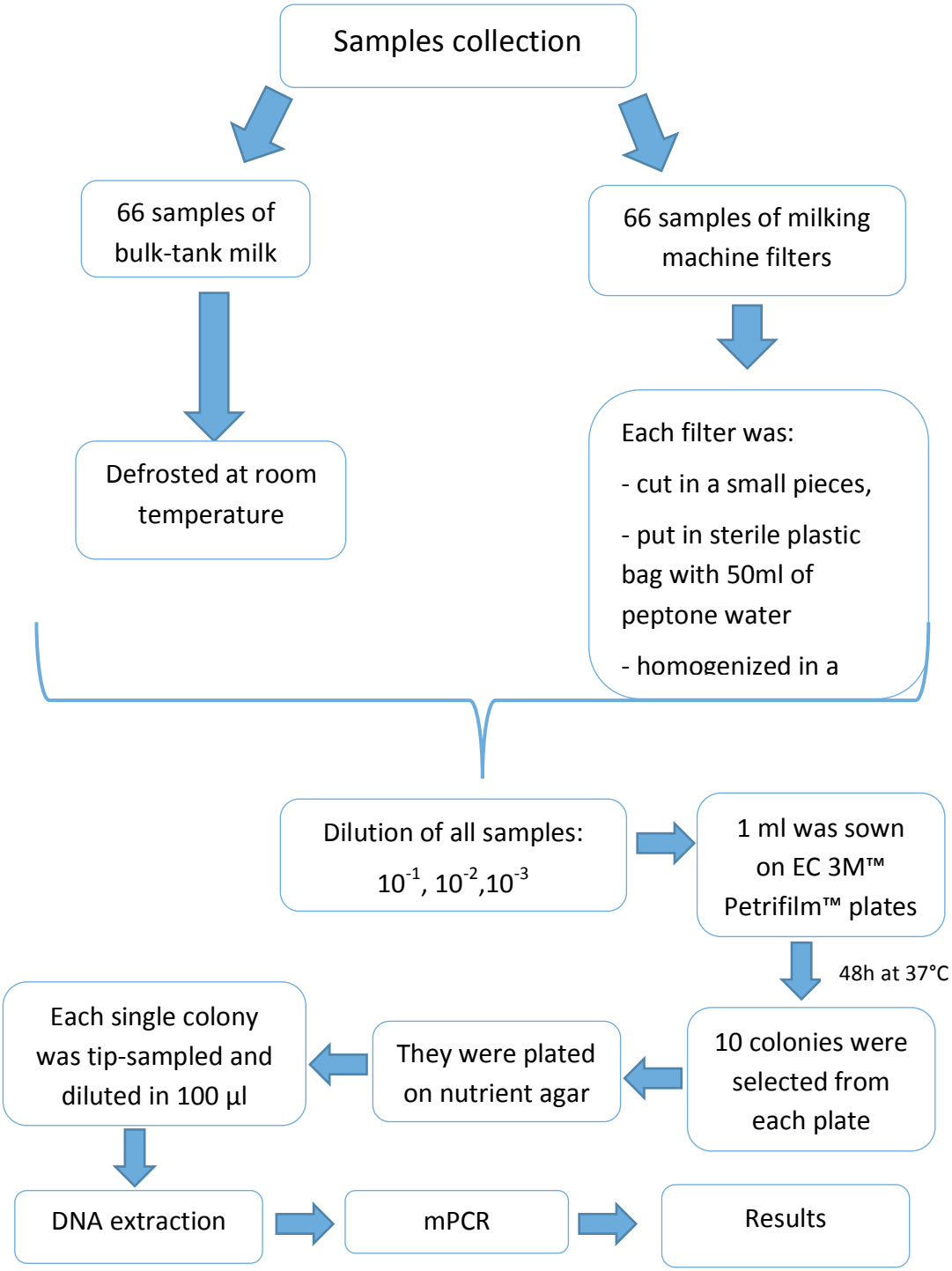
To achieve this, the classical isolation and identification techniques have been coupled with a molecular diagnostic approach.

The implementation of such combined microbiological and biomolecular approach allowed the selective identificationof *E. coli* from other bacteria on specific media and the profiling of virulence factors , leading to a rapid and sensitive identification of pathogenic strains, as a necessary step for a proper characterization of the host and human exposure, and inherent health risk.

3.3 Materials and methods

3.3.1 Overview of the experimental design

The experimental design involved several steps from the collection of the samples in dairy cows to biological analysis, as follows



The collection of the samples and the microbiological isolation was done in collaboration with the Infectious Diseases Unit of the Veterinary Medicine Department (DIMEVET), University of Milan.

3.3.2 Collection of samples

The samples were collected during the milking in seven different dairy farms located In Lombardy (closed to Lecco and Como) and Trentino-Alto Adige.

The samples included:

- → 66 bulk-tank milk;
- → 66 milking machine filters.

The filter (similar to a sock) is placed in the duct that leads the milk from the milking room to the milk collection tank. The material of the filter is similar to a tissue with a variable weight of 60 to 80 g/m^2 and its capacity is optimal for milking about 200 beef, with satisfactory breast hygiene and good milking practices.The filterhas the function of blocking debris, large particles of organic material and foreign objects (hair, vegetable fibers, dirt, etc.), ensuring that milk is free of macroscopically visible contaminant, but it is not able to reduce the number of bacteria present in the milk.

The samples collected were refrigerated within eight hours from milking. Once in the laboratory, they were frozen and stored at -20 °C until further processing.

3.3.3 Microbiological isolation

The samples were treated differently if there were filter or milk.

The milk samples were defrosted at room temperature and they were diluted as follows: 10^{-1}, 10^{-2} and 10^{-3}/ml.

The Milking machine filters were chopped sterile, introduced in plastic bags with 50 mL of sterile peptone water and processed in Stomacher for 4 minutes. The obtained preparation was brought to the following dilutions: 10^{-1}, 10^{-2} and 10^{-3}/ml.

After dilution, all samples were sown on plates for the enumeration of *E. coli* (EC 3M ™ Petrifilm ™) and the plates were incubated for 48 hours at 37 °C. The plates were then checked for bacterial growth, and the colonies of *E. coli* can appear blue or red-blue and with or without

one or more gas production. Indeed, the colonies that appear blue or red-blue were *E. coli* which produce β-glucuronidase. Instead, the colonies that produce one or more gas production were *E. coli* which ferment lactose. Then *E. coli* colonies were picked up randomly from each plate to a maximum of 10 colonies and these were plated on nutrient agar. Each single colony was tip-sampled and placed in an 0,5 ml Eppendorf diluted in 100 µl of molecular grade water.

3.3.4 DNA extraction

The extraction of the samples was performed using alternative cycles of hot and cold break: 15 minutes at 95 °C and 15 minutes at -80°C. This method allowed the cells lysis and DNA extrusion from bacterial cells.

3.3.5 Conventional PCR

Conventional PCR runs were performed on T100™ Thermal Cycler-Bio-Rad. The reactions were carried out in a total volume of 20µl containing 2µl of DNA, 1X Taq buffer (containing 1.5mM MgCl2), 0.2 mM dNTPs, 0.2-0.4 µM of each primer, and 1.25 U TaqPromega; H_20 was added to reach a total volume of 20 µl. The thermal protocol for the PCR was as follows: denaturation step at 95° C for 10 min; 35 amplification cycles at 95°C for 30 s, annealing T °C depending on each primer pair for 30 s and 72°C for 30s; and the final elongation step at 72°C for 10 min. Primers sequences, amplicons size, concentration and annealing temperatures for each primer pair for PCR amplification are reported in **Table 3.1**. The amplification products were run on 2% agarose gels and visualized under a trans-illuminator.

Table 3.2: Primers sequences, amplicon size and annealing temperature

PCR specifies	PCR format	Target	Primer sequences (5'-3')	Amplicon Size	Concentr (µM
EAEC	Multiplex PCR Conventional,	*aggR*	F: GCAATCAGATTAARCAGCGATACA R: CATTCTTGATTGCATAAGGATCTGG	425	0.2
EIEC		ipaH	F: TTCCTTGACCGCCTTTCCG R: AGCCACCCTCTGAGAGTAC	611	0.2
ETEC		eltB	F: CGGCGTTACTATCCTCTC R: CCATACTGATTGCCGCAAT	336	0.2
	Multiplex PCR	estIa	F: CCTCTTTTAGYCAGACARCTGAATCASTTG R: CAGGCAGGATTACAACAAAGTTCACAG	157	0.4
		estIb	F: TGTCTTTTTCACCTTTCGCTC R: CGGTACAAGCAGGATTACAACAC	171	0.2
ExPEC	Conventional, multiplex	focG	F: CGTACCTGTACCATTGGTAATGGAGG R: TGAATTAATACTTCCCGCACCAGC	366	0.2
		kpsMII	F: GCGCATTTGCTGATACTGTTG R: GGGAACATGATGCAGGAGATG	452	0.2
		papA	F: ATGGCAGTGGTGTCTTTTGGTG R: CGTCCCACCATACGTGCTCTTC	717	0.2
		sfaS	F: GTCTCTCACCGGATGCCAGAATAT R: GCATTACTTCCATCCCTGTCCTG	138	0.2
		afa	F: GGCAGAGGGCCGGCAACAGGC R: CCCGTAACGCGCCAGCATCTC	594	0.2
		hlyD	F: CTCCGGTACGTGAAAAGGAC R: GCCCTGATTACTGAAGCCTG	904	0.2
		iutA	F: ATCGGCTGGACATCATGGGAAC R: CGCATTTACCGTCGGGAACGG	314	0.2

ExPEC was defined as a strain having three or more of these genes (Jang et al. 2013).

3.4 Results and discussion

From the samples of milk and milking machine filters, after the selection on EC 3M ™ Petrifilm ™, 149 colonies (65 form the milk and 84 from the filter) were collected which appeared as *E. coli*. The colonies were then planted on agar soil, then each single colony was tip-sampled and placed in an 0,5 ml Eppendorf diluted in 100 µl of molecular water to perform the conventional PCR.

Before the analysis of unknown samples, each gene-specific PCR assay was tested on positive control samples provided by the Istituto Zooprofilatico Sperimentale delle Venezie, Italy. The agarose gel-electrophoresis run confirmed the effective amplification of the gene target with the expected amplicon size, as shown in **Figure 3.2**.

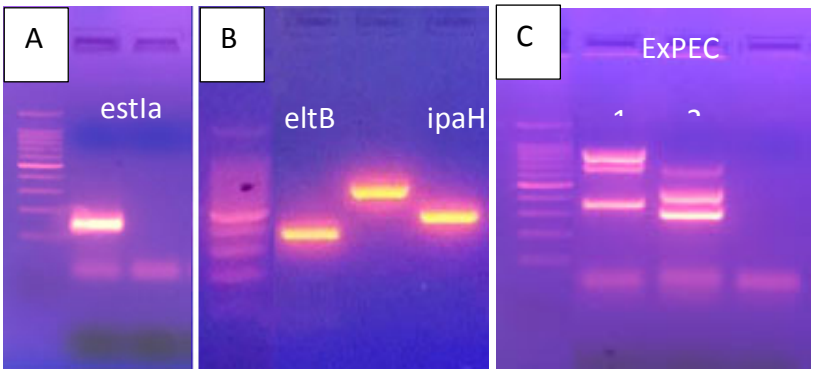


Figure 3.2. Agarose-gel electrophoresis of PCR products. **A**, amplification of estIa (157 bp). **B**, Amplification of eltB, ipaH and aggR (336 bp,611 bp and 425 bp, respectively) and. **C**, Amplification lane 1: from the top to the bottom hlyD, papA and focG (901 bp, 717 bp and 366 bp, respectively); amplification line 2: from the top to the bottom papA, kpsMII and iutA (717 bp, 452 bp and 314 bp, respectively).

The results obtained after conventional PCR performed on the DNA preparation from the selected colonies are reported in **Table 3.2.**

Table 3.3. Pathotype identification of the selected col onies through Conventional PCR analysis of virulence genes. The ID indicated by M are bulk-tank milk samples, while ID indicated by F are milking machine filter samples. The positive samples for the pathotype gene are indicated with a "+" symbol. Each sample could have from 1 to 10 colonies analyzed depending on EC 3M ™ Petrifilm ™ results.

Colonies		Virulence genes											Patothype
ID	Number	aggR	ipaH	eltB	estA	focG	papA	afa	iutA	kpsMII	sfaS	hlyD	
M3	1	-	-	-	-	-	-	-	-	-	-	-	
	2	-	-	-	-	-	-	-	-	-	-	-	
M6	3	-	-	-	-	-	-	-	-	-	-	-	
	4	-	-	-	-	-	-	-	-	-	-	-	
	5	-	-	-	-	-	-	-	-	-	-	-	
	6	-	-	-	-	-	-	-	-	-	-	-	
M7	7	-	-	-	-	-	-	-	-	-	-	-	
M8	8	-	-	-	-	-	-	-	-	-	-	-	
	9	-	-	-	-	-	-	-	-	-	-	-	
	10	-	-	+	-	-	-	-	-	-	-	-	ETEC
	11	-	-	-	-	-	-	-	-	-	-	-	
	12	-	-	-	-	-	-	-	-	-	-	-	
M9	13	-	-	-	-	-	-	-	-	-	-	-	
	14	-	-	-	-	-	-	-	-	-	-	-	
M12	15	-	-	-	-	-	+	-	-	-	-	-	
	16	-	-	+	-	+	-	-	-	-	-	-	ETEC
M24	17	-	-	-	-	-	-	-	-	-	-	-	
	18				-	+	-	-	-	-	-	-	
	19	-	-	-	-	-	-	-	-	-	-	-	
	20	-	-	+	-	-	-	-	-	-	-	-	ETEC
	21	-	-	-	-	-	-	-	-	-	-	-	
	22	-	-	-	-	-	-	-	-	-	-	-	
M28	23	-	-	-	-	-	-	+	-	-	-	-	
	24	-	-	-	-	-	-	+	-	-	-	-	
M29	25	-	-	-	-	-	-	-	-	-	-	-	
M31	26	-	-	-	-	-	-	-	-	-	-	-	
	27	-	-	+	-	-	-	-	-	-	-	-	ETEC
	28	-	-	-	-	-	-	-	-	-	-	-	
M33	29	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
	30	-	-	-	-	-	-	-	-	-	-	+	

	31	-	-	-	-	-	-	-	-	-	-	-	
	32	-	+	-	-	-	-	-	-	-	-	+	EIEC
	33	-	-	-	-	-	-	-	-	-	-	+	
	34	-	-	+	-	-	-	-	-	-	-	+	ETEC
	35	-	-	+	-	-	-	-	-	-	-	+	ETEC
	36	-	+	+	-	-	-	-	-	-	-	+	ETEC/EIEC
	37	-	-	-	-	-	-	-	-	-	-	+	
	38	-	-	-	-	-	-	-	-	-	-	-	
M34	39	-	-	-	-	-	-	-	-	-	-	-	
	40	-	-	-	-	-	-	-	-	-	-	-	
	41	-	-	+	-	-	-	-	-	-	-	-	ETEC
	42	-	-	+	-	-	-	-	-	-	-	-	ETEC
	43	-	-	-	-	-	-	-	-	-	-	-	
	44	-	-	-	-	-	-	-	+	-	-	-	
	45	-	-	+	-	-	-	-	-	-	-	-	ETEC
	46	-	-	-	-	-	-	-	-	-	-	-	
	47	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
	48	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
M35	49	-	-	-	-	-	-	-	-	-	-	-	
	50	+	-	+	-	-	-	-	-	-	-	-	ETEC/EAEC
M38	51	-	-	+	-	-	-	-	-	-	-	-	ETEC
	52	-	+	-	-	-	-	-	-	-	-	-	EIEC
	53	-	-	+	-	-	-	-	-	-	-	-	ETEC
	54	-	-	+	-	-	-	-	-	-	-	-	ETEC
	55	-	-	-	-	-	-	-	-	-	-	-	
	56	-	-	+	-	-	-	-	-	-	-	-	ETEC
M39	57	-	-	-	-	-	-	-	-	-	-	-	
	58	-	-	+	-	-	-	-	-	-	-	-	ETEC
M42	59	-	-	+	-	-	-	-	-	-	-	-	ETEC
	60	+	-	+	-	-	-	-	-	-	-	-	ETEC/EAEC
	61	-	-	-	-	-	-	-	-	-	-	-	
	62	-	-	-	-	-	+	-	+	-	-	-	
	63	-	-	-	-	-	-	-	-	-	-	-	
	64	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
M44	65	-	-	-	-	-	-	-	-	-	-	-	
F1	66	-	-	+	-	-	-	-	-	-	-	-	ETEC

	67	-	-	-	-	-	-	-	-	-	-	-	
	68	-	-	-	-	-	-	-	-	-	-	-	
F5	69	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
	70				-	-	-	-	-	-	-	-	
F6	71	-	-	-	-	-	-	-	-	-	-	-	
	72	-	-	+	-	-	-	-	-	-	-	-	ETEC
F7	73	-	-	+	-	-	-	-	-	-	-	-	ETEC
	74	-	-	+	-	-	-	-	-	-	-	-	ETEC
	75	-	-	+	-	-	-	-	-	-	-	-	ETEC
	76	-	-	-	-	-	-	-	-	-	-	-	
	77	-	-	-	-	-	-	-	-	-	-	-	
	78	-	-	+	-	-	-	-	-	-	-	-	ETEC
	79	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
F9	80	-	+	-	-	-	-	-	-	-	-	-	EIEC
	81	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
	82	-	+	-	-	-	-	-	-	-	-	-	EIEC
	83	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
F10	84	-	-	-	-	-	-	-	-	-	-	-	
	85	-	-	-	-	-	-	-	-	-	-	-	
F12	86	-	-	-	-	-	-	-	-	-	-	-	
F13	87	-	-	+	-	-	-	-	-	-	-	-	ETEC
	88	-	-	+	-	-	-	-	-	-	-	-	ETEC
	89	-	-	-	-	-	-	-	-	-	-	-	
	90	-	-	-	-	-	-	-	-	-	-	-	
	91	-	-	+	-	-	-	-	-	-	-	-	ETEC
	92	-	-	-	-	-	-	-	-	-	-	-	
F18	93	-	-	-	-	-	-	-	-	-	-	-	
	94	-	-	+	-	-	-	-	-	-	-	-	ETEC
	95	-	+	+	-	-	-	-	+	-	-	-	ETEC/EIEC
	96	-	-	-	-	-	-	-	+	-	-	-	
	97	-	-	-	-	-	-	-	+	-	-	-	
	98	-	-	-	-	-	-	-	+	-	-	-	
	99	-	-	-	-	-	-	-	+	-	-	-	
	100	-	-	-	-	-	-	-	+	-	-	-	
	101	+	-	-	-	-	-	-	-	-	-	-	EAEC
	102	-	-	-	-	-	-	-	-	-	-	-	

F24	103	-	-	-	-	-	-	-	-	-	-	-	
	104	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
	105	-	-	+	-	-	-	-	-	-	-	-	ETEC
F30	106	-	-	-	-	-	-	-	-	-	-	-	
F33	107	-	-	-	-	-	-	-	-	-	-	-	
	108	-	-	-	-	-	-	-	-	-	-	-	
	109	-	-	-	-	+	-	-	-	-	+	-	
	110	-	-	-	-	-	-	-	-	-	-	-	
	111	-	-	-	-	-	-	-	-	-	-	-	
	112	-	-	-	-	-	-	-	-	-	-	-	
	113	-	-	-	-	-	-	-	-	-	-	-	
F38	114	-	-	-	-	-	-	-	-	-	-	-	
	115	-	-	-	-	-	-	-	-	-	-	-	
	116	-	-	-	-	-	-	-	-	-	-	-	
	117	-	-	-	-	-	-	-	-	-	-	-	
	118	-	-	-	-	-	-	-	-	-	-	-	
	119	-	-	-	-	-	-	-	-	-	-	-	
	120	-	-	-	-	-	-	-	-	-	-	-	
	121	-	-	+	-	-	-	-	-	-	-	-	ETEC
	122	-	-	+	-	-	-	-	-	-	-	-	ETEC
	123	-	-	-	-	-	-	-	-	-	-	-	
	124	+	-	-	-	-	-	-	-	+	-	-	EAEC
F40	125	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
	126	-	-	-	-	-	-	-	-	-	-	-	
	127	-	-	-	-	-	-	-	-	-	-	-	
F42	128	-	+	-	-	-	+	-	+	-	-	-	EIEC
F45	129	-	-	-	-	-	-	-	-	-	-	-	
	130	-	+	-	-	-	-	-	-	-	-	-	EIEC
	131	-	-	-	-	-	-	-	-	-	-	-	
	132	-	-	-	-	-	-	-	-	-	-	-	
	133	-	-	-	-	-	-	-	-	-	-	-	
	134	-	-	-	-	-	-	-	-	-	-	-	
	135	-	-	+	-	-	-	-	-	-	-	-	ETEC
	136	-	-	-	-	-	-	-	-	-	-	-	
F46B	137	-	-	-	-	-	-	-	-	-	+	-	
	138	-	-	-	-	-	-	-	-	-	-	-	

	139	-	-	-	-	-	-	-	-	-	-	-	
	140	-	-	-	-	-	-	-	-	-	-	-	
	141	-	-	-	-	-	-	-	-	-	-	-	
	142	-	-	-	-	-	-	-	-	-	-	-	
	143	-	-	-	-	-	-	-	-	-	-	-	
	144	-	-	-	-	-	-	-	-	-	-	-	
	145	-	-	-	-	-	-	-	-	-	-	-	
	146	-	-	+	-	-	-	-	-	-	-	-	ETEC
	147	-	-	+	-	-	-	-	-	-	-	-	ETEC
F66	148	-	-	-	-	-	-	-	-	-	-	-	
F67	149	-	-	-	-	-	-	-	-	-	-	-	

ExPEC was not detected in these colonies. The comparison of the data obtained from the same milking samples after analysis of the bulk-tank milk and the corresponding filter underlines the different *E. coli* detection power in almost the samples (**Table 3.3**).In **Table 3.4**, the pathogenic strains detected in the two different matrices are summarized.

Table 3.4 Comparison of PCR results of bulk-tank milk and milking machine filters in the same samples.

ID	Bulk-tank milk	Milking machine filters
1		ETEC
5		ETEC/EIEC
6		ETEC
7		ETEC
8	ETEC	ETEC/EIEC
9		ETEC/EIEC
13		ETEC
18		ETEC/EIEC/EAEC
21	ETEC	
24	ETEC	ETEC/EIEC
31	ETEC	
33	ETEC/EIEC	
34	ETEC/EIEC	
35	ETEC/EAEC	
38	ETEC/EIEC	ETEC/EAEC
39	ETEC	
40		ETEC/EIEC
42	ETEC/EIEC/EAEC	EIEC
45		ETEC/EIEC
46		ETEC

Table 3.5. Overview of the pathotypes identified through conventional PCR on E. coli-positive samples.

E. coli pathotype	Bulk-tank milk	Milking machine filters
ETEC	10	13
EIEC	4	6
EAEC	2	2

3.5 Discussion

The transmission of several pathogens (bacteria, fungi and viruses) to humans can occur through food in most, if not all, cases.. Thus, food safety is a global issue, and furthermore an increase in import and export of food products could lead to introduction and establishment of new diseases in geographical areas that have never experienced such foodborne pathogens (Yang et al. 2017).

Milk can be a good source of foodborne pathogens since it carries a variety of microorganisms. Indeed, it is a direct contact with contaminated sources in the milk stall from the environment and infected animals.

Last year Ribeiro et al. (2016), published a study highlighting the correlation between the pathogenic *E. coli* in raw-milk cheese in Brazil and the risk to public health. The innovative parts of this project that is the detection of intestinal and extraintestinal *E. coli* in the bulk-tank milk and in filter milking machine.

These results underline the different detection pattern of pathogenic *E. coli* in the bulk-tank milk with respect to milking machine filter.

Indeed, when the molecular assay is targeted to the milking machine filters, more pathogens are detected when compared to the bulk-tank samples, with special regard to EIEC strains. The analysis of milk filters could be an useful tool to assess the presence of a risk of contamination by *E. coli* pathotypes both at food and environmental level.

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Appendix

During my PhD course, I also took part in the following two research projects:

1. **High-resolution melting analysis of gyrA codon 84 and grlA codon 80 mutations conferring resistance to fluoroquinolones in Staphylococcus pseudintermedius isolates from canine clinical samples**

Staphylococcus pseudintermedius is an opportunistic pathogen of dogs and cats. A high-resolution melting analysis (HRMA) protocol was designed and tested on 42 clinical isolates with known fluoroquinolone (FQ) susceptibility and gyrA codon 84 and grlA codon 80 mutation status. The HRMA approach was able to discriminate between FQ-sensitive and FQ-resistant strains and confirmed previous reports that the main mutation site associated with FQ resistance in S. pseudintermedius is located at position 251 (Ser84Leu) of gyrA. Routine, HRMA-based FQ susceptibility profiles may be a valuable tool to guide therapy. The FQ resistance-predictive power of the assay should be tested in a significantly larger number of isolates.

The results were published in the following paper:

Loiacono, M., Martino, P. A., Albonico, F., **Dell'Orco, F**., Ferretti, M., Zanzani, S., & Mortarino, M. (2017). High-resolution melting analysis of gyrA codon 84 and grlA codon 80 mutations conferring resistance to fluoroquinolones in Staphylococcus pseudintermedius isolates from canine clinical samples. *Journal of Veterinary Diagnostic Investigation*, *29*(5), 711–715. https://doi.org/10.1177/1040638717712330.

2. **18S rRNA V12 and V9 metabarcoding analysis of night and day diet of *Sardina pilchardus* from the Bay of Biscay.**

Metabarcoding approach is highly sensitive and able to detect traces of DNA in community samples, that methodology due to the combination of the highest taxonomic resolution in a cost- and time-effective. The 18S V12 and V9 metabarcoding was used to characterize the day and night diet of European sardine (*Sardina pilchardus*) from the Bay of Biscay. The data set obtained is able to discriminate the different alimentation of the fish.

The results are being prepared for publication as follows.

Dell'Orco F., Albaina A., Aguirre M., Abad D., Zarraonaindia I., Estonba A. 18S rRNA V12 and V9 metabarcoding analysis of night and day diet of *Sardina pilchardus* from the Bay of Biscay. Manuscript in preparation.

Attendance to courses, seminars, workshops and scientific meetings (AA 2015-17):

- Visiting period: From June 25th to July 8st 2017 to Bieneinstitut Kirchhain, in Kirchhain (Germany) under the surveillance of Dr. Ralph Büchler. The research activities were focused to learn and apply apical breeding techniques to be used in the genetic selection of *Varroa* resistance. In particular, training will focus on the detection of phenotypes such as production, resistance to disease and docility.
- Visiting period: From October 11th 2016 to January 31th 2017 to University of Basque Country/ Euskal Herriko Unibertsitatea (UPV / EHU), in Bilbao (Spain) under the surveillance of Prof. And one Estonba. The research activities were to learn and to apply genetic techniques, in the field of metabarcoding approach.
- Obligatory course: "Communication 3", held by Dott. Ettore Galanti, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: January 21 2016, days in February 8 and 22 in total 12 hours
- Obligatory course: "Statistica 3", held by Dott.ssa Elisabetta Sala, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: from November 21 2016 to Dicember 15 2016, in total 12 hours;
- Scientific Meeting: "CRISPR genome editing: From high-throughput screening to disease models", to Lundsgaard auditorium, Panum Institutet, Copenhagen (Denmark), September 22-23 2016, in total 11 hours;
- Scientific Meeting: "Seventh EurBee Congress of Apidology", to university of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania, September 7-9 2016, in total 24 hours;
- Workshop: "Le pesti delle api: attualità e prospettive", to faculty of Veterinary Medicine, UNIMI, February 26 2016 for 8 hours;
- Obligatory course: "Inglese 2", held by Dott.ssa Gigliola Canepa with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: from January 29 2016 to April 15 2016, in total 40 hours;
- Obligatory course: "Statistica 2", held by Dott.ssa Elisabetta Sala, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: from February 1 2016 to April 04 2016, in total 24 hours;
- Obligatory course: "Communication 2", held by Dott. Ettore Galanti, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: days in march 2-9-23 2016, in total 16 hours;
- Ongoing evaluation, January 18 2016, time 9:30. Lesson class 12, Faculty of Medicine Veterinary, UNIMI, 5 hours;

- Ongoing evaluation: Vas Day, days in June 8– 10 2016, in total 18 ore.
- Workshop: "Emergenza da *Aethina tumida* in apicoltura: conoscenze attuali e prospettive per il coontrollo", to Università degli studi di Milano, faculty of Veterinary Medicine, october 26 2015 for 8 hours;
- Theoric course:" Corso di allevamento di api regine", by Elio Bonfanti in Lecco. Period of lessons: Days 20 and 21 february 2015, 8 hours a day, 16 hours in total;
- Course of "VSH- varroa-sensitive hygiene", Aspromiele Beekeepers Association, Alessandria. March 06 2015, for 8 hours;
- Course: "Next Generation Sequencing procedures", at Insubria Università of Busto Arsizio (Va). Period of lessons: Days June 15-16-17 2015, 8 hours a day, 24 hours in total;
- Course: "Genetic association studies in field of animal infectious disease, held by Dott.ssa Giulietta Minozzi, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: in June 17-18-23-24 201), in total 16 hours;
- Course: "Imaging", held by Dott.ssa Valentina Lodde, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: days in September 14- 15-17-18-21 2015, in total 16 ore;
- Obligatory course: "Statistica 1", held by Dott.ssa Elisabetta Sala, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons from November 24 2014 to February 09 2015, in total 32 hours;
- Obligatory course: "Banche dati" held by Dott.ssa Angela Moccia, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: days in June 04 - 11 2015, every day 4 hours;
- Obligatory course: "Inglese 1", held by Dott.ssa Gigliola Canepa with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: days January 26 to 18 2015, in total 40 hours;
- Obligatory course: "Comunicazione e Public Speaking", held by Dott. Ettore Galanti, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: days in march 4-11-25 2015, in total 16 hours;
- Ongoing evaluation, January 22 2015, time 9:30. Lesson class 10, Faculty of Medicine Veterinary, UNIMI, 5 hours;
- Ongoing evaluation: Vas Day, days in July 15 – 16 – 17 2015, in total 18 hours.

Publications:

Dell'Orco F., Formato G. Mortarino M. Diagnostic tools for honeybee nosemosis: an update. Submitted manuscript, Veterinaria Italiana

Dell'Orco F., Loiacono M., Albonico A., Zanzani S., Cersini A., Formato G., Colombo M., Mortarino M.. Detection and quantitation of Nosema ceranae in honey bees by real time PCR coupled to High Resolution Melting Analysis**.** Submitted manuscript**,** Parasitology Research.

Dell'Orco F., Facchini E., Rizzi R., Mortarino M. The expression ratio between Act5 and Obp-4 is a marker of hygienic behavior in honeybees. Manuscript to be submitted.

Dell'Orco F., Albaina A., Aguirre M., Abad D., Zarraonaindia I., Estonba A. 18S rRNA V12 and V9 metabarcoding analysis of night and day diet of *Sardina pilchardus* from the Bay of Biscay. Manuscript in preparation.

Mortarino M., Blonda M., Zanzani S., **Dell'Orco F**., Facchini E., Rizzi R. Neem (Azadirachta indica) oil as a sustainable tool against Varroa destructor. Proceedings of the 26th Conference of the Word Association for the Advancement of Veterinary Parasitology. Kuala Lumpur, 4th-8th September 2017.

Loiacono M., Martino P. A., Albonico F., **Dell'Orco F**., Ferretti M., Zanzani S., Mortarino M.. 2016. High-resolution melting analysis of gyrA codon 84 and grlA codon 80 mutations conferring resistance to fluoroquinolones in Staphylococcus pseudintermedius isolates from canine clinical samples. Journal of Veterinary Diagnostic Investigation, 29(5), 711–715. https://doi.org/10.1177/1040638717712330.

Dell'Orco F., Loiacono M., Albonico F., Zanzani S., Cersini A., Formato G., Facchini E., Colombo M., Mortarino M.. 2016. Real time PCR coupled to High Resolution Melting Analysis for detection and quantitation of Nosema ceranae in honey bees. Poster communication, the 7th European Conference of Apidology, page218, 7-9 September 2016.

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Congress of the Italian Society of Parasitology, page 210, 21-24.

Formenti N., Albonico F.**,** **Dell'Orco F.**, Loiacono M., Trogu T., Pombi M., Cavallero S., Ferrari N., Mortarino M., Lanfranchi P.. 2016. Multiplex PCR assay for the identification of Haemonchus contortus and Teladorsagia circumcincta in alpine chamois (Rupicapra r. rupicapra). Oral communication, XXIX Congress of the Italian Society of Parasitology, page 109, 21-24.

Dell'Orco F., Facchini E., Cilia G. Rizzi R., Mortarino M.. 2016. Candidate molecular markers of hygienic behaviour in honeybees (Apis mellifera): an expression study. International Journal of Health, Animal Science & Food Safety, Vol. 2s, Proceedings of the Veterinary and Animal Science Days.

Dell'Orco F., Loiacono M., Albonico, F., Minozzi G., Pagnacco, G., Mortarino M.. (2015). MicroRNA expression correlated with hygienic behaviour in honeybees. International Journal of Health, Animal Science & Food Safety, Vol. 2s, Proceedings of the Veterinary and Animal Science Days.

1.2.4 Gene expression analysis: ASS2016 and ASS2017 vs HB score

The Ct values obtained for each target genes after qRT-PCR, were used to calculate the ΔCt of each gene pair. For each sample, the ΔCt is the difference between the Ct of one target gene and the Ct of another target gene, as reported in **Table 1.6** for ASS2016 and **Table 1.7** for ASS2017.

Table 1.6: ΔCt values of ASS2016. The colonies with Higher HB score are typed in bold.

ASS2016	ΔCt(Obp4-Act5C)	ΔCt(Obp4-Mblk-1)	ΔCt(Obp4-Rpl32)	ΔCt(Act5C-Mblk1)	ΔCt(Act5C-Rpl32)	ΔCt(Mblk1-Rpl32)
14	**5.06**	**0.52**	**3.65**	**-4.54**	**-1.41**	**3.13**
LS10	**3.42**	**-0.68**	**1.03**	**-4.1**	**-2.39**	**1.71**
13	**4.27**	**-0.07**	**3.61**	**-4.34**	**-0.66**	**3.68**
LS12	**4.55**	**0.57**	**6.32**	**-3.98**	**1.77**	**5.75**
LS11	**3.66**	**-1.25**	**4.65**	**-4.91**	**0.99**	**5.9**
LS8	1.54	-0.68	4.06	-2.22	2.52	4.74
LS13	1.53	-1.77	4.42	-3.3	2.89	6.19
11	1.39	-1.22	3.12	-2.61	1.73	4.34
12	2.56	-1.44	5.92	-4	3.36	7.36
10	0.8	-2.68	5.5	-3.48	4.7	8.18
9	2.3	-0.81	2.57	-3.11	0.27	3.38
8	2.23	-0.68	4.17	-2.91	1.94	4.85
7	0.74	-3.02	5.15	-3.76	4.41	8.17

Table 1.7: ΔCt values of ASS samples of 2017. The colonies with Higher HB score are typed in bold.

ASS 2017	ΔCt (Act5C-Mblk1)	ΔCt (Act5C-Rpl32)	ΔCt (Act5C-Obp3)	ΔCt (Act5C-Obp18)	ΔCt (Act5C-Obp16)	ΔCt (Mblk1-Rpl32)	ΔCt (Mblk1-Obp3)	ΔCt (Mblk1-Obp4)	ΔCt (Mblk1-Obp16)	ΔCt (Mblk1-Obp18)	ΔCt (Rpl32-Obp3)	ΔCt (Rpl32-Obp4)	ΔCt (Rpl32-Obp16)	ΔCt (Rpl32-Obp18)	ΔCt (Obp3-Obp4)	ΔCt (Obp3-Obp16)	ΔCt (Obp4-Act5C)	ΔCt (Obp3-Obp18)	ΔCt (Obp4-Obp16)	ΔCt (Obp4-Obp18)	ΔCt (Obp16-Obp18)
8B	**-5.01**	**1.09**	**-2.77**	**-0.54**	**-0.16**	**6.1**	**2.24**	**3.97**	**4.85**	**4.47**	**-3.86**	**-2.13**	**-1.25**	**-1.63**	**1.73**	**2.61**	**1.04**	**2.23**	**0.88**	**0.5**	**-0.38**
3B	**-4.9**	**1.89**	**-1.86**	**-0.88**	**-1.11**	**6.79**	**3.04**	**4.56**	**3.79**	**4.02**	**-3.75**	**-2.23**	**-3**	**-2.77**	**1.52**	**0.75**	**0.34**	**0.98**	**-0.77**	**-0.54**	**0.23**
6B	**-5.95**	**1.84**	**-2.42**	**-1.06**	**-1.43**	**7.79**	**3.53**	**4.55**	**4.52**	**4.89**	**-4.26**	**-3.24**	**-3.27**	**-2.9**	**1.02**	**0.99**	**1.40**	**1.36**	**-0.03**	**0.34**	**0.37**
5B	**-4.83**	**2.56**	**-1.74**	**-1.26**	**-1.62**	**7.39**	**3.09**	**4.01**	**3.21**	**3.57**	**-4.3**	**-3.38**	**-4.18**	**-3.82**	**0.92**	**0.12**	**0.82**	**0.48**	**-0.8**	**-0.44**	**0.36**
11B	**-2.11**	**1.26**	**-0.06**	**-0.31**	**-1.66**	**3.37**	**2.05**	**2.32**	**0.45**	**1.8**	**-1.32**	**-1.05**	**-2.92**	**-1.57**	**0.27**	**-1.6**	**-0.21**	**-0.25**	**-1.87**	**-0.52**	**1.35**
1B	-4.52	2.75	-2.64	-1.61	-1.92	7.27	1.88	4.11	2.6	2.91	-5.39	-3.16	-4.67	-4.36	2.23	0.72	0.41	1.03	-1.51	-1.2	0.31
2B	-3.68	2.9	-0.76	-0.06	-0.68	6.58	2.92	4.11	3	3.62	-3.66	-2.47	-3.58	-2.96	1.19	0.08	-0.43	0.7	-1.11	-0.49	0.62
9B	-4.44	2.19	-1.15	-1.72	-0.89	6.63	3.29	5.51	3.55	2.72	-3.34	-1.12	-3.08	-3.91	2.22	0.26	-1.07	-0.57	-1.96	-2.79	-0.83
13B	-7.44	2.06	-4.59	0.77	-3.38	9.5	2.85	6.93	4.06	8.21	-6.65	-2.57	-5.44	-1.29	4.08	1.21	0.51	5.36	-2.87	1.28	4.15
10B	-6.21	2.24	-2.05	0.03	-0.26	8.45	4.16	6.59	5.95	6.24	-4.29	-1.86	-2.5	-2.21	2.43	1.79	-0.38	2.08	-0.64	-0.35	0.29
7B	-5.31	2.4	-1.26	-1.89	-1.79	7.71	4.05	5.75	3.52	3.42	-3.66	-1.96	-4.19	-4.29	1.7	-0.53	-0.44	-0.63	-2.23	-2.33	-0.1
14B	-3.77	1.02	-0.81	0.11	-1.11	4.79	2.96	3.15	2.66	3.88	-1.83	-1.64	-2.13	-0.91	0.19	-0.3	0.62	0.92	-0.49	0.73	1.22
4B	-5.52	1.9	-2.34	-0.58	-0.76	7.42	3.18	4.61	4.76	4.94	-4.24	-2.81	-2.66	-2.48	1.43	1.58	0.91	1.76	0.15	0.33	0.18

In particular, the analysis of ASS2016 highlights the correlation of ΔCt between Obp4 and Act5C with respect to HB scores (R^2 = 0.74) and p value < 0.05 (**Figure 1.6**).

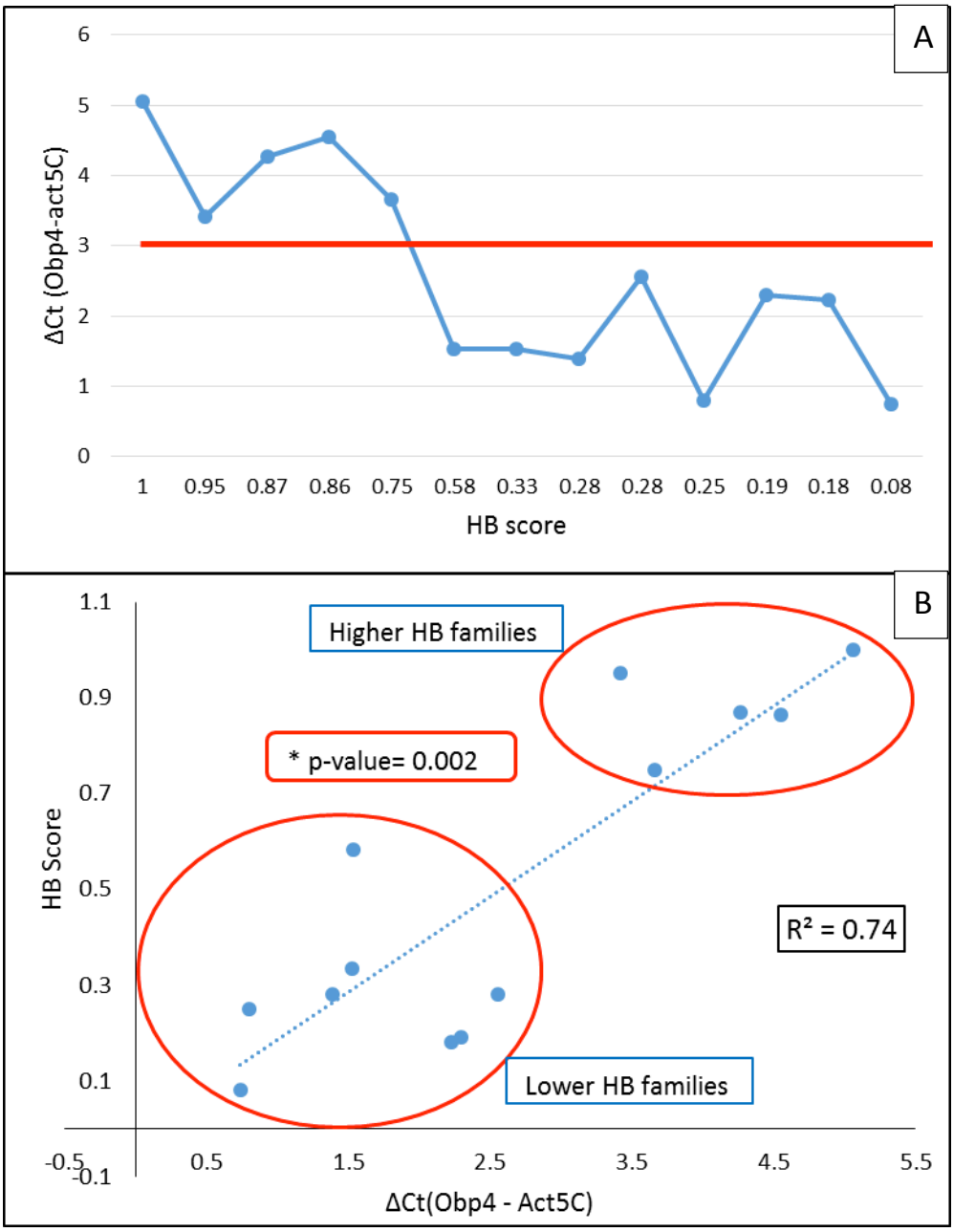

Figure 1.6: Analysis of ΔCt(Obp4-Act5C) versus HB score in ASS2016. Panel A: descriptive graph. Panel B: correlation analysis.

The ΔCt(Obp4-Act5C) of ASS2017 was the only expression parameter whose difference was nearly significant after statistical analysis (p-value= 0,06).

Then, the HB score and ΔCt(Obp4-Act5C) data obtained from ASS2016 and ASS2017 were

combined into a single data set (**Table 1.8**). The threshold for Higher HB score was then set over/equal to 0,85.

Table 1.8: Combined ASS2016 and ASS2017 data set. The colonies with Higher HB score are typed in bold.

Samples ID	HB score	ΔCt(Obp4-Act5C)
14	**1**	**5.06**
8B	**0.98**	**1.04**
LS10	**0.95**	**3.42**
3B	**0.90**	**0.34**
13	**0.87**	**4.27**
LS12	**0.86**	**4.55**
6B	**0.86**	**1.4**
5B	**0.85**	**0.82**
11B	0.84	-0.21
1B	0.79	0.41
2B	0.77	-0.43
LS11	0.75	3.66
9B	0.71	-1.07
13B	0.69	0.51
10B	0.64	-0.38
7B	0.63	-0.44
4B	0.63	0.91
LS8	0.58	1.54
LS13	0.33	1.53
14B	0.33	0.62
11	0.28	1.39
12	0.28	2.56
10	0.25	0.8
9	0.19	2.3
8	0.18	2.23
7	0.08	0.74

The combined ASS2016 and ASS2017 dataset was analyzed using the median test. The result showed that the ΔCt(Obp4-Act5C) median value of the colonies with the Higher-HB score was indeed higher (2,41) compared to the colonies with the Lower HB score (0.77) and to the whole set of colonies (0,97) (**Figure 1.7**). The statistical analysis was performed using Mann-Whitney U test, that confirmed the statistically significant difference (p value = 0.04) between the two sets of colonies with Higher and Lower HB score as for ΔCt(Obp4-Act5C).

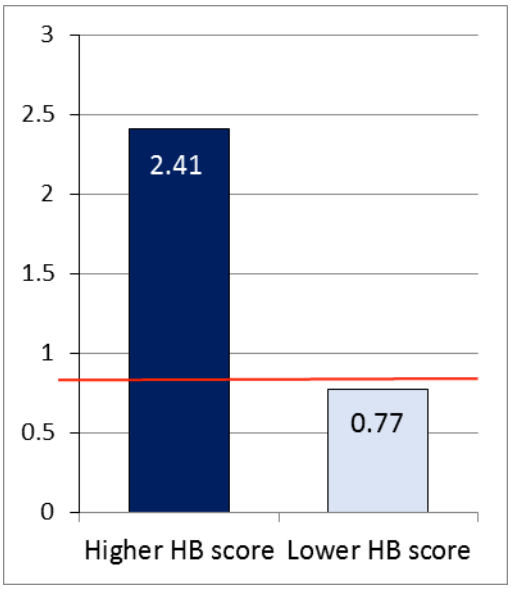

Figure 1 Errore. Lo stile non è definito.**7:** Median values of ΔCt(Obp4-Act5C) for Higher HB and Lower HB score colonies.

1.2.5Comparison of ASS2016 and MSS2016

1.2.5.1 Gene expression analysis of MSS2016 vs ASS2016

As already detailed, the Ct values obtained for each target genes after qRT-PCR, were used to calculate the ΔCt of each gene pair. For each sample, the ΔCt is the difference between the Ct of one target gene and the Ct of another target gene, as reported in **Table 1.9**

Table 1.9: ΔCt values calculated for MSS2016. The colony with higher HB score is typed in bold.

MSS2016	ΔCt(Obp4-Act5C)	ΔCt(Obp4-Mblk-1)	ΔCt(Obp4-Rpl32)	ΔCt(Act5c-Mblk1)	ΔCt(Act5c-Rpl32)	ΔCt(Mblk1-Rpl32)
25	**2.57**	**2.81**	**6.17**	**0.24**	**3.6**	**3.36**
26	0.82	-0.36	4.66	-1.18	3.84	5.02
24	3	4.1	7.59	1.1	4.59	3.49
22	0.59	0.12	3.88	-0.47	3.29	3.76
17	0.76	1.5	6.97	0.74	6.21	5.47
19	2.08	1.18	5.54	-0.9	3.46	4.36
27	1.89	-1.11	7.79	-3	5.9	8.9
20	-0.81	-0.12	5.23	0.69	6.04	5.35
18	0.78	0.45	4.65	-0.33	3.87	4.2
23	1.43	0.7	4.03	-0.73	2.6	3.33

The results of MSS2016 highlights the loss of correlation between ΔCt(Obp4-Act5C) and HB scores (**Figure 1.8**).

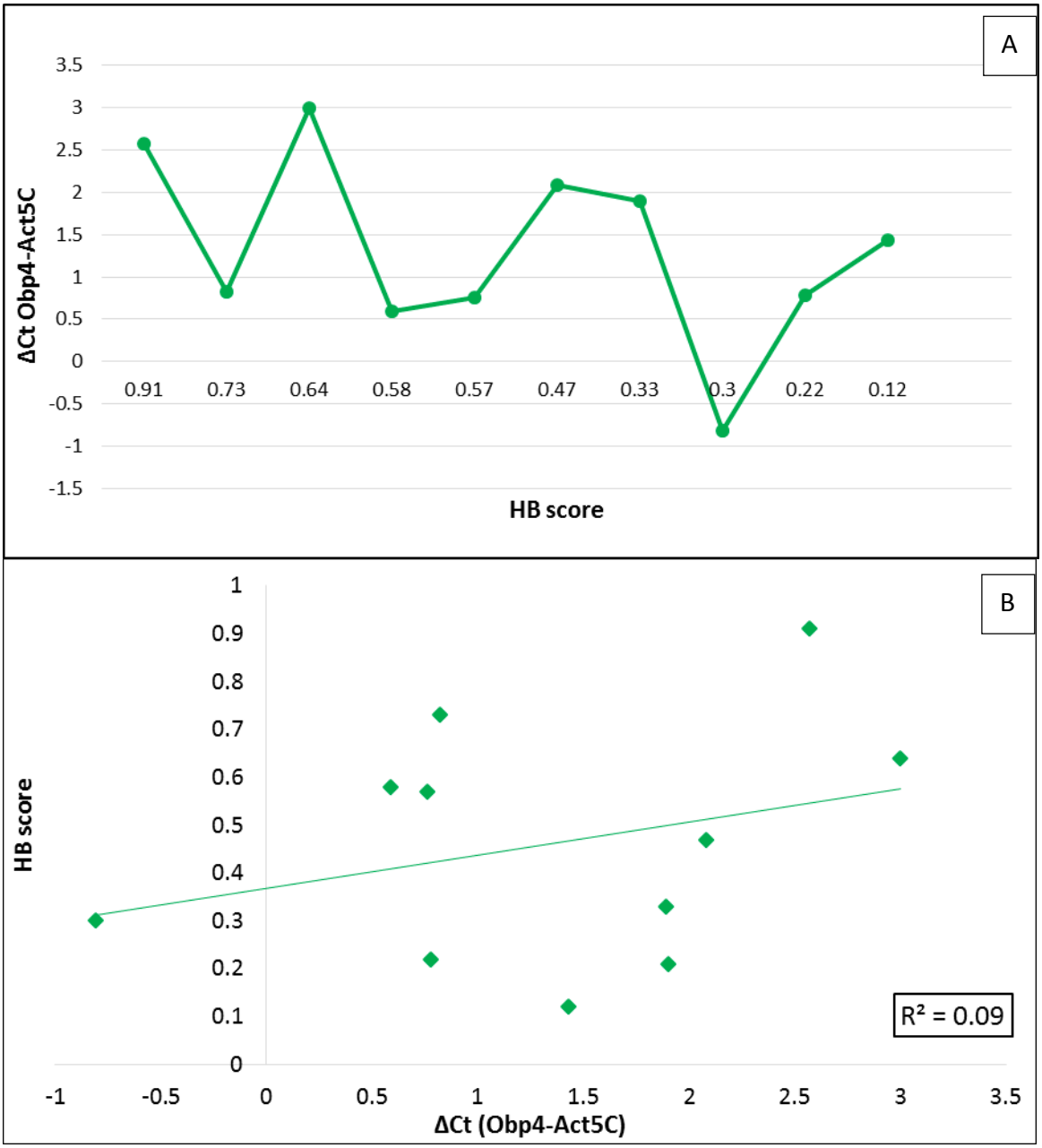

Figure 1.8: Analysis of ΔCt(Obp4-Act5C) versus HB score in MSS2016. Panel A: descriptive graph. Panel B: correlation analysis.

Then, the HB score and ΔCt(Obp4-Act5C) data obtained from ASS2016 and MSS2016 were combined into a single data set (**Table 1.10**). The threshold for Higher HB score was then set over/equal to 0,75. In this dataset, a low correlation was found between HB score and ΔCt(Obp4-Act5C) (**Figure 1.9)**.

Table 1.10: Combined ASS2016 and MSS2016 data set. The colonies with Higher HB score are typed in bold.

Samples ID	HB score	ΔCt(Obp4-Act5C)
14	**1**	**5.06**
LS10	**0.95**	**3.42**
25	**0.91**	**2.57**
13	**0.87**	**4.27**
LS12	**0.86**	**4.55**
LS11	**0.75**	**3.66**
26	0.73	0.82
24	0.64	3
LS8	0.58	1.54
22	0.58	0.59
17	0.57	0.76
19	0.47	2.08
LS13	0.33	1.53
27	0.33	1.89
20	0.3	-0.81
11	0.28	1.39
12	0.28	2.56
10	0.25	0.8
18	0.22	0.78
9	0.19	2.3
8	0.18	2.23
23	0.12	1.43
7	0.08	0.74

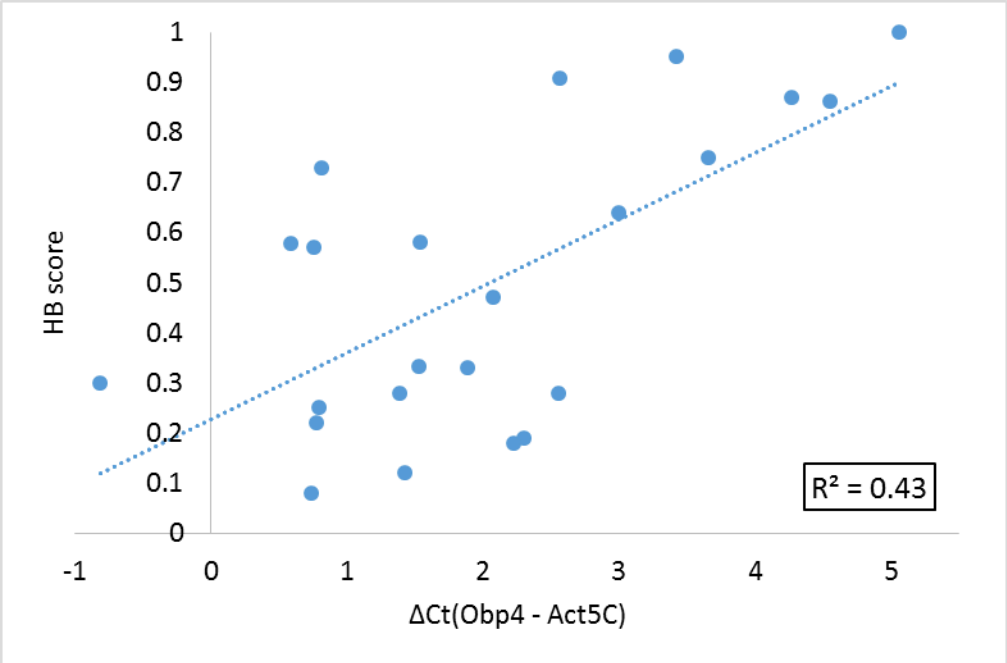

Figure 1.9: Correlation analysis between HB score and ΔCt(Obp4-Act5C) data from combined ASS2016 and MSS2016 dataset.

1.3 Conclusion

In the *Apis mellifera,* HB is a heritable phenotype that confers colony level resistance against brood diseases, including the damaging parasitic mite by *Varroa destructor*. Currently, the beekeepers measure the HB score in their honeybee colonies using methods like the pin-prick test or freeze-killed brood (FKB) assays, during their in-field tests. These methods are inherently imprecise, time consuming and resource intensive for the breeders, thus limiting their suitability for widespread application. The availability of molecular biomarkers for the hygienic phenotype could fasten the recognition of this trait and the breeding process. Currently, the genetic and biochemical elements driving the manifestation of this behavior are largely unknown. Once identified, such genes can be potentially used as biomarkers to aid identification and selection of colonies resistant to disease. More generally, gene expression analysis can provide a valid tool to identify coding genes that are known controlling behavioral processes.

The present work was characterized by a transversal approach: from in-field phenotypic characterization of HB, to expression profiling of selected cerebral coding (RNAm) genes, with the aim to assess differential expression of such genes between selected honeybee colonies with the Higher and the Lower HB, respectively. The gene targets of this work have been selected from previous literature evidences as a subset of honeybee nurse brain coding genes that may be involved in HB intensity: Act5C, a cytoskeletal gene involved in memory formation; Obp4, as a brain-specific olfactory gene and also expressed in antennae; Obp3, Obp16 and Obp18 as olfactory genes being expressed in a wide range of organs and tissues with different patterns; Mblk-1, as a transcription factor specifically expressed in mushroom body. Beyond, Rpl32 was targeted as a housekeeping gene with stable expression in honeybees as reported in previous studies.

Le Conte et al., (2010) reported the analysis of brain transcriptome of highly Varroa-hygienic bees and the identification of a set of genes involved in social immunity including some Obps. The function of these candidate genes did not seem to support higher olfactory sensitivity in hygienic bees. Thus the correlation between Obps and HB could be linked to other, still unknown functional mechanisms behind the binding of olfactory molecules. The results of this thesis highlight the prominent significance of Act5 and Obp4 among the analyzed candidate

markers as a distinguishable tools to infer the hygienic feature of a honeybee colony. In particular, the evaluation of a candidate gene panel in nurse brains allowed to clarify a significant relationship between Obp4 and Act5C vs HB. More in details, ΔCt of Obp4 and Act5C was a discriminating parameter for the colonies with higher HB score with respect to the colonies with lower HB. The results showed repeatability in two different sampling years on a total 26 colonies.

As reported in literature, HB is influenced by external environmental factors, as the abundance of nectar and other unknown factors, and it is not constant in time in each hive (Xonis et al., 2015). Noteworthy, when comparing years 2016 and 2017, the Obp4 and Act5C gene pair showed somewhat different expression levels. The variability of the relationship between Obp4 and HB score could be correlated to the different nectar flow in April 2017 compared to April 2016 in the apiary site (Lodi) and related honeybee colony functional activity (during April 2016, the nectar flow in Lodi was indeed markedly higher compared to April 2017). The different abundance of potential OBPs ligands (mainly, small hydrophobic volatile molecules of 10-20 carbon atoms, as reported by Zhou, 2010) could affect the expression of OBPs with functional involvement in metabolic pathways not linked to brood cleaning. In particular, this could be likely especially for Obp4, since its expression in brain can be associated with odorous stimuli attracting honeybees looking for pollen and nectar. Besides, a different balance between younger (nurses less than three weeks of age) and older honeybees is also appreciable during strong nectar flows, mainly due both to impaired egg deposition by the queen (due to scarcity of empty cells) and to an increased need for foragers. Consequently, HB should be considered a dynamic, rather than a static, phenotype being heavily affected by a variety of environmental stressors. The appreciable effect of seasonal conditions on the level of HB phenotype could be a reason for the inconstant correlation with OBP4 and/or Act5 that has been observed in the present thesis. Notwithstanding this, the overall results obtained from the pooled 2016-2017 sample set confirmed the suitability of ΔCt(Obp4-Act5C) as a marker to infer HB condition.

From the perspective point of view, the identification of colonies with high HB trait using molecular markers should be based on repeated measurements during the brood season. Evaluation of more honeybee colonies for large-scale in-field validation of marker-assisted HB-selection is also necessary. As a further objective for the future, technical improvements such

as the setup of a new duplex protocol allowing measurement of both candidate genes (Obp4 and AC5C) through a single analysis would be highly advisable.

The present study was reported as a manuscript to be submitted for publication:
(**Dell'Orco F**., Facchini E., Rizzi R., Mortarino M. The expression ratio between Act5 and Obp-4 is a marker of hygienic behavior in honeybees. Manuscript to be submitted)

Chapter 2: Detection and quantitation of *Nosema ceranae* in honey bees by Real Time PCR coupled to High Resolution Melting Analysis

2.1. Materials and methods

2.1.1. Overview of the experimental design

The experimental design involved several steps from the collection of the samples to biological analysis, as follows:

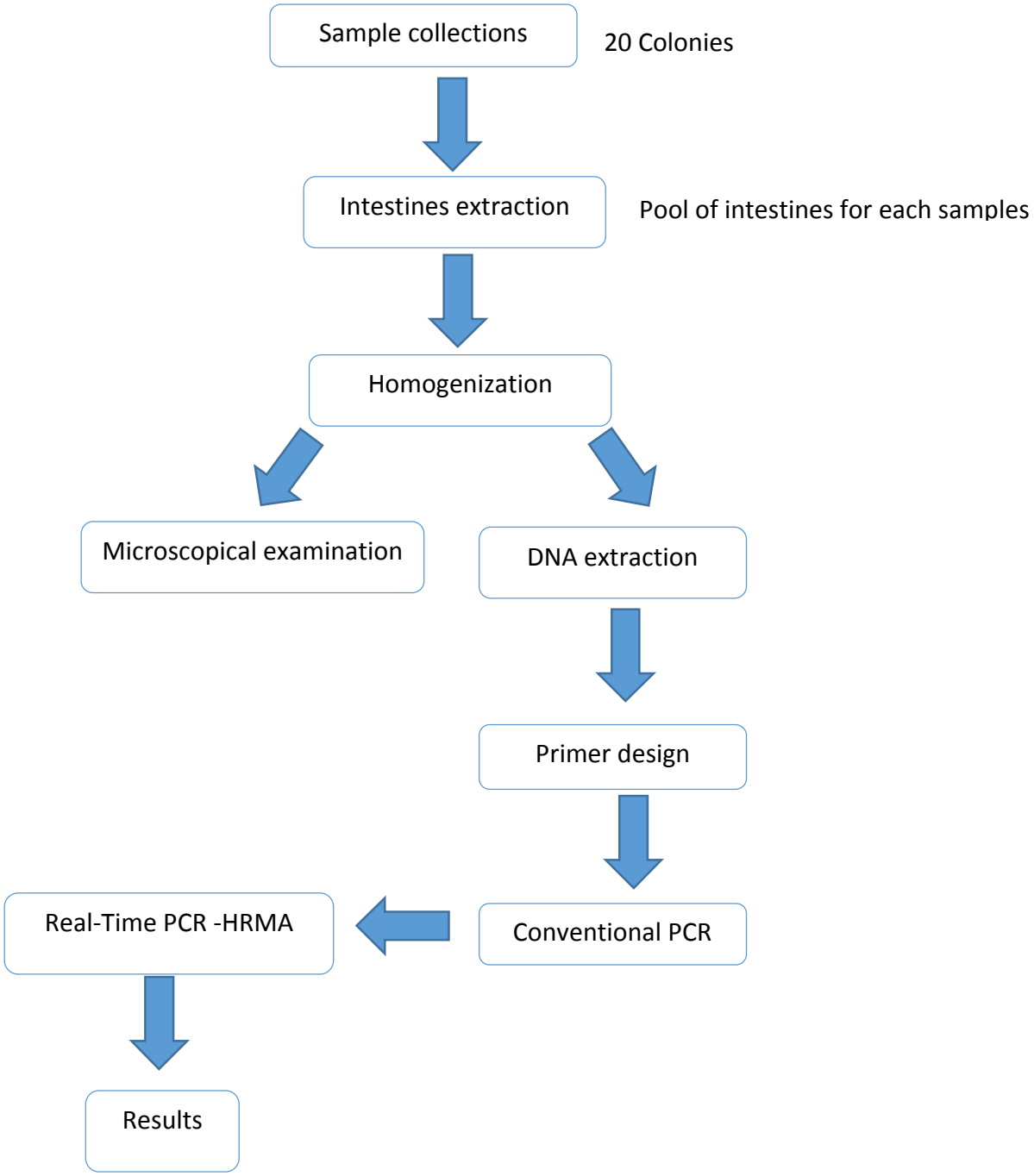

Figure 2.1: Experimental design of *Nosema* spp project.

2.1.2. Honey bee sample collection

For the setup of the qPCR-HRMA assay for *Nosema spp* (named as " HRMNosem"), 20 samples were collected in-field during summer months from honeybee colonies already known as infected by *Nosema spp*. From each colony, 25 foraging bees were collected at the hive entrance.

2.1.3. Honey bee sample processing and DNA extraction

Honeybee samples were processed for microscopical spore counting and genomic DNA (gDNA) extraction following a published protocol with some modifications (Bourgeois et al. 2010). Briefly, from each colony sample the intestines of the honey bee were collected and pooled. Then the intestine pool samples were omogenized in 5 ml of double-distilled water and filtered using a metal tea strainer. After two centrifugations at 800 g for 6 minutes, the pellets were resuspended in 1 ml of double-distilled water. At this step, the spore load for each sample was assessed using the method described in the OIE reference manual ((OIE) 2013). Then a 500 µl aliquot was sonicated for three cycles of 10 seconds each (output=2) using a Sonifier 250 instrumentation , at setting output=2 (Branson Ultrasonics, Danbury, CT, U.S.A). Then 1 ml of TNNT lysis buffer (0,5% SDS, 0.5% Tween 20, 0.5% Nonidet P-40, 10mM NaOH, 10mM Tris [pH 7.2]) and 8 µl of 20 mg/ml proteinase K were added to each sample. After overnight incubation at 56 °C, the proteinase K was heat-inactivated at 95 °C for 10 min. After this step, DNA was isolated using the standard phenol-chloroform-isoamyl alcohol extraction followed by ethanol precipitation (J. Sambrook, Fritsch, Maniatis, Molecular Cloning: A Laboratory Manual 2nd Edition, Vol. 3, pages E3 - E4; Cold Spring Harbor Laboratory Press 1989). Then the DNA pellet was resuspended in 50 µl of PCR-grade water and then stored at −20 ◦C before analysis by real-time PCR. For each sample, total DNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and adjusted to 20 ngr/µl using PCR-grade water. Positive control samples of *N. ceranae* and *N. apis* were provided from the archival of Istituto Zooprofilattico Sperimentale of Lazio and Tuscany, Rome. Honeybee samples previously known as free from *Nosema* spp infection were also included as negative controls. All used chemicals and reagents were from Sigma (Sigma-Aldrich S.r.l., Milano, Italy).

2.1.4. Primers design

Nosema spp ribosomal gene 16S was chosen as gene target. The nucleotide sequences of 16S ribosomal RNA gene from *N. ceranae* (FJ789797.1, FJ789789.1, FJ789785.1, FJ789799.1, FJ789786.1, FJ789788.1, FJ789791.1, DQ374656.2, DQ673615.1, FJ789795.1, EU045844.1, U26533.1) and *N. apis* (FJ789796.1, FJ789790.1) were obtained from GenBank (available at http://www.ncbi.nlm.nih.gov/genbank/) and aligned using the online version of Clustal Omega software (available at http://www.ebi.ac.uk/Tools/msa/clustalo/). The following primer pair: HRMNosem forward (HRMNosemF): 5' –AGGGGCGAAACTTGACCTAT- 3' and reverse (HRMNosemR): 5' – GCATCAATCATCATACACACTC- 3', was designed on conserved regions of the alignement, and substantially avoiding polymorphic regions that have been reported in *N. ceranae* 16S rRNA gene sequence (Sagastume et al. 2014) (**Figure 2.2**). The expected amplicon size was 162 bp for *N. ceranae* and 160 bp for *N. apis*. The specificity of primer sequences for *Nosema* species was confirmed using the Basic Local Alignment Search Tool program (available at http://blast.ncbi.nlm.nih.gov/).

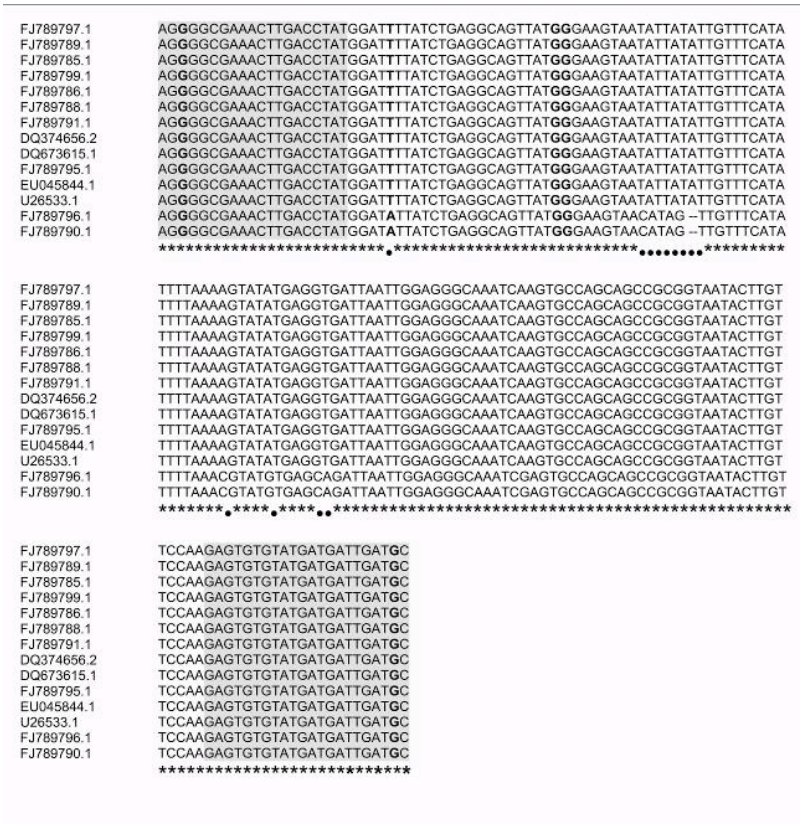

Figure 2.2. Alignment of partial *Nosema* ribosomal 16S gene sequences of *N. ceranae* (FJ789797.1, FJ789789.1, FJ789785.1, FJ789799.1, FJ789786.1, FJ789788.1, FJ789791.1, DQ374656.2, DQ673615.1, FJ789795.1, EU045844.1, U26533.1) and *N. apis* (FJ789796.1, FJ789790.1). Asterisks indicate identity; dots indicate sequence variations; gray areas indicate primer sequences (HRMNosemF and HRMNosemR) targeting conserved regions with no intra-species variability and with maximized interspecies variability design.

2.1.5. Conventional PCR

The designed primer pair (HRMNosemF and HRMNosemR) was first tested by conventional PCR as follows: 20 ng of extracted gDNA, 1U GoTaq® G2 DNA Polymerase (Promega Corporation, Madison, WI, USA), 1X Green GoTaq® Reaction Buffer (Promega Corporation), 0.2 mM PCR Nucleotide Mix (Promega Corporation), 0.3 mM of each primer pairs and PCR-grade water to a final volume of 20 µl. The thermal protocol was set as follows: 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 sec and elongation at 72°C for 30 sec. The amplification ended with a final elongation step at 72°C for 7 min. The amplification was performed on iCycler thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). Amplification products were run on 2% agarose gel and UV-visualized.

2.1.6. Real-time PCR - High Resolution Melting Analysis (HRMA)

Amplification product melting analysis is not a novel concept and exploits a fundamental property of DNA, called melting: the separation of the two strands of DNA with heat. PCR product melting analysis in combination with real-time PCR that was first introduced with the LightCyclers and the stability of DNA duplexes was detected using intercalating dye, like SYBR Green I. This is able to detect primer-dimers or other non-specific products. This procedure, called Low Resolution Melting has been performed for over a decade. A Low Resolution Melt curve is produced when the temperature increases, normally in steps of 0.5 °C increment per second, thereby gradually denaturing a previously amplified DNA target. Since SYBR Green I is only fluorescent when bound to dsDNA, fluorescence decreases as the double strand of the DNA amplicon is denatured. The melting profile depends on the length, GC content, sequence and heterozygosity of the amplified target. The highest rate of fluorescence decrease is normally at the melting temperature of the DNA sample (Tm). The Tm is defined as the temperature at which 50% of the DNA sample is doublestranded and 50% is single-stranded. The Tm is typically higher for DNA fragments that are longer and/or have a high GC content. The fluorescence data from low resolution melting curves can easily be used to derive the Tm by plotting the derivative of fluorescence vs temperature (-dF/dT against T) as reported in **Figure 2.3** (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

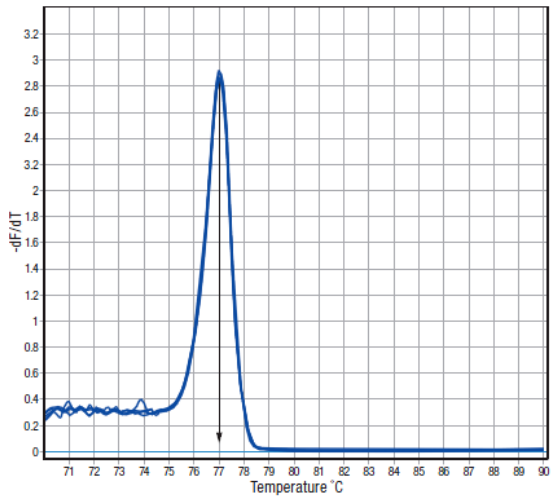

Figure 2.3: Low Resolution Melt profile derivative plot (-dF/dT against T). The steepest slope is easily visualized as a melt peak. In this example the Tm of the amplicon is 77 °C (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

The principle of High Resolution Melting Analysis (HRMA) is the same as a Low Resolution Melt, except that the temperature difference between each fluorescence reading is reduced. During a Low Resolution Melt curve analysis, the temperature increases are typically in 0.5 °C steps, but for HRMA this is reduced to 0.008 - 0.2 °C increments, depend on the property of the instrument.

Briefly, the first step of the HRM protocol consists in the amplification of the region of interest (usually, through a real-time PCR protocol), in the presence of a specialized double-stranded DNA (dsDNA) binding dye. This particular dye is highly fluorescent when bound to dsDNA and inadequately fluorescent in the unbound state. This change allows to monitor the DNA amplification during PCR (as in quantitative PCR). After completion of the PCR step, the amplified target is gradually denatured by increasing the temperature in small increments, in order to produce a characteristic melting profile; this is termed melting analysis. The DNA sequence is gradually heat-denatured, the dye is released from the amplicon and therefore resulting in a decrease of fluorescence. If tuned in the correct way, this process allows a much more detailed analysis of the melting behavior. HRMA is thus able to detect a single change in the nucleotide sequence. HRMA uses low-cost dyes and requires less optimization than similar systems based on TaqMan chemistry and fluorescence resonance energy transfer (FRET) probes. Besides, HRMA is a simpler and more cost-effective way to characterize multiple samples (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com). HRMA sensitivity and reliability has been improved with the use of a variety of new dsDNA intercalating dyes and the availability of new instruments and more sophisticated analysis software.

There are a variety of dsDNA intercalating dyes, which have particular different characteristics. The conditions of the dyes used for HRMA are different from dyes typically used for standard quantitative PCR (qPCR) assays. Factors critical in qPCR, such as the signal-to-noise ratio and amplification efficiency, are not essential requirements for HRMA. Instead, the dye must provide detailed information on the melting behavior of an amplified target. Ideally the dye should not bind preferentially to pyrimidines or purines, change the Tm of the amplicon, or inhibit DNA amplification (High Resolution Melt Analysis, Application Guide,

www.kapabiosystem.com). The three main classes of dsDNA binding dyes currently available are:

- **Non-saturating dyes.** SYBR® Green I is the most common non-saturating dsDNA intercalating dye. This type of dye is not very suitable to be used in HRMA. SYBR® Green I stabilizes the dsDNA when used at high concentrations, but it inhibits DNA polymerase. In contrast, low concentrations are not distributed in an appropriate way in the strand of DNA that results in poor discrimination-based difference (**Figure 2.4**). For a good performance the dye must be used at the right concentration.

- **Saturating dyes.** These dyes, unlike the previous ones do not inhibit the DNA polymerase and do not alter the Tm of the fragment. These dyes in contrast to other can be added in higher amounts so that the entire sequence is full of dye. Unfortunately this dye is not able to redistribute during melting because the dsDNA is saturated. More accurate examination of the melting performance is therefore possible, as reported in **Figure 2.4**. Dyes such as SYTO9® and LCGreen® can be used at the saturating concentrations required for HRMA.

- **Release-on-demand dyes.** The "release-on-demand" class of dyes, which include EvaGreen®, can be added at non-saturating concentrations. This characteristic depends on the new method of fluorescence emission. In fact, the dye signal turns off when the fluorescent is free in solution. After the dye is tied in with the filament dsDNA, when the temperature drops the dye starts to emit the signal. This allows non-saturating concentrations of the dye to be used, ensuring that there is no PCR inhibition, whilst the unique dye chemistry provides highly sensitive HRMA analysis.

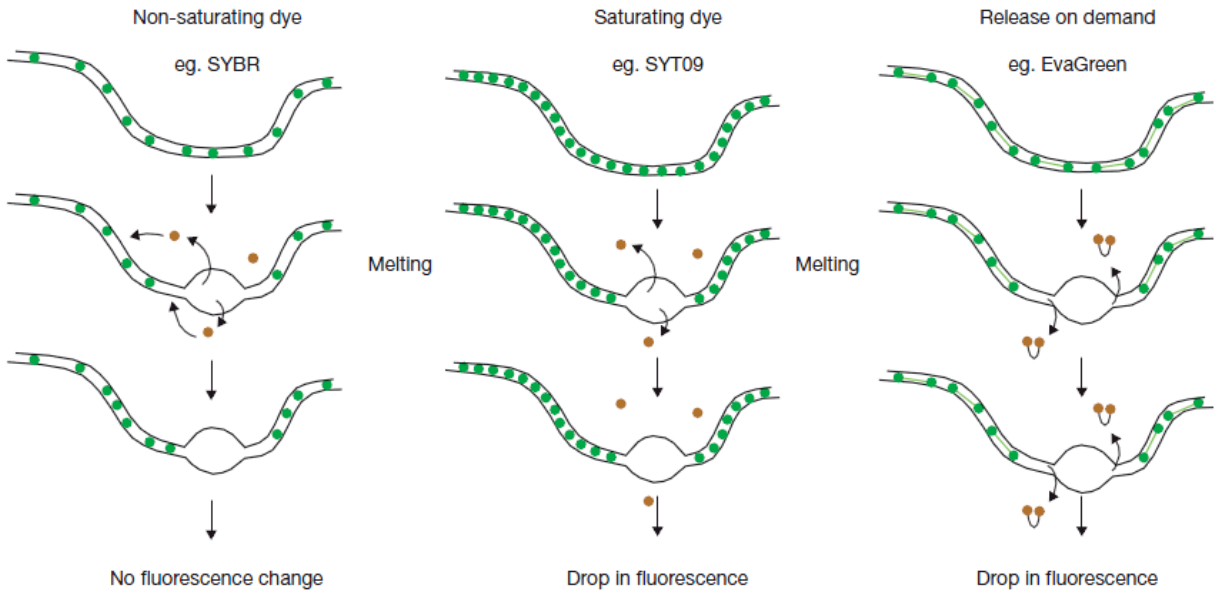

Figure 2.4: Non-saturating, saturating and 'release-on-demand' dsDNA intercalating dyes (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com). Melting of the duplex as the temperature increases releases the intercalated dyes. At non-saturating concentrations the dye rapidly rebinds to regions that remain double stranded; consequently there is no drop in fluorescence. Saturating and 'releaseon-demand' dyes do not redistribute from the melted regions of single-stranded DNA back to dsDNA, resulting in a reduction of fluorescence. This difference gives dyes such as EvaGreenR the high sensitivity required for HRM analysis.

The HRMA is a very sensitive technique and for this reason the development of the method is crucial. There are several factors that influence the behavior of melting, as genomic DNA (gDNA) quality, the background pattern, the concentration of $MgCl_2$ and polymerase inhibitors. Small differences in the initial configuration can significantly impact the final results. Achieving specific amplification is critical to the success of an assay, since any non-specific amplification will greatly impair the melt analysis. The following most important aspects are listed (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com):

- **DNA quality and quantity:** One of the main factors that can affect the quality of the results is the relationship between DNA samples and PCR preparation. Unsuitable purification techniques can distort the signal. The presence of salts can change the melting behavior of the PCR product, and may result in poor reproducibility, low sensitivity and incorrect genotype calls.

Buffer carryover from the template DNA will not only modify the Tm and melting behavior during the HRM, but can encourage also nonspecific amplification during PCR.

To avoid possible bias in the results, some tricks may be used, as for example: a) to extract the DNA to be analyzed using always the same kit, b) to check the concentration of salts in the kit because any excess can distort the signal, and c) to check that the DNA concentration is equal for all samples. **Table 2.1** shows the recommended concentration of DNA for the different types of targets. At low concentrations of the target, there is a greater possibility of incorporating a mutation early in the PCR that will affect the melting behavior. By contrast, high concentrations of DNA can result in high fluorescence due to an increase in dsDNA intercalation of dye. The positive control(s) for all genotypes should be included if possible, preferably at the same concentration of their corresponding samples. Control DNA should also be eluted and/or diluted in the same buffer of the samples.

Table 2.1: Recommended concentration of DNA for different types of template (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com)

Type of DNA	Recommended amount for HRM analisys/reaction
Genomic DNA (gDNA)	10 ng - 100 pg
Plasmid DNA (1-10 kb)	1 ng - 10 fg
Amplicon DNA	10 pg - 1 fg

- **Primer design:** the primers for HRM are not easy to draw, since they should adhere to several requirements. In fact, it should be noted that the HRM analysisis is preceded by a qPCR, then amplicons can not be very long to ensure high sensitivity. Potential primer sets and full-length amplicons can then be tested for specificity using a BLAST search. Factors such as the position of the primer, the need for a GC region on the 3 'end, the presence of any secondary structures in the primers or target, and the specificity of the predicted amplification should be carefully considered. Software as Primer3 or Primer3Plus can be very useful for drawing of primer HRMA, because the user can specify the location, the length and any other characteristics that are needed.

After the primers have been designed, the following factors must be checked:

- ✓ The presence of a GC ‘clamp’ on the 3’ end. This is not essential, but usually improves amplification efficiency.

- ✓ The specificity of the primers and the presence of known sequence variations. A BLAST search with both primer sets and the expected amplification product should be performed. If possible, sequence variations should also be checked.
- ✓ The secondary structure of the primers and the expected amplification product. This should be examined using appropriate softwares, for example, m-fold, http://mfold.rna.albany.edu/?q=mfold/dna-folding-form, or operon, http://www.operon.com/tools/oligo-analysis-tool.aspx. Amplification of any secondary structure within the product may result in unusual melting profiles. Primers may need to be redesigned to avoid areas of secondary structure.
- ✓ Amplicon size. This must be optimal for the specific application, typically between 100 - 300 bp.

- **Primer concentration and purification:** the concentration of primer does not significantly affect the overall result in HRMA, however the recommended primer concentration to avoid nonspecific amplification is between 0.05 and 0.5 µM final. For normal purposes, it can be used the final concentration of 0.3 µM as recommended by the Evagreen's manufacturers.

- **Effect of $MgCl_2$ on HRM analysis:** different concentrations of $MgCl_2$ influence the efficiency of amplification, as is the case with conventional PCR. The amount of $MgCl_2$ leads to reduced non-specific amplification, and allows a clearer distinction of sequence variations. It is important to realize that the optimum $MgCl_2$ concentration for amplification efficiency Is not necessarily optimal sensitivity for HRM. The effect of $MgCl_2$ is shown in **Figure 2.5**. As the concentration of $MgCl_2$ is increased, the HRM difference graphs change distinctively.

- **Amplicon length:** in HRMA it is difficult to specify the optimal length of the amplicon, since there are many factors to be considered. For example, one factor is the number of nucleotide changes in the sequence, i.e. the less variations there are, the shorter should be the amplicon. Normally the amplicon length can vary from 50 to 300 bp. Clearer discrimination is usually seen with shorter amplicons. However, small amplicons can result in lower fluorescence values, presumably because of decreased incorporation dye. By contrast, very long amplicons are allowed to create complex structures resulting in melting behaviours that can be misinterpreted in the end. When screening for unknown sequence differences, longer

amplicons (typically 200-500 bp) can be used. This is useful in gene scanning or determining the variation within at population (e.g., viral) and reduces the number of required primer sets. Furthermore, too short amplicons are difficult to sequence.

- **Effect of PCR enhancers on HRM analysis:** PCR enhancers are commonly added to end-point PCR in order to help reducing non-specific amplification and the number of reaction cycles, and increasing yield. These enhancers function by assisting the melting and annealing of primers and templates, and can affect HRMA performance. For GC-rich amplicons, the addition of DMSO appears not to affect the HRMA significantly, and is recommended if amplification is difficult. Overall, the addition of PCR enhancers is not recommended as they usually detract from HRM performance.

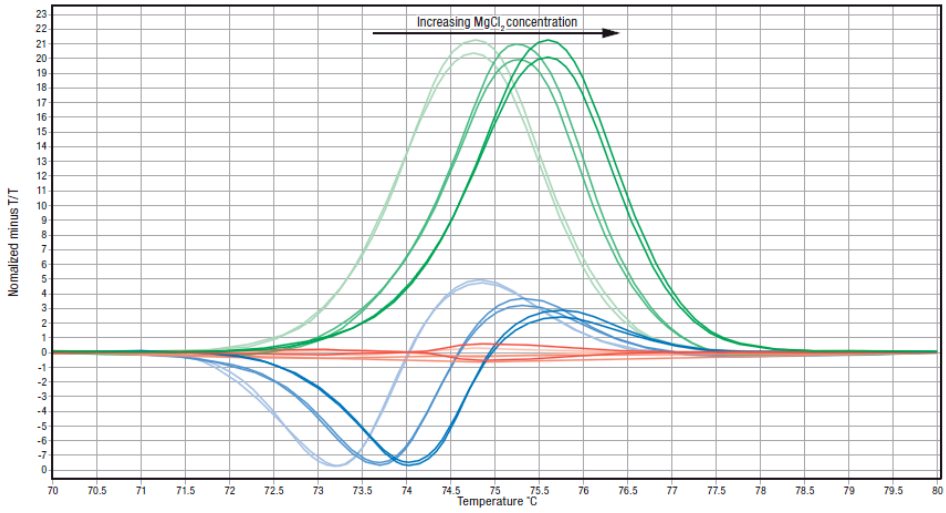

Figure 2.5. Effect of increasing $MgCl_2$ on melting behavior. Three Difference Graphs are overlayed to demonstrate the dependence of amplicon melting on $MgCl_2$ concentration. The effect of $MgCl_2$ is most pronounced in the heterozygote samples (blue), a result of the magnesium stabilizing the annealing of mispairs. The $MgCl_2$ concentrations are 1.5 mM, 2.5 mM and 3.5 mM final. G/G = green, G/A = blue, and A/A = red (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

The capacity of HRM analysis depends upon the sensitivity of the instrument and the nature of the PCR reagents used. Certain instruments have been designed specifically for HRM analysis. These can be summarized into two distinct classes:

1) **Block based instruments** – Samples are placed in a block for cycling and a scanning head or stationary camera is used for detection.
2) **Rotary** – Samples reside in a single chamber and spin past an optical detector.

There are advantages and disadvantages with each class, and the users should choose the type of instrument that best suits their needs. As a general rule, for high sensitivity and reproducibility a rotary-based instrument should be used, whilst for high-throughput and ease-of-handling, block-type instruments are optimal. The HRM run can vary considerably between instruments; some take considerably longer than others. Every instrument should be calibrated regularly (as recommended by the manufacturer, typically every 6 months), kept in a clean area on a secure and stable benchtop, and checked daily for dust buildup around the optics. The computer hardware and software must be capable of handling the large quantities of data usually generated during a HRM experiment. Analysis of the data is relatively easy with the appropriate software. HRM specific software uses various algorithms to analyze the data and display the results in user-friendly formats to help discriminate between sequence variants. Additional software packages, designed for more detailed analysis, are available from some instrument manufacturers (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

Data analysis is typically straightforward, with the correct software, allowing multiple samples to be analyzed at the same time. Low resolution melt plots are often viewed as a derivative plot (−dF/dT against T); however, HRM raw melting curves must be analyzed differently. It is important to know what to look for when interrogating results; unforeseen errors made during setup can be identified at this point.

Amplification plots. For HRMA, the reproducibility of amplification is more important than efficiency amplification, compared with qPCR. As an example, the HRM analysis can still be performed if the product amplifies later than expected (for example due to limitations in the design of primers), provided that all the samples are constants, the no template control (NTC) does not amplify and the product is specific.

Raw data melt curve. The data collected during the analysis HRM has a variety of (pre-melt) initial values of fluorescence. These variations make it difficult to interpret the results, even if different genotype groups can be visible. **Figure 2.6** shows how to analyze the HRM results and a 124 bp product covering a Type IV SNP is used as an example (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com). The panel A of this figure illustrates how the selection of pre-and post-melt regions is used to align data. It is important to adjust these bars

so that the melt region is not selected. If the preor post-melt regions are not clearly defined, it is possible to repeat the HRM run only (without repeating the amplification step), and adjust the temperature range as required. Pre- and post-melt regions must be selected for each primer set, by positioning the parallel double-bars as shown.

Normalization data. If the data is normalized correctly, it will appear as shown in the panel B of the **Figure 2.6**. This is termed 'Normalization Data'. Here the fluorescence variance seen in the Figure has been eliminated, and only the temperature range between the outer bars of the pre- and post-melt regions is shown. The genotypes are now more distinct, but the two homozygote samples (in red and blue) are still difficult to distinguish.

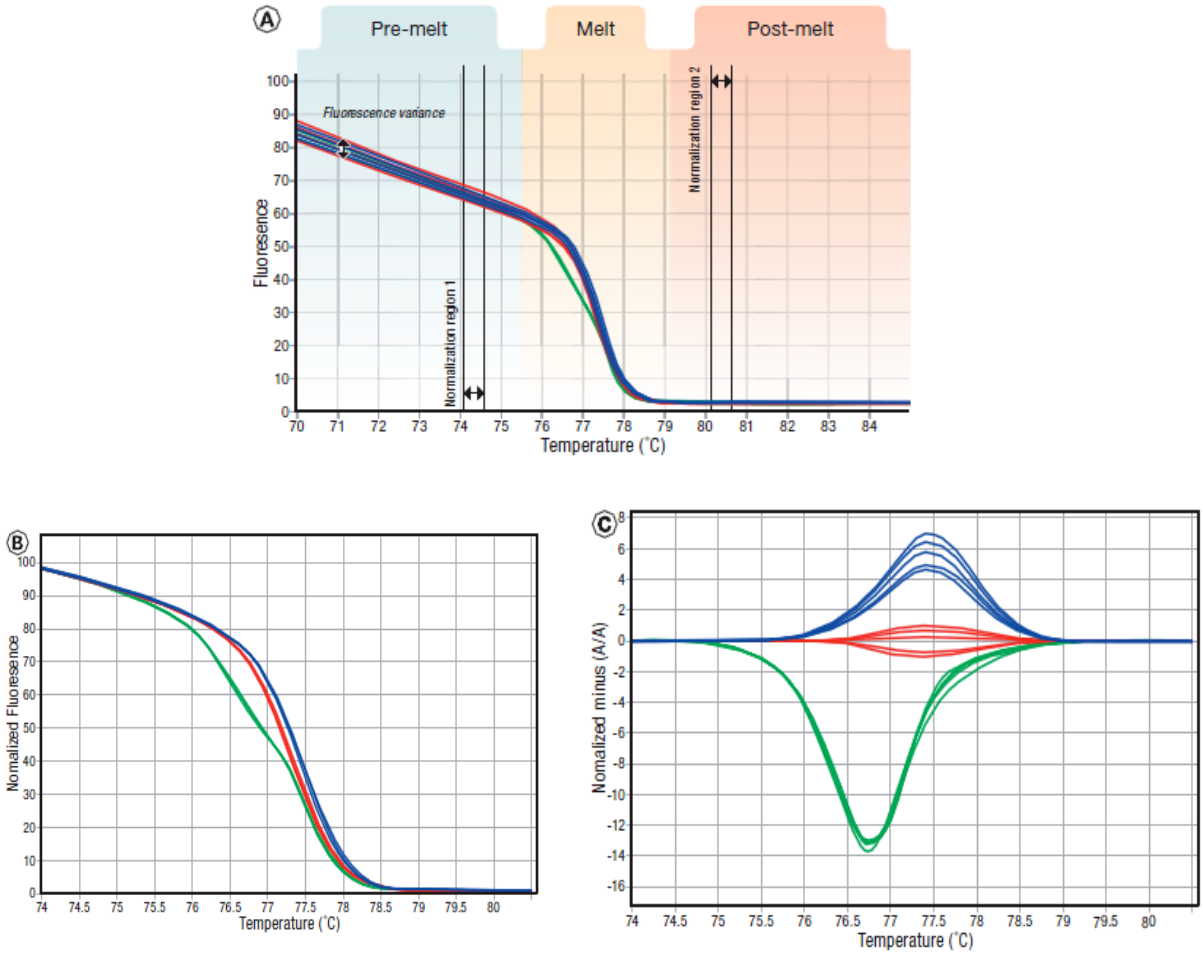

Figure 2.6. Analysis of HRM data from a type 4 SNP (A/T). Different genotypes are highlighted in different colors. A/A = red, A/T = green, and T/T = blue. **A.** Raw data showing the pre-melt, melt and post-melt regions. Notice the fluorescence variance and the positioning of the pre- and post-melt identification bars. **B.** Normalization Data derived from the raw data plots in A. Positioning of the pre-melt, and post-melt bars provides a more detailed view of the melt region. **C.** Difference Graph derived

from the Normalization Data (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

Difference graph. Some HRM software applications allow calculation of the difference plot. This is achieved by subtracting the normalized fluorescence data of a user-defined genotype from that of each of the other samples in the HRM analysis. In panel C of **Figure 2.6**, the A/A genotype has been selected as a baseline (any genotype can be selected, but usually one of the homozygotes is used). The position of each sample relative to the baseline is plotted against the temperature. This output of HRM analysis is an aid for visualizing the normalization data. Automatic calling of genotypes of sequence variants can usually be performed with most instrumentation softwares; sometimes confidence ratings are given. However, checking the difference to confirm the genotype is recommended.

The gDNA extracted was tested by real-time PCR using an EcoTM Real-Time PCR System (Illumina, Inc., San Diego, CA, USA). The reaction mix was prepared as follows: 20 ng of extracted gDNA, 7.5 µl Supermix SsoFast EvaGreen mix (Bio-Rad Laboratories Inc.), 0.3 µM of each primer and PCR-grade water to a total volume of 15 µl. PCR-grade water was used as blank sample in each experiment. Each sample was tested in duplicate. The thermal protocol for the real time PCR was 95°C for 600 s, 45 cycles of 95°C for 15 s and 60°C for 30s. The High Resolution Melting protocol was 95°C for 15 s, 55°C for 15 s, ramping to 95°C with continuous fluorescence data acquisition at each 0.1°C increment and a final step at 95°C for 15 s. The Eco_v4.0 software was used to analyze the HRM curves. Raw melting-curve data were normalized by setting the pre-melt (initial fluorescence) and post-melt (final fluorescence) signals of all samples to relative values of 100%, and 0% respectively.

2.1.7. Amplicon cloning and sequencing

The performance of the qPCR-HRMA method for the identification and quantification of *Nosema ceranae* was analyzed by building reference curves obtained from ten-fold serial dilutions of the corresponding cloned amplicon. Briefly, conventional PCR amplification products from positive control *N. ceran*ae and *N. apis* DNA were run on 2% ethidium bromide agarose gel electrophoresis and purified using QiaquickTM Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The concentration of purified amplicons was spectrophotometrically measured using using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). Each

quantitated amplicon was ligated to the plasmid pGEM T-easy at a concentration of 50 ng/µl (Promega Corporation) as reported in manufacturer's instructions. Then, DH5α competent cells were transformed with the corresponding plasmid at 3:1 concentration. After an overnight incubation at 37° C, the obtained colonies were screened for presence of the amplicon-ligated plasmidby conventional PCR using T7-SP6 plasmid-specific primers (pGEM® Vectors). One positive colony for each target was grown and the corresponding plasmid clone was purified using Qiaprep™ Spin Miniprep kit (Qiagen) and spectrophotometrically quantitated. The plasmid clones were then sequenced using T7 and SP6 primes and ABI technology in order to confirm the identity of each ligated target. The performance of the amplification was evaluated through the analysis of ten-fold serial dilutions of the corresponding plasmid from 10^6 to 10^1 copies/well. Mixed reconstructed samples containing different concentrations of *N. ceranae* and *N. apis* cloned DNA samples (10:1, 1:1, and 1:10 respectively) were also prepared in order to test the capability of the assay to distinguish mixed infection with different parasite load ratios.

The performance of the qPCR-HRMA method was assessed by comparing the results obtained from the same samples with the reference PCR method that is currently utilized in conventional and real time PCR for the identification and differentiation of *N. ceranae* and *N. apis* (Martin-Hernandez et al., 2007; Fries et al., 2013). This method is based on the primer pairs 218MITOC and 321APIS for the amplification of *N. ceranae* and *N. apis* respectively.

2.1.8. Statistics analysis

Pearson's correlation coefficient was calculated as a measure of the strength of the linear relationship between two variables using Social Science Statistics online tool (http://www.socscistatistics.com/tests/pearson/Default.aspx).

2.2. Results

The new qPCR-HRMA method was tested both in conventional and real-time PCR, demonstrating high specificity and sensitivity for the target sequences. All samples showed a reliable and reproducible amplification signal. The amplicons were visualized on agarose gel electrophoresis at the expected size: 162 bp for *N. ceranae*, 160 bp for *N. apis* and 160/2 bp for the mixed samples, with undetectable non-specific amplication or primer dimers. No amplification was obtained from *Nosema*-negative sample, confirming the specificity of the protocol (**Figure 2.7.**). The DNA sequencing confirmed the identity of the corresponding *Nosema* amplicons (data not shown).

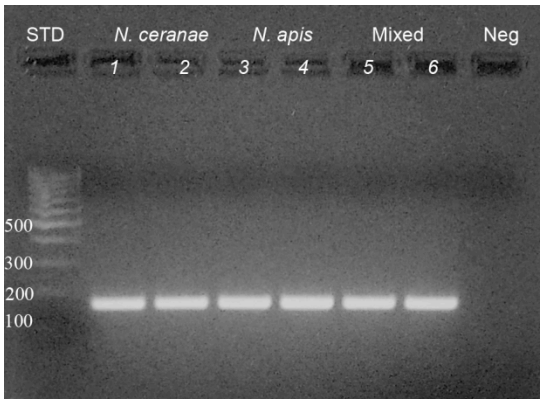

Figure 2.7. Agarose gel electrophoresis performed after conventional PCR. STD = 100 bp molecular weight marker; lines 1, 2 = *N. ceranae* positive control samples, lines 3, 4 = *N. apis* positive control samples, lines 5, 6 = 1:1 mixed samples; Neg = negative control.

The HRMA that was performed after real time PCR allowed the clear discrimination of the two species based on the different melting temperature of the two amplicons. In the normalized plot, the *N. ceranae* pure sample was clearly distinguishable from the *N. apis* pure sample and the mixed samples respectively . In addition, the differential plot allowed sample classification by subtracting the corresponding curves from a reference curve, i.e., the mixed 1:1 sample *N. ceranae/N. apis* (**Figure 2.8.**).

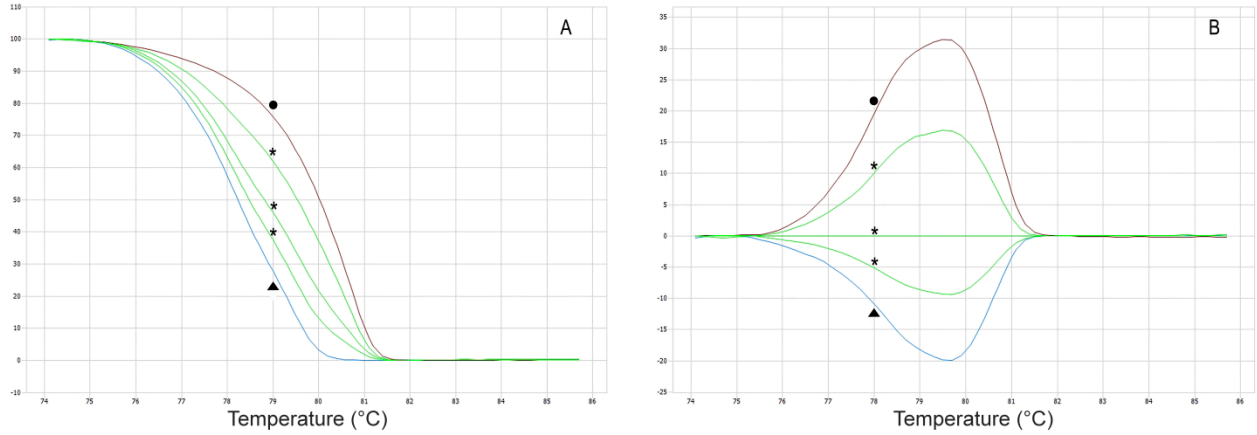

Figure 2.8. Quantitative PCR-HRMA of *Nosema* positive samples. **A**, Normalized and temperature shifted melting curves; **B**, differential plot of the same curves. From bottom to top, three different types of samples are distinguishable in both panels: the sample with pure *N. ceranae* infection (indicated by triangles), the samples with mixed *N. ceranae*/*N .apis* infection (indicated by asterisks) and the sample with pure *N. apis* infection (indicated by dots). The mixed samples correspond to a *N. ceranae*/*N. apis* copy number ratio of 10:1, 1:1 and 1:10 from bottom to top, respectively. In panel B, the mixed sample corresponding to a 1:1 *N. ceranae*/*N. apis* copy number ratio was used as a reference curve.

The real time PCR amplification of 10-fold serial dilutions of *N. ceranae* target clones allowed the set up of the reference curve, and a comparable amplification efficiency between HRMNosem and 218MITOC assay respectively was shown (**Figure 2.9.**) The reference curve for HRMNosem and 218MITOC methods were also used to quantitate the *N. ceranae* target DNA in the 20 honeybee samples that were analyzed with both methods. The corresponding values obtained with the two methods for the same positive samples underwent correlation analysis. The resulting plot showed a very good correlation (R=0.9) between the *N. ceranae* load calculated with the new HRMA-based method and the reference method based on 218MITOC primers pair (**Figure 2.10.**).

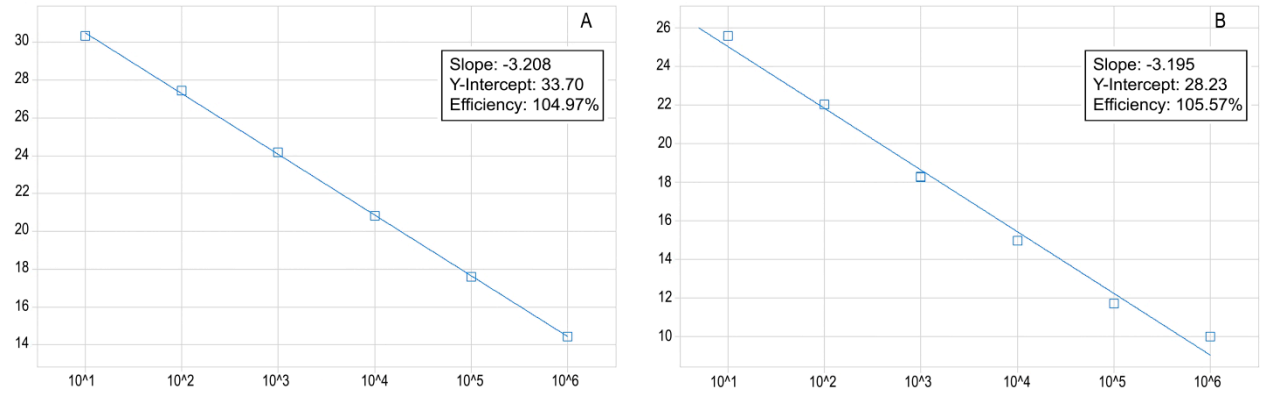

Figure 2.9. Reference curves for *N. ceranae* using HRMANosem method (panel A) and 218MITOC method (panel B) on a target plasmid serial diluition ranging from 10^6 to 10^1 copies/well.

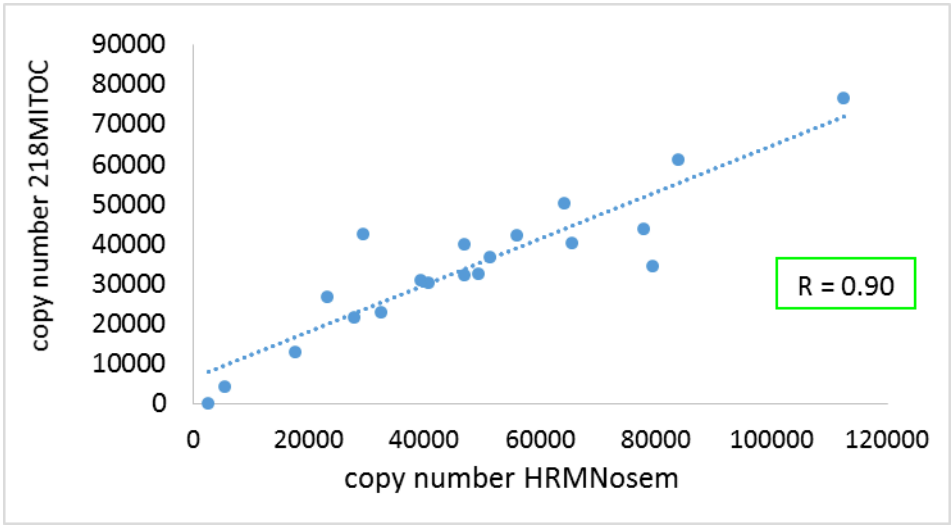

Figure 2.10. Correlation analysis of the *N. ceranae* load evaluated with HRMNosem and 218MITOC methods.

The tested samples ranged from 49166.67 to 1800000.00 mean spores/honeybee as determined by microscopical counting. The comparison between the mean number of spores per honeybee and the DNA copy number showed a moderate (R=0.64) correlation between the values obtained by the HRMNosem assay and by the microscopical method from the same samples (**Figure 2.11.**).

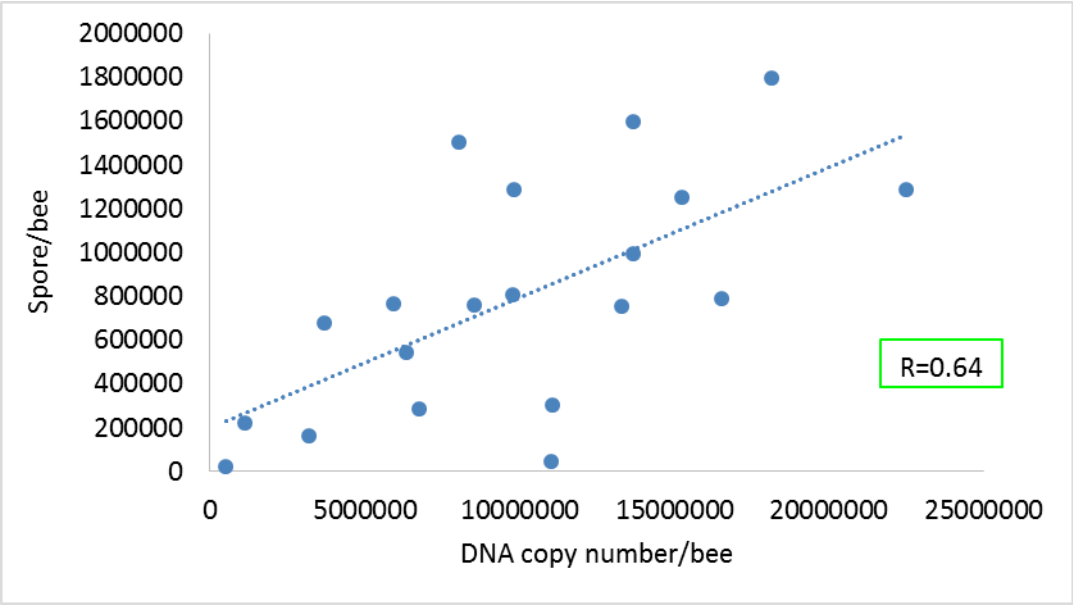

Figure 2.11. Correlation analysis between molecular (by HRMNosem method) and microscopic assessment of *N. ceranae* load for each sample

2.3. Discussion

N. ceranae and *N. apis* are honeybee parasites widely circulating inside the apiaries around the world. The infestation of *A. mellifera* by *N. apis* represents a real danger to the health of the beehive. The type A nosemiasis caused by this parasite is a notifiable disease under the provisions of the Veterinary Police Regulations (Botías et al. 2013). Only recently, *N. ceranae* has been widely admitted as a pathogen of the honey bee. At present the dynamics of the infection and the pathological consequences it may have on the host are not well defined and give rise to controversial opinions among experts in the sector. The rapid diffusion of the parasite caused emerging concern and for this reason careful monitoring of this phenomenon is required (Martin-Hernandez et al. 2007). To take precautionary measures that are efficient and durable it is first necessary to make an accurate assessment of the pathogen at the apiary level. To do this it is important to develop methods of certain identification of the parasite that are able to distinguish the two species of *Nosema* correctly and to provide information on the degree of infestation of honeybees. Positive identification of *Nosema* spp. may be established by standard light microscopical detection of the typical spores in the honey bee ventriculus or faeces. Microscopical examination can be routinely performed on individual or pooled honey bee samples. Due to age-related infection pattern, the highest proportions of infected honey bees are usually found in foragers, thus older workers are generally sampled from the hive entrance (Meana et al. 2010). Due to the high variability in spore load among workers, a relevant (at least 25-60 honey bees) sample size is usually recommended. After grinding the honey bees or their abdomen or ventriculus in a reliable liquid medium, spores are usually detected in the macerate at 400x magnification. Giemsa's staining or phase contrast light microscopy can be used to improve differential diagnosis with yeast cells, and spores and cysts from similar microorganisms (Fries et al. 2013). Spore count has been suggested as a marker of the severity of the infection especially regarding *N. ceranae*, even if the relationship between spore count and the health of the colony has been questioned (Meana et al. 2010). A compound microscope using 400 X magnification is sufficient for observing *Nosema* spp. spores in macerated bee preparations. During microscopical analysis, it is possible to perform spore counting by using a standard haemacytometer. The proportion of infected honey bees into the colony can show a good correlation with the average spore counts in pooled samples (Fries et

al. 2013). Nevertheless, under standard microscopical examination the spores of the two microsporidia, even if slighty different in size, are not easily distinguishable, especially whem mixed infections occur. Electronic microscopy can rely on the different number of polar filament coins to distinguish between the two species, but this technique is clearly not reliable for routine in-field diagnosis.

Molecular analysis can be suitable tool for highly sensitive and cost-effective pathogen species identification both for laboratory and field purposes. In the last years, several molecular protocols have been published for the identification of *N. ceranae* and *N. apis*, including conventional species-specific PCR allowing separate identification of the two pathogens, and PCR-RFLP and multiplex PCR allowing amplification for the two species simultaneously (Martin-Hernandez et al. 2007; Paxton et al. 2007; Chen et al. 2008; Carletto et al. 2013). Most published real time PCR (qRT-PCR)-based assays are capable of simultaneous identification and quantification of both species in the same reaction, using separate primers pairs and species-specific probes (Chen et al. 2009; Bourgeois et al. 2012). The OIE recommend a qualitative multiplex PCR method as the reference protocol for molecular diagnosis of *Nosema* (Martin-Hernandez et al. 2007; (OIE) 2008). This method was also adapted for duplex-real time PCR with melting curve analysis, thus improving the sensitivity, specificity and speed of the original assay (Burgher-MacLellan et al. 2010).

The present study validated a new qPCR-HRMA protocol assay for infection assessment of the two *Nosema* species affecting honey bees by comparison with the results obtained with two largely used reference methods. Taken together, the results of the present study support qPCR-HRMA as an alternative method for parasite identification and load estimation. This assay is promising for extensive routine use in in-field diagnosis as a quick and sensitive single step protocol to identify both Nosema species using an unique primer pair and saturating dye and avoiding the use of expensive sequence-specific probes. Thus, this assay has some advantages compared to already existing reference methods, not requiring multiplex methods or time-consuming post amplification techniques like agarose gel amplification and/or DNA sequencing. Specific features of this assay are a shorter analysis time, a reduced cost, and a comparable amplification efficiency for both targets, especially useful in case of simultaneous infection with the two *Nosema* species.

The present study was reported as a manuscript currently submitted for publication (**Dell'Orco F.**, Loiacono M., Albonico F., Zanzani S., Cersini A., Formato G., Colombo M., Mortarino M.. Detection and quantitation of *Nosema ceranae* in honey bees by real time PCR coupled to High Resolution Melting Analysis**.** Parasitology Research, submitted manuscript)

As for Milk

Chapter 3: Identification of pathogenic *Escherichia coli* in bulk-tank milk and filter milking machine through molecular profiling of virulence genes

3.1 Introduction

3.1.1 Escherichia coli

Domain	Bacteria
Phylum	Proteobacteria
Class	Gamma-Proteobacteria
Order	Enterobacteriales
Family	*Enterobacteriaceae*
Genus	*Escherichia*
Species	*E. coli*

Escherichia coli is a member of the genus *Escherichia* within the family *Enterobacteriaceae*. It is a gram-negative, rod-shaped, mostly motile and facultative anaerobic bacterial species (Edwards and Ewing 1962). *E. coli* can live in two different habitat during his life cycle: The first one is host-associated in the lower intestine of warm-blooded animal/human, in which it establishes commensal associations (Macfarlane and Gibson 1997); the second one is non-host-associated (Savageau 1983). Indeed, *E. coli* is released in the environment through faecal deposition, in which it can survive for long periods and potentially replicate on algae in water and soils in tropical, subtropical and temperate climates (Ishii and Sadowsky 2008).

Beside these habitats, certain strains have the potential to cause a wide spectrum of intestinal and extra-intestinal diseases such as urinary tract infection, septicaemia, meningitis, and pneumonia in humans and animals (Michael S. Donnenberg 2002). *E. coli* is frequently used as a faecal indicator bacterium (FIB) for assessing water quality, and the survival and growth of *E. coli* in the environment raises concerns regarding the use of this bacterium for indicating faecal contamination. In addition, since some *E. coli* strains and serotypes can cause human diseases, understanding the ecology of this bacterium is important to prevent infection and spread of this pathogen to food, soil, and water (Jang et al. 2017).

The first information about the genome sequence of *E. coli* was in 1997, the last available analysis allowing the division into seven major phylogenetic groups: A, B1, B2, C, D, E, and F,

with the remaining unclassified subtypes placed in an eighth group, *Escherichia* cryptic clade *I* (Herzer et al. 1990; Clermont et al. 2013). These groups encompass saprophytic (A) and pathogenic (in particular B2, D) types and are often considered to be the result of long evolution time.

The phenotypes of the different *E. coli* organisms types are related to genomic differences and the patterns of gene expression, while the genomic islands define the different behavioral types (Touchon et al. 2009). In immunocompromised hosts or when gastrointestinal barriers are disrupted, even nonpathogenic strains of *E. coli* can cause infection (Cullor and Smith 1996)

- **Morphological structure**

The cell wall of *E. coli* is composed of a thin peptidoglycan layer and an outer membrane. The external cell membrane contains phospholipids, membrane proteins and lipopolysaccharide (LPS). LPS comprises lipid-A, the lipopolysaccharide core and repeated polysaccharide units called O-antigens and the toxic effects are caused by lipid-A (Cullor and Smith 1996). The polysaccharide layer called capsule can be divided into on three types of somatic (O), capsular (K) and flagellar (H) antigens (Cullor and Smith 1996). Different *E. coli* serotypes are defined by combination of O and H antigens, among which more than 700 are identified (Kaper et al. 2004; Ishii and Sadowsky 2008). *E. coli* strains also diverge in other phenotypic characteristics, such as carbon utilization patterns, antibiotic resistance profiles, flagellar motility, ability to form biofilms, and the ability to cause diseases. The *E. coli* adaptability to specific hosts would lose fitness in other environments, that phenotypic variation could explain the ecological adaptation(Franz and van Bruggen 2008).

Major groups of *E. coli* virulence factors include adhesins, toxins, polysaccharide capsules and O-antigens, proteins secreted into host cells and other mechanisms to resist killing by complement or to bind iron ions. Bacteria do not produce virulence factors continually but only when intercepting particular signals from the host or environment (China and Goffaux 1999).
The horizontal gene transfer plays an important role in the acquisition of new genes in *E. coli* (Doi et al. 2012), while genetic mutations can also contribute to the *E. coli* phenotypic diversity such as carbon metabolism (Cooper and Lenski 2000). High genotypic diversity has been identified in *E. coli* based on the repetitive extragenic palindromic PCR (rep-PCR) DNA

fingerprinting (Byappanahalli et al. 2012; Jang et al. 2015) and pulsed field gel electrophoresis (Johnson et al. 2013). Such genotypic diversity appears to be common among environmental strains (Ishii and Sadowsky 2008).

3.1.2 Pathotypes of *E. coli*

The virulence factors can be present in the *E. coli* genome or on plasmids (Harel and Martin 1999).

The environmental conditions can influence *E. coli* genomic traits of the bacterium (Tenaillon et al. 2010). In the host-animal intestine, *E. coli* population structure is determined by the conditions of the gastrointestinal tract, which in turn, is influenced by host physiology and diet. Similarly, the genetic structures of *E. coli* populations in the natural environment are shaped by abiotic and biotic environmental factors (van Elsas et al. 2011).

Pathogenic *E. coli* can be divided into two groups: intestinal and extraintestinal pathogens.

The major intestinal pathogens include:

- enteroaggregative *E. coli* (EAEC),
- enteroinvasive *E. coli* (EIEC),
- enterotoxigenic *E. coli* (ETEC).

The major extraintestinal pathogens include:

- neonatal meningitis-associated *E. coli* and sepsis-causing *E. coli* (NMEC)(Dale and Woodford 2015).
- uropathogenic *E. coli* (UPEC)
- urinary tract infections (UTIs)

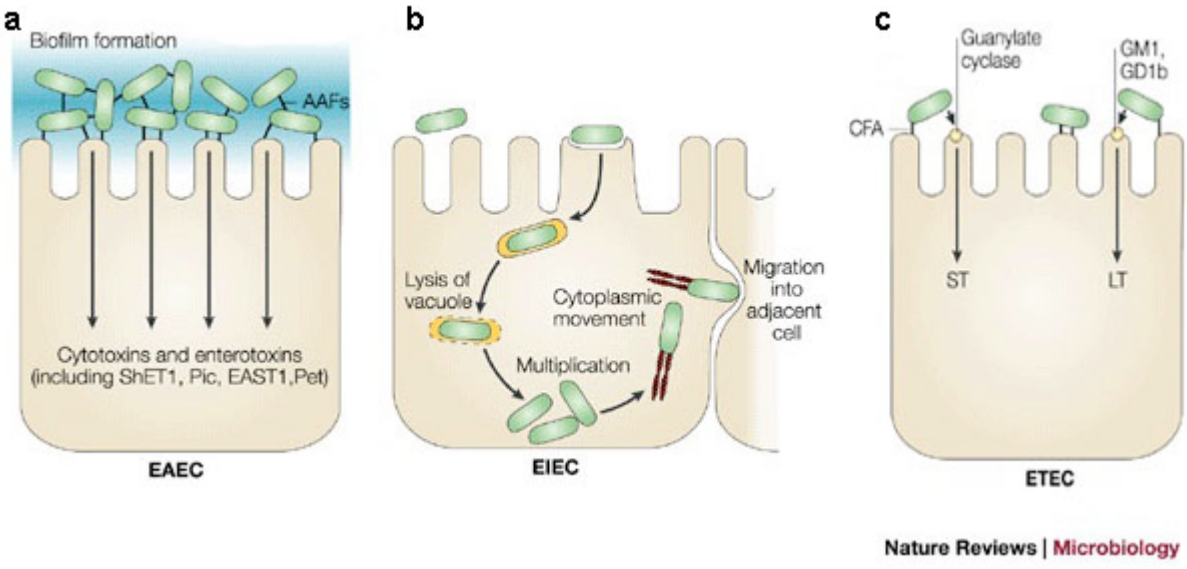

Figure 3.1.: Pathogenic schema of intestinal E. coli. a) EAEC adheres to small and large bowel epithelia in a thick biofilm and elaborates secretory enterotoxins and cytotoxins. b) EIEC invades the colonic epithelial cell, lyses the phagosome and moves through the cell by nucleating actin microfilaments. The bacteria might move laterally through the epithelium by direct cell-to-cell spread or might exit and re-enter the baso-lateral plasma membrane. c) Similarly, ETEC adheres to small bowel enterocytes and induces watery diarrhoea by the secretion of heat-labile (LT) and/or heat-stable (ST) enterotoxins (Kaper, 2004).

- **Enteroaggregative *E. coli* (EAEC)**

The first identification of EAEC was in 1987, being defined by its aggregative patterns of adherence to cultured cells (Nataro et al. 1987). EAEC strains are important causative agents of traveler's watery diarrhea with mucus accompanied by fever, vomiting, and abdominal pain (Okhuysen and Dupont 2010).

The invasion process can be divided into three steps. First, the aggregative adherence fimbriae of EAEC allows the colonization of the intestinal mucosa and induces IL-8 release. Second, it produces a mucus-mediated biofilm on the enterocyte surface. Third, EAEC releases the toxins, causing the inflammatory response, intestinal secretion and mucosal toxicity (Pereira et al. 2008). In particular, the cytotoxins can stimulate neutrophil transmigration across the epithelium, which can itself lead to tissue disruption and fluid secretion (Kaper et al. 2004) (**Figure 3.1.**).

EAEC has a pattern known as autoaggregative, in which bacteria adhere to each other in a 'stacked-brick' configuration (Nataro and Kaper 1998), indeed EAEC produces plasmid-encodeds aggregative adherence virulence factors as:

- ✓ aggregative adherence fimbriae I (AAF/I; aggA),
- ✓ aggregative adherence fimbria II (AAF/II; aafA),
- ✓ aggregative adherence fimbria III (AAF/III; agg3A),
- ✓ aggregative adherence fimbria IV (AAF/IV; agg4A),
- ✓ aggregative adherence fimbria V (AAF/V; agg5A),
- ✓ plasmid-encoded toxin (Pet), that can lead to cytoskeletal changes and epithelial-cell;
- ✓ enteroaggregative heat stable toxin 1 (EAST1; astA) which causes the watery diarrhoea
- ✓ dispersin (aap), a protein which increases the spread of infection across the mucosal surface or the penetration of the mucous layer (Sheikh et al. 2002),
- ✓ transcriptional activator AggR (aggR) regulon, that increases faecal concentrations of IL-8 and IL-1 (Jiang et al. 2002).
- ✓ enterotoxin ShET2
- ✓ *Shigella* enterotoxin 1 (ShET1), it seems contributing to the secretory diarrhea (Kaper, Nataro, and Mobley 2004; Aslani et al. 2011; Chaudhuri et al. 2010; Jønsson et al. 2015)

The typical symptoms caused by EAEC are typical of strains carrying AggR and AggR-regulated genes, indeed the term 'atypical EAEC' is used for strains lacking the AggR regulon (Kaper et al. 2004).

The infection treatments are oral rehydration therapy and antimicrobial therapy, but the antibiotic resistance is increasing worldwide (Aslani et al. 2011). The detection of EAEC is on the detection of aggregative adherence (AA). The assays utilized are the cell culture (HEp-2 assay) (Cravioto et al. 1979) and DNA probe (Nataro et al. 1985). A PCR assay with primers derived from the AA probe sequence shows similar sensitivity and specificity (Nataro and Kaper 1998).

- **Enteroinvasive *E. coli* (EIEC)**

EIEC strains are biochemically, genetically, and pathogenetically closely related to *Shigella* spp.. Several studies have shown that *Shigella* and *E. coli* are taxonomically indistinguishable at the species level (Wei et al. 2003). EIEC can be distinguished from *Shigella* by a few minor

biochemical tests, but these pathotypes share essential virulence factors. EIEC strains are generally lysine decarboxylase negative, non- motile, and lactose negative (Brenner et al. 1973).

EIEC causes an invasive inflammatory colitis, and occasionally dysentery, but in most cases watery diarrhea. EIEC is invasive, but dissemination of the organism past the submucosa is rare.

The infection starts with epithelial cell penetration, followed by lysis of the endocytic vacuole, intracellular multiplication, directional movement through the cytoplasm and extension into adjacent epithelial cells. Movement within the cytoplasm is mediated by nucleation of cellular actin into a 'tail' that extends from one pole of the bacterium (**Figure 3.1.**) (Kaper et al. 2004).

Much of EIEC pathogenesis seems to be the result of the multiple effects of plasmid-borne type III secretion system (Sansonetti et al. 2000). It secretes different protein:

- ✓ IpaA, involved in binding the vinculin and induces actin depolymerization, thereby helping to organize the extensions that are induced by IpaC into a structure that enables bacterial entry;
- ✓ IpaB, involved in binding the signaling protein CD44 and the macrophage caspase 1, causing apoptosis and release of IL-1 from macrophages;
- ✓ IpaC, involved in actin polymerization, which leads to the formation of cell extensions by activating the GTPases Cdc42 and Rac;
- ✓ IpgD, involved in the reorganization of host-cell morphology by uncoupling the cellular plasma membrane from the actin cytoskeleton, which leads to membrane blebbing (Kaper et al. 2004).

The type III secretion system apparatus, which is encoded by mxi and spa genes, enables the insertion of a pore containing IpaB and IpaC proteins into host cell membranes.

Although the extensively characterized type III secretion system is essential for the invasiveness characteristic of EIEC and Shigella species, this process is also contributed by the plasmid-encoded serine protease SepA, the chromosomally encoded aerobactin iron-acquisition system and other secreted proteases that are encoded by genes present on pathogenicity islands.

The detection of EIEC is on the ability of the strain to invade epithelial cells and to spread from cell to cell to distinguish from *Shigella* spp (Sereny test) (Kopecko 1994), enzyme-linked immunosorbent assay (ELISA) to detect the ipaC gene (Inv) (Pal et al. 1997) and DNA probes

(Wood et al. 1986; Gomes et al. 1987). A PCR and multiple PCR assay with primers derived from ial gene (Frankel et al. 1989, 1990).

- **Enterotoxigenic *E. coli* (ETEC)**

ETEC strains were first recognized as causes of diarrheal disease in piglets, with lethal infection in newborn animals (Alexander 1994). Studies of ETEC in piglets first elucidated the mechanisms of disease, including the existence of two plasmid- encoded enterotoxins (ST and LT). The descriptions of ETEC in humans reported that certain *E. coli* isolates, first in children, following in adult with diarrhea, elicited fluid secretion by the intestine (DuPont et al. 1971). ETEC are also an important cause of diarrhoeal disease in animals and these animal strains express fimbrial intestinal colonization factors, which are not found in human ETEC strains.

The pathogenesis begins when the ETEC colonizes the surface of the small bowel mucosa using fibrillar colonization (CF) factors and elaborates enterotoxins, which give rise to intestinal secretion.

ETEC enterotoxins can be divided in two groups: the heat-labile enterotoxins (LTs) and the heat-stable enterotoxins (STs). ETEC strains can express LT and ST alone, or both. LT is a potent mucosal adjuvant, independently of its toxic activity (Pizza et al. 2001) and has been incorporated into numerous vaccine candidates containing a variety of antigens, thus resulting in increased antibody responses to these antigens. STs are small, single-peptide toxins that include two unrelated classes — STa and STb — which differ in both structure and mechanism of action. Indeed, STa toxin can affect humans (Nataro and Kaper 1998), while STb toxin can affect animals (Dubreuil 1997) ETEC is largely a pathogen of developing countries, and it is well known that these countries typically have a low rate of colon cancer. So the high prevalence of ETEC in developing countries might have a protective effect against this important disease, and indicates that infectious diseases might exist in a complex evolutionary balance with their human populations (Kaper et al. 2004).

The detection of ETEC is related on the expression and analyses enterotoxins LT and ST. The assays developed are the immunoassays (Honda et al. 1981), including a radioimmunoassay for ST (Giannella et al. 1981), ELISA (Yolken et al. 1977; Cryant 1990) and latex agglutination (Ito et al. 1983). The molecular techniques developed are DNA probes to detect LT- and ST-encoding

genes in stool and environmental samples (Nataro and Kaper 1998). Also, the “multiplex” PCR assay (Lang et al. 1994; Stacy-Phipps et al. 1995) in which several PCR primers are combined to detect one of several different diarrheagenic *E. coli* pathotypes in a single reaction.

- **Extraintestinal pathogenic *E. coli* (EXPEC)**

The ExPEC strains predominantly cluster in the B2 and D phylogenetic groups while APEC strains have also expanded into C and F groups (Johnson et al. 2001; Nicolas-Chanoine et al. 2007; Totsika et al. 2011). ExPECs colonize and infect a wide range of host species, using different virulence factors (VFs) that are not restricted to the ExPEC pathotype. ExPECs respond to environmental stimuli using several signaling networks; the best characterized of these signaling systems are two-component systems (Breland et al. 2017). The common VFs for ExPEC are pili assembled by the chaperone-usher pathway (CUP), protein adhesins, toxins, iron acquisition systems, transport systems, and other non-essential factors (Russo and Johnson 2000).

ExPEC strains colonizes different tissues: UPEC, urethra, bladder and kidneys; NMEC, brain and nervous tissues; UTIs, urinary tract. Despite the existence of these classes of ExPEC, there is great overlap in the variety of VFs expressed by each class, making these designations rather artificial (Russo and Johnson, 2000).

ExPEC infections in all afflicted populations are typically treated with antibiotics, although this practice can cause multi-drug resistance, thus affecting the healthcare setting and making more difficult eradication of ExPEC strains (Breland et al. 2017).

The detection of the ExPEC is based on VFs of the different strains(Russo and Johnson 2000).

3.2 Aim

The identification of *E. coli* strains is important for public health since it can reproduce in animals, humans and the environment. The routine detections and differentiations of intestinal and extraintestinal *E. coli* are usually based on a combination of biochemical tests; serotyping, phenotypic assays based on virulence characteristics and molecular detection methods. The detention of the causative agent is very important to infer the inherent sensitivity of the host animals to specific virulent strains and for the setup of an effective control strategy.

The aim of this work was to evaluate the presence and virulence profile of pathogenic *E. coli* in bulk-tank milk compared to milking machine filters samples on a number of samples collected in dairy farms.

To achieve this, the classical isolation and identification techniques have been coupled with a molecular diagnostic approach.

The implementation of such combined microbiological and biomolecular approach allowed the selective identification of *E. coli* from other bacteria on specific media and the profiling of virulence factors, leading to a rapid and sensitive identification of pathogenic strains, as a necessary step for a proper characterization of the host and human exposure, and inherent health risk.

3.3 Materials and methods

3.3.1 Overview of the experimental design

The experimental design involved several steps from the collection of the samples in dairy cows to biological analysis, as follows

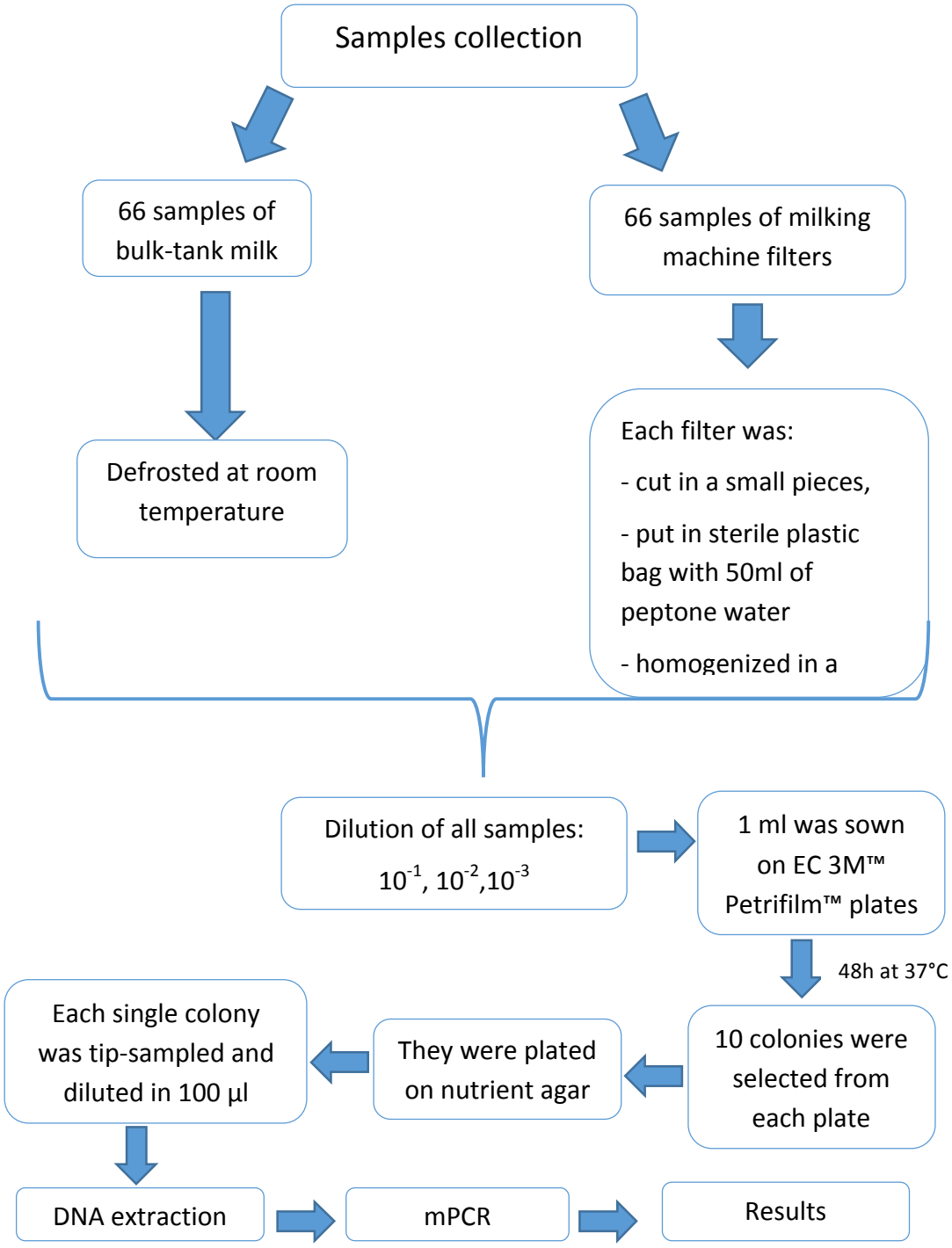

Figure 3.2.: experimental design of *E. coli* project.

The collection of the samples and the microbiological isolation was done in collaboration with the Infectious Diseases Unit of the Veterinary Medicine Department (DIMEVET), University of Milan.

3.3.2 Collection of samples

The samples were collected during the milking in seven different dairy farms located In Lombardy (closed to Lecco and Como) and Trentino-Alto Adige.

The samples included:

- → 66 bulk-tank milk;
- → 66 milking machine filters.

The filter (similar to a sock) is placed in the duct that leads the milk from the milking room to the milk collection tank. The material of the filter is similar to a tissue with a variable weight of 60 to 80 g/m^2 and its capacity is optimal for milking about 200 beef, with satisfactory breast hygiene and good milking practices. The filter has the function of blocking debris, large particles of organic material and foreign objects (hair, vegetable fibers, dirt, etc.), ensuring that milk is free of macroscopically visible contaminant, but it is not able to reduce the number of bacteria present in the milk.

The samples collected were refrigerated within eight hours from milking. Once in the laboratory, they were frozen and stored at -20 °C until further processing.

3.3.3 Microbiological isolation

The samples were treated differently if there were filter or milk.

The milk samples were defrosted at room temperature and they were diluted as follows: 10^{-1}, 10^{-2} and 10^{-3}/ml.

The Milking machine filters were chopped sterile, introduced in plastic bags with 50 mL of sterile peptone water and processed in Stomacher for 4 minutes. The obtained preparation was brought to the following dilutions: 10^{-1}, 10^{-2} and 10^{-3}/ml.

After dilution, all samples were sown on plates for the enumeration of *E. coli* (EC 3M ™ Petrifilm ™) and the plates were incubated for 48 hours at 37 °C. The plates were then checked for bacterial growth, and the colonies of *E. coli* can appear blue or red-blue and with or without

one or more gas production. Indeed, the colonies that appear blue or red-blue were *E. coli* which produce β-glucuronidase. Instead, the colonies that produce one or more gas production were *E. coli* which ferment lactose. Then *E. coli* colonies were picked up randomly from each plate to a maximum of 10 colonies and these were plated on nutrient agar. Each single colony was tip-sampled and placed in an 0,5 ml Eppendorf diluted in 100 µl of molecular grade water.

3.3.4 DNA extraction

The extraction of the samples was performed using alternative cycles of hot and cold break: 15 minutes at 95 °C and 15 minutes at -80°C. This method allowed the cells lysis and DNA extrusion from bacterial cells.

3.3.5 Conventional PCR

Conventional PCR runs were performed on T100™ Thermal Cycler-Bio-Rad. The reactions were carried out in a total volume of 20µl containing 2µl of DNA, 1X Taq buffer (containing 1.5mM MgCl2), 0.2 mM dNTPs, 0.2-0.4 µM of each primer, and 1.25 U TaqPromega; H_20 was added to reach a total volume of 20 µl. The thermal protocol for the PCR was as follows: denaturation step at 95° C for 10 min; 35 amplification cycles at 95°C for 30 s, annealing T °C depending on each primer pair for 30 s and 72°C for 30s; and the final elongation step at 72°C for 10 min. Primers sequences, amplicons size, concentration and annealing temperatures for each primer pair for PCR amplification are reported in **Table 3.1**. The amplification products were run on 2% agarose gels and visualized under a trans-illuminator.

Table 3.1: **Primers sequences, amplicon size and annealing temperature**

PCR specifies	PCR format	Target	Primer sequences (5'-3')	Amplicon Size	Concentr (µM
EAEC	Multiplex PCR Conventional,	*aggR*	F: GCAATCAGATTAARCAGCGATACA R: CATTCTTGATTGCATAAGGATCTGG	425	0.2
EIEC		ipaH	F: TTCCTTGACCGCCTTTCCG R: AGCCACCCTCTGAGAGTAC	611	0.2
ETEC		eltB	F: CGGCGTTACTATCCTCTC R: CCATACTGATTGCCGCAAT	336	0.2
	Multiplex PCR	estIa	F: CCTCTTTTAGYCAGACARCTGAATCASTTG R: CAGGCAGGATTACAACAAAGTTCACAG	157	0.4
		estIb	F: TGTCTTTTTCACCTTTCGCTC R: CGGTACAAGCAGGATTACAACAC	171	0.2
ExPEC	Conventional, multiplex	focG	F: CGTACCTGTACCATTGGTAATGGAGG R: TGAATTAATACTTCCCGCACCAGC	366	0.2
		kpsMII	F: GCGCATTTGCTGATACTGTTG R: GGGAACATGATGCAGGAGATG	452	0.2
		papA	F: ATGGCAGTGGTGTCTTTTGGTG R: CGTCCCACCATACGTGCTCTTC	717	0.2
		sfaS	F: GTCTCTCACCGGATGCCAGAATAT R: GCATTACTTCCATCCCTGTCCTG	138	0.2
		afa	F: GGCAGAGGGCCGGCAACAGGC R: CCCGTAACGCGCCAGCATCTC	594	0.2
		hlyD	F: CTCCGGTACGTGAAAAGGAC R: GCCCTGATTACTGAAGCCTG	904	0.2
		iutA	F: ATCGGCTGGACATCATGGGAAC R: CGCATTTACCGTCGGGAACGG	314	0.2

ExPEC was defined as a strain having three or more of these genes (Jang et al. 2013).

3.4 Results and discussion

From the samples of milk and milking machine filters, after the selection on EC 3M™ Petrifilm™, 149 colonies (65 from the milk and 84 from the filter) were collected which appeared as *E. coli*. The colonies were then planted on agar soil, then each single colony was tip-sampled and placed in an 0,5 ml Eppendorf diluted in 100 µl of molecular water to perform the conventional PCR.

Before the analysis of unknown samples, each gene-specific PCR assay was tested on positive control samples provided by the Istituto Zooprofilatico Sperimentale delle Venezie, Italy. The agarose gel-electrophoresis run confirmed the effective amplification of the gene target with the expected amplicon size, as shown in **Figure 3.3**.

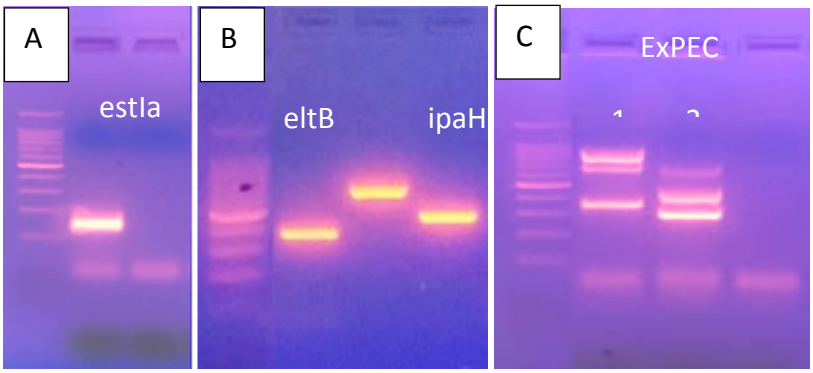

Figure 3.3. Agarose-gel electrophoresis of PCR products. **A**, amplification of estIa (157 bp). **B**, Amplification of eltB, ipaH and aggR (336 bp,611 bp and 425 bp, respectively) and. **C**, Amplification lane 1: from the top to the bottom hlyD, papA and focG (901 bp, 717 bp and 366 bp, respectively); amplification line 2: from the top to the bottom papA, kpsMII and iutA (717 bp, 452 bp and 314 bp, respectively).

The results obtained after conventional PCR performed on the DNA preparation from the selected colonies are reported in **Table 3.2.**

Table 3.2. Pathotype identification of the selected col onies through Conventional PCR analysis of virulence genes. The ID indicated by M are bulk-tank milk samples, while ID indicated by F are milking machine filter samples. The positive samples for the pathotype gene are indicated with a "+" symbol. Each sample could have from 1 to 10 colonies analyzed depending on EC 3M™ Petrifilm™ results.

Colonies		Virulence genes											Patothype
ID	Number	aggR	ipaH	eltB	estA	focG	papA	afa	iutA	kpsMII	sfaS	hlyD	
M3	1	-	-	-	-	-	-	-	-	-	-	-	
	2	-	-	-	-	-	-	-	-	-	-	-	
M6	3	-	-	-	-	-	-	-	-	-	-	-	
	4	-	-	-	-	-	-	-	-	-	-	-	
	5	-	-	-	-	-	-	-	-	-	-	-	
	6	-	-	-	-	-	-	-	-	-	-	-	
M7	7	-	-	-	-	-	-	-	-	-	-	-	
M8	8	-	-	-	-	-	-	-	-	-	-	-	
	9	-	-	-	-	-	-	-	-	-	-	-	
	10	-	-	+	-	-	-	-	-	-	-	-	ETEC
	11	-	-	-	-	-	-	-	-	-	-	-	
	12	-	-	-	-	-	-	-	-	-	-	-	
M9	13	-	-	-	-	-	-	-	-	-	-	-	
	14	-	-	-	-	-	-	-	-	-	-	-	
M12	15	-	-	-	-	-	+	-	-	-	-	-	
	16	-	-	+	-	+	-	-	-	-	-	-	ETEC
M24	17	-	-	-	-	-	-	-	-	-	-	-	
	18				-	+	-	-	-	-	-	-	
	19	-	-	-	-	-	-	-	-	-	-	-	
	20	-	-	+	-	-	-	-	-	-	-	-	ETEC
	21	-	-	-	-	-	-	-	-	-	-	-	
	22	-	-	-	-	-	-	-	-	-	-	-	
M28	23	-	-	-	-	-	-	+	-	-	-	-	
	24	-	-	-	-	-	-	+	-	-	-	-	
M29	25	-	-	-	-	-	-	-	-	-	-	-	
M31	26	-	-	-	-	-	-	-	-	-	-	-	
	27	-	-	+	-	-	-	-	-	-	-	-	ETEC
	28	-	-	-	-	-	-	-	-	-	-	-	
M33	29	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
	30	-	-	-	-	-	-	-	-	-	-	+	

	31	-	-	-	-	-	-	-	-	-	-	-	
	32	-	+	-	-	-	-	-	-	-	-	+	EIEC
	33	-	-	-	-	-	-	-	-	-	-	+	
	34	-	-	+	-	-	-	-	-	-	-	+	ETEC
	35	-	-	+	-	-	-	-	-	-	-	+	ETEC
	36	-	+	+	-	-	-	-	-	-	-	+	ETEC/EIEC
	37	-	-	-	-	-	-	-	-	-	-	+	
	38	-	-	-	-	-	-	-	-	-	-	-	
M34	39	-	-	-	-	-	-	-	-	-	-	-	
	40	-	-	-	-	-	-	-	-	-	-	-	
	41	-	-	+	-	-	-	-	-	-	-	-	ETEC
	42	-	-	+	-	-	-	-	-	-	-	-	ETEC
	43	-	-	-	-	-	-	-	-	-	-	-	
	44	-	-	-	-	-	-	-	+	-	-	-	
	45	-	-	+	-	-	-	-	-	-	-	-	ETEC
	46	-	-	-	-	-	-	-	-	-	-	-	
	47	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
	48	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
M35	49	-	-	-	-	-	-	-	-	-	-	-	
	50	+	-	+	-	-	-	-	-	-	-	-	ETEC/EAEC
M38	51	-	-	+	-	-	-	-	-	-	-	-	ETEC
	52	-	+	-	-	-	-	-	-	-	-	-	EIEC
	53	-	-	+	-	-	-	-	-	-	-	-	ETEC
	54	-	-	+	-	-	-	-	-	-	-	-	ETEC
	55	-	-	-	-	-	-	-	-	-	-	-	
	56	-	-	+	-	-	-	-	-	-	-	-	ETEC
M39	57	-	-	-	-	-	-	-	-	-	-	-	
	58	-	-	+	-	-	-	-	-	-	-	-	ETEC
M42	59	-	-	+	-	-	-	-	-	-	-	-	ETEC
	60	+	-	+	-	-	-	-	-	-	-	-	ETEC/EAEC
	61	-	-	-	-	-	-	-	-	-	-	-	
	62	-	-	-	-	-	+	-	+	-	-	-	
	63	-	-	-	-	-	-	-	-	-	-	-	
	64	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
M44	65	-	-	-	-	-	-	-	-	-	-	-	
F1	66	-	-	+	-	-	-	-	-	-	-	-	ETEC

	67	-	-	-	-	-	-	-	-	-	-	-	
	68	-	-	-	-	-	-	-	-	-	-	-	
F5	69	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
	70				-	-	-	-	-	-	-	-	
F6	71	-	-	-	-	-	-	-	-	-	-	-	
	72	-	-	+	-	-	-	-	-	-	-	-	ETEC
F7	73	-	-	+	-	-	-	-	-	-	-	-	ETEC
	74	-	-	+	-	-	-	-	-	-	-	-	ETEC
	75	-	-	+	-	-	-	-	-	-	-	-	ETEC
	76	-	-	-	-	-	-	-	-	-	-	-	
	77	-	-	-	-	-	-	-	-	-	-	-	
	78	-	-	+	-	-	-	-	-	-	-	-	ETEC
	79	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
F9	80	-	+	-	-	-	-	-	-	-	-	-	EIEC
	81	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
	82	-	+	-	-	-	-	-	-	-	-	-	EIEC
	83	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
F10	84	-	-	-	-	-	-	-	-	-	-	-	
	85	-	-	-	-	-	-	-	-	-	-	-	
F12	86	-	-	-	-	-	-	-	-	-	-	-	
F13	87	-	-	+	-	-	-	-	-	-	-	-	ETEC
	88	-	-	+	-	-	-	-	-	-	-	-	ETEC
	89	-	-	-	-	-	-	-	-	-	-	-	
	90	-	-	-	-	-	-	-	-	-	-	-	
	91	-	-	+	-	-	-	-	-	-	-	-	ETEC
	92	-	-	-	-	-	-	-	-	-	-	-	
F18	93	-	-	-	-	-	-	-	-	-	-	-	
	94	-	-	+	-	-	-	-	-	-	-	-	ETEC
	95	-	+	+	-	-	-	-	+	-	-	-	ETEC/EIEC
	96	-	-	-	-	-	-	-	+	-	-	-	
	97	-	-	-	-	-	-	-	+	-	-	-	
	98	-	-	-	-	-	-	-	+	-	-	-	
	99	-	-	-	-	-	-	-	+	-	-	-	
	100	-	-	-	-	-	-	-	+	-	-	-	
	101	+	-	-	-	-	-	-	-	-	-	-	EAEC
	102	-	-	-	-	-	-	-	-	-	-	-	

F24	103	-	-	-	-	-	-	-	-	-	-	-	
	104	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
	105	-	-	+	-	-	-	-	-	-	-	-	ETEC
F30	106	-	-	-	-	-	-	-	-	-	-	-	
F33	107	-	-	-	-	-	-	-	-	-	-	-	
	108	-	-	-	-	-	-	-	-	-	-	-	
	109	-	-	-	-	+	-	-	-	-	+	-	
	110	-	-	-	-	-	-	-	-	-	-	-	
	111	-	-	-	-	-	-	-	-	-	-	-	
	112	-	-	-	-	-	-	-	-	-	-	-	
	113	-	-	-	-	-	-	-	-	-	-	-	
F38	114	-	-	-	-	-	-	-	-	-	-	-	
	115	-	-	-	-	-	-	-	-	-	-	-	
	116	-	-	-	-	-	-	-	-	-	-	-	
	117	-	-	-	-	-	-	-	-	-	-	-	
	118	-	-	-	-	-	-	-	-	-	-	-	
	119	-	-	-	-	-	-	-	-	-	-	-	
	120	-	-	-	-	-	-	-	-	-	-	-	
	121	-	-	+	-	-	-	-	-	-	-	-	ETEC
	122	-	-	+	-	-	-	-	-	-	-	-	ETEC
	123	-	-	-	-	-	-	-	-	-	-	-	
	124	+	-	-	-	-	-	-	-	+	-	-	EAEC
F40	125	-	+	+	-	-	-	-	-	-	-	-	ETEC/EIEC
	126	-	-	-	-	-	-	-	-	-	-	-	
	127	-	-	-	-	-	-	-	-	-	-	-	
F42	128	-	+	-	-	-	+	-	+	-	-	-	EIEC
F45	129	-	-	-	-	-	-	-	-	-	-	-	
	130	-	+	-	-	-	-	-	-	-	-	-	EIEC
	131	-	-	-	-	-	-	-	-	-	-	-	
	132	-	-	-	-	-	-	-	-	-	-	-	
	133	-	-	-	-	-	-	-	-	-	-	-	
	134	-	-	-	-	-	-	-	-	-	-	-	
	135	-	-	+	-	-	-	-	-	-	-	-	ETEC
	136	-	-	-	-	-	-	-	-	-	-	-	
F46B	137	-	-	-	-	-	-	-	-	-	+	-	
	138	-	-	-	-	-	-	-	-	-	-	-	

	139	-	-	-	-	-	-	-	-	-	-	-	
	140	-	-	-	-	-	-	-	-	-	-	-	
	141	-	-	-	-	-	-	-	-	-	-	-	
	142	-	-	-	-	-	-	-	-	-	-	-	
	143	-	-	-	-	-	-	-	-	-	-	-	
	144	-	-	-	-	-	-	-	-	-	-	-	
	145	-	-	-	-	-	-	-	-	-	-	-	
	146	-	-	+	-	-	-	-	-	-	-	-	ETEC
	147	-	-	+	-	-	-	-	-	-	-	-	ETEC
F66	148	-	-	-	-	-	-	-	-	-	-	-	
F67	149	-	-	-	-	-	-	-	-	-	-	-	

ExPEC was not detected in these colonies. The comparison of the data obtained from the same milking samples after analysis of the bulk-tank milk and the corresponding filter underlines the different *E. coli* detection power in almost the samples (**Table 3.3**).In **Table 3.4**, the pathogenic strains detected in the two different matrices are summarized.

Table 3.3. Comparison of PCR results of bulk-tank milk and milking machine filters in the same samples.

ID	Bulk-tank milk	Milking machine filters
1		ETEC
5		ETEC/EIEC
6		ETEC
7		ETEC
8	ETEC	ETEC/EIEC
9		ETEC/EIEC
13		ETEC
18		ETEC/EIEC/EAEC
21	ETEC	
24	ETEC	ETEC/EIEC
31	ETEC	
33	ETEC/EIEC	
34	ETEC/EIEC	
35	ETEC/EAEC	
38	ETEC/EIEC	ETEC/EAEC
39	ETEC	
40		ETEC/EIEC
42	ETEC/EIEC/EAEC	EIEC
45		ETEC/EIEC
46		ETEC

Table 3.4. Overview of the pathotypes identified through conventional PCR on E. coli-positive samples.

E. coli pathotype	Bulk-tank milk	Milking machine filters
ETEC	10	13
EIEC	4	6
EAEC	2	2

3.5 Conclusion

The transmission of several pathogens (bacteria, fungi and viruses) to humans can occur through food in most, if not all, cases. Thus, food safety is a global issue, and furthermore an increase in import and export of food products could lead to introduction and establishment of new diseases in geographical areas that have never experienced such foodborne pathogens (Yang et al. 2017).

Milk can be a good source of foodborne pathogens since it carries a variety of microorganisms. Indeed, it is a direct contact with contaminated sources in the milk stall from the environment and infected animals.

Last year Ribeiro et al. (2016), published a study highlighting the correlation between the pathogenic *E. coli* in raw-milk cheese in Brazil and the risk to public health. The innovative parts of this project that is the detection of intestinal and extraintestinal *E. coli* in the bulk-tank milk and in filter milking machine.

These results underline the different detection pattern of pathogenic *E. coli* in the bulk-tank milk with respect to milking machine filter.

Indeed, when the molecular assay is targeted to the milking machine filters, more pathogens are detected when compared to the bulk-tank samples, with special regard to EIEC strains. The analysis of milk filters could be an useful tool to assess the presence of a risk of contamination by *E. coli* pathotypes both at food and environmental level.

Overall discussion and conclusions

The molecular techniques used in this PhD thesis have been developed with the main aim to support the standard methods for the monitoring and control of some pathogens that can cause injury to farmed animal or man, in particular those reported for *V. destructor* in (Dietemann et al. 2012), for *Nosema* spp in OIE, 2013 and for *E. coli* in Nataro 1998.

In particular, the Real Time PCR used for the identification of biomarkers for hygienic behavior in honeybees was introduced as a complementary test in the field activity during which the standard methods were used for the selection of hygienic families. The standard methods are inherently inaccurate since subjected to individual variability, while the use of biomarkers can make the family selection most specific, with the purpose to help the beekeepers to breed the strongest families. Still relying on the Real Time PCR protocol, a coupled protocol with HRMA allowed the identification and quantification of *N. ceranae* and *N. apis* in a fast, precise and sensitive way. The perspective of using such protocol for field purpose should be favorably considered, as the most used standard techniques are based on microscopical examination and mPCR, which are less accurate and sensitive for different reasons. In particular, microscopical analysis requires high experience in the recognition of spores, in particular those from *N. ceranae*, whereas mPCR is not a very sensitive method when compared to Real Time PCR with usually higher rate of falsely positive or falsely negative results. When fully implemented into the field, the Real Time PCR-HRMA approach can ensure a more sensitive and specific monitoring and an earlier diagnosis of *Nosema* spp in the hive. This would allow to decrease the number of sick / dead families that can be presently observed due to the underlying spread of the fungus in the apiary.

Furthermore, the combination of microbiology and conventional PCR makes the identification of *E. coli* more precise and accurate, especially for the identification of the pathogenic strain through virulence factors. The innovative part of this diagnosis is the identification of pathogenic strains in milk, which usually reside in faeces and water. The use of bulk-tank milk and milking machine filter provides a better picture of the dairy conditions during the milking phase, which is among the most delicate ones in dairy farming. The implementation of this combined analysis protocol can be very important also for the protection of human health, as raw milk is used for fresh cheeses (es crescenza) and these pathogens can infect humans, even

if their risk is currently underestimated. This innovative protocol for the monitoring of pathogens like *E. coli* through a "bulk approach" within a risky dairy activity can support an earlier diagnosis before than being performed on individual animals.

The above molecular techniques, even if performed on two clearly different types of farmed animal species (honeybees vs dairy cows), are both relevant for typing, intended as the identification of different species within a genus, or strains within a species. Molecular typing, in particular, can facilitate the differentiation of isolated pathogens compared to classical methods based on the phenotype, such as serotyping or the antibiogram, and at the same time is rapid, highly reproducible, easy to perform and interpret (Sabat et al. 2013). The use of the correct molecular approach on a certain sample provides more reliable results, which would not be provided at the same extent with the classical methodologies, helping the actual monitoring of the risk in the field. In particular, certainly regarding E. coli but potentially also *Nosema* spp, the virulence factor profiling has the potential to clarify if the target strain is dangerous or not. Such factors can be present in the bacterial genome or at the plasmid level, both detectable using conventional PCR/Real Time PCR; or at genomic level with mutations, which are detectable by a Real Time PCR coupled with HRMA. The latter molecular technique, can simultaneously genotype the pathogen and detect a specific antibiotic resistance patterns. Currently, antibiotic resistance is a very important issue, because the excessive use of antibiotics and/or in an incorrect way is leading several pathogens to loose their antibiotic susceptibility. The three major categories that antibiotic resistance include are: change in the binding site between the target gene and the antibiotic, in this way there is a inhibition of antibiotic function; production of enzymes that destroy the antibiotic and decreased concentration of the antibiotic in the cytoplasm (Walsh 2000). The underlying molecular mechanisms include small mutations of the antibiotic target genes (mostly through SNPs and/or insertion and deletion of a small number of bases) or acquisition of entire gene traits mainly at the plasmid level. In the first case, Real Time PCR coupled to HRMA would be a very profitable technique in the routine analysis to detect resistance linked to specific SNPs and other small mutations at the gene level, as briefly reported among the activities of the present thesis regarding the *gyrA*-mediated antibiotic resistance to fluoroquinolones in *Staphilococcus pseudointermedius* (Loiacono et al. 2017). In the second case, gene-specific PCR could be a very

valuable tool for field monitoring. Overall, both molecular approaches can help to avoid selection of resistance strains and decrease the use of antibiotics.

As a conclusion, the present work clearly shows that molecular typing, especially if properly integrated with standard techniques, provides a suitable tool both to understand the risk linked to a specific pathogen and to help the design of control strategies even within very different animal product supply systems like beekeeping and dairy cow farming.

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Appendix

During my PhD course, I also took part in the following two research projects:

1. **High-resolution melting analysis of gyrA codon 84 and grlA codon 80 mutations conferring resistance to fluoroquinolones in Staphylococcus pseudintermedius isolates from canine clinical samples**

Staphylococcus pseudintermedius is an opportunistic pathogen of dogs and cats. A high-resolution melting analysis (HRMA) protocol was designed and tested on 42 clinical isolates with known fluoroquinolone (FQ) susceptibility and gyrA codon 84 and grlA codon 80 mutation status. The HRMA approach was able to discriminate between FQ-sensitive and FQ-resistant strains and confirmed previous reports that the main mutation site associated with FQ resistance in S. pseudintermedius is located at position 251 (Ser84Leu) of gyrA. Routine, HRMA-based FQ susceptibility profiles may be a valuable tool to guide therapy. The FQ resistance-predictive power of the assay should be tested in a significantly larger number of isolates.

The results were published in the following paper:

18S rRNA V12 and V9 metabarcoding analysis of night and day diet of *Sardina pilchardus* from the Bay of Biscay.

Metabarcoding approach is highly sensitive and able to detect traces of DNA in community samples, that methodology due to the combination of the highest taxonomic resolution in a cost- and time-effective. The 18S V12 and V9 metabarcoding was used to characterize the day and night diet of European sardine (*Sardina pilchardus*) from the Bay of Biscay. The data set obtained is able to discriminate the different alimentation of the fish.

The results are being prepared for publication as follows.

Dell'Orco F., Albaina A., Aguirre M., Abad D., Zarraonaindia I., Estonba A. 18S rRNA V12 and V9 metabarcoding analysis of night and day diet of *Sardina pilchardus* from the Bay of Biscay. Manuscript in preparation.

Attendance to courses, seminars, workshops and scientific meetings (AA 2015-17):

- Visiting period: From June 25th to July 8st 2017 to Bieneinstitut Kirchhain, in Kirchhain (Germany) under the surveillance of Dr. Ralph Büchler. The research activities were focused to learn and apply apical breeding techniques to be used in the genetic selection of *Varroa* resistance. In particular, training will focus on the detection of phenotypes such as production, resistance to disease and docility.
- Visiting period: From October 11th 2016 to January 31th 2017 to University of Basque Country/ Euskal Herriko Unibertsitatea (UPV / EHU), in Bilbao (Spain) under the surveillance of Prof. And one Estonba. The research activities were to learn and to apply genetic techniques, in the field of metabarcoding approach.
- Obligatory course: "Communication 3", held by Dott. Ettore Galanti, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: January 21 2016, days in February 8 and 22 in total 12 hours
- Obligatory course: "Statistica 3", held by Dott.ssa Elisabetta Sala, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: from November 21 2016 to Dicember 15 2016, in total 12 hours;
- Scientific Meeting: "CRISPR genome editing: From high-throughput screening to disease models", to Lundsgaard auditorium, Panum Institutet, Copenhagen (Denmark), September 22-23 2016, in total 11 hours;
- Scientific Meeting: "Seventh EurBee Congress of Apidology", to university of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania, September 7-9 2016, in total 24 hours;
- Workshop: "Le pesti delle api: attualità e prospettive", to faculty of Veterinary Medicine, UNIMI, February 26 2016 for 8 hours;
- Obligatory course: "Inglese 2", held by Dott.ssa Gigliola Canepa with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: from January 29 2016 to April 15 2016, in total 40 hours;
- Obligatory course: "Statistica 2", held by Dott.ssa Elisabetta Sala, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: from February 1 2016 to April 04 2016, in total 24 hours;
- Obligatory course: "Communication 2", held by Dott. Ettore Galanti, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: days in march 2-9-23 2016, in total 16 hours;
- Ongoing evaluation, January 18 2016, time 9:30. Lesson class 12, Faculty of Medicine Veterinary, UNIMI, 5 hours;

- Ongoing evaluation: Vas Day, days in June 8– 10 2016, in total 18 ore.
- Workshop: "Emergenza da *Aethina tumida* in apicoltura: conoscenze attuali e prospettive per il coontrollo", to Università degli studi di Milano, faculty of Veterinary Medicine, october 26 2015 for 8 hours;
- Theoric course:" Corso di allevamento di api regine", by Elio Bonfanti in Lecco. Period of lessons: Days 20 and 21 february 2015, 8 hours a day, 16 hours in total;
- Course of "VSH- varroa-sensitive hygiene", Aspromiele Beekeepers Association, Alessandria. March 06 2015, for 8 hours;
- Course: "Next Generation Sequencing procedures", at Insubria Università of Busto Arsizio (Va). Period of lessons: Days June 15-16-17 2015, 8 hours a day, 24 hours in total;
- Course: "Genetic association studies in field of animal infectious disease, held by Dott.ssa Giulietta Minozzi, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: in June 17-18-23-24 201), in total 16 hours;
- Course: "Imaging", held by Dott.ssa Valentina Lodde, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: days in September 14- 15-17-18-21 2015, in total 16 ore;
- Obligatory course: "Statistica 1", held by Dott.ssa Elisabetta Sala, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons from November 24 2014 to February 09 2015, in total 32 hours;
- Obligatory course: "Banche dati" held by Dott.ssa Angela Moccia, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: days in June 04 - 11 2015, every day 4 hours;
- Obligatory course: "Inglese 1", held by Dott.ssa Gigliola Canepa with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: days January 26 to 18 2015, in total 40 hours;
- Obligatory course: "Comunicazione e Public Speaking", held by Dott. Ettore Galanti, with passing the final exam. Faculty of Medicine Veterinary, UNIMI. Period of lessons: days in march 4-11-25 2015, in total 16 hours;
- Ongoing evaluation, January 22 2015, time 9:30. Lesson class 10, Faculty of Medicine Veterinary, UNIMI, 5 hours;
- Ongoing evaluation: Vas Day, days in July 15 – 16 – 17 2015, in total 18 hours.

Publications:

Dell'Orco F., Formato G. Mortarino M. Diagnostic tools for honeybee nosemosis: an update. Manuscript accepted to Veterinaria Italiana

Dell'Orco F., Loiacono M., Albonico A., Zanzani S., Cersini A., Formato G., Colombo M., Mortarino M.. Detection and quantitation of Nosema ceranae in honey bees by real time PCR coupled to High Resolution Melting Analysis**.** Submitted manuscript**,** Parasitology Research.

Dell'Orco F., Facchini E., Rizzi R., Mortarino M. The expression ratio between Act5 and Obp-4 is a marker of hygienic behavior in honeybees. Manuscript to be submitted.

Dell'Orco F., Albaina A., Aguirre M., Abad D., Zarraonaindia I., Estonba A. 18S rRNA V12 and V9 metabarcoding analysis of night and day diet of *Sardina pilchardus* from the Bay of Biscay. Manuscript in preparation.

Mortarino M., Blonda M., Zanzani S., **Dell'Orco F**., Facchini E., Rizzi R. Neem (Azadirachta indica) oil as a sustainable tool against Varroa destructor. Proceedings of the 26th Conference of the Word Association for the Advancement of Veterinary Parasitology. Kuala Lumpur, 4th-8th September 2017.

Loiacono M., Martino P. A., Albonico F., **Dell'Orco F**., Ferretti M., Zanzani S., Mortarino M.. 2016. High-resolution melting analysis of gyrA codon 84 and grlA codon 80 mutations conferring resistance to fluoroquinolones in Staphylococcus pseudintermedius isolates from canine clinical samples. Journal of Veterinary Diagnostic Investigation, 29(5), 711–715. https://doi.org/10.1177/1040638717712330.

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Formenti N., Albonico F.**, Dell'Orco F.**, Loiacono M., Trogu T., Pombi M., Cavallero S., Ferrari N., Mortarino M., Lanfranchi P.. 2016. Multiplex PCR assay for the identification of Haemonchus contortus and Teladorsagia circumcincta in alpine chamois (Rupicapra r. rupicapra). Oral communication, XXIX Congress of the Italian Society of Parasitology, page 109, 21-24.

Dell'Orco F., Facchini E., Cilia G. Rizzi R., Mortarino M.. 2016. Candidate molecular markers of hygienic behaviour in honeybees (Apis mellifera): an expression study. International Journal of Health, Animal Science & Food Safety, Vol. 2s, Proceedings of the Veterinary and Animal Science Days.

Dell'Orco F., Loiacono M., Albonico, F., Minozzi G., Pagnacco, G., Mortarino M.. (2015). MicroRNA expression correlated with hygienic behaviour in honeybees. International Journal of Health, Animal Science & Food Safety, Vol. 2s, Proceedings of the Veterinary and Animal Science Days.