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Thesis’ title: Modelling the risk of bacterial infections, within the framework of the evaluation of the health and welfare of dairy cows

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Abstract

Among the main strategies proposed by FAO for sustainable livestock production are improving the efficiency of livestock farming and the use of local resources. This research work addressed both challenges with two case studies.

The first deals with the evaluation of four health indicators (HI) limiting the productivity of dairy cattle farming, and it used data collected within the framework of the dairy herd improvement program (DHI) in the Piedmont region over a 5-year period. The incidence, prevalence and cure rate of mastitis, the incidence of hyperketonemia, the duration of the calving interval and the culling rate of fresh cows were estimated for more than 1200 Piedmontese herds. For each HI, risk factors acting at individual animal level were evaluated and the impact of farm management was estimated. The geographic distribution of each HI was explored to identify areas where the risk was higher and *foci* of aggregation of high or low rates of HIs. Finally, an attempt was made to use the indicators as an early warning system for the risk of poor welfare (PWR).

The large dataset allowed a precise estimation of individual-cow risk factors and to determine that at least 20% of the risk, depending on the HI, derived from herd management. DHI did not include information about herd type, limiting the comprehension of its impact on risk factors. On the other hand, the used model was general enough to permit herd benchmarking on a regional scale. The results suggest that along with high-producing herds in lowlands, other worse-performing and less controlled herds remained in marginal territories. The lack of information about those last led to the poor performance of HI as screening tools for PWR, as more is needed to train the detection model. However, HI can be used to confirm the absence of PWR thanks to the high specificity of the analysis.

The second case study regarded the comparison of dairy cattle farms in marginal areas of the northern Apennines. Sixteen farms from three neighboring areas were analyzed, collecting information on herd management, antimicrobial use and the prevalence of intramammary infections. From cows' milk analysis, mastitis pathogens were isolated and subsequently evaluated for susceptibility to antimicrobials.

Although it was not possible to distinguish geographic areas based on interview results, they evidenced some differences and a widespread lack of awareness about herd health problems. Antimicrobial use was significantly higher in the highest producing herds, and intramammary infections were the most

frequent reason for antimicrobial administration. Nonetheless, antimicrobial resistance was at a low level in all herds. The microbiological results highlighted that pathogens with different epidemiology characterized different geographical areas, not due to environmental factors, but to management characteristics of the farms. The least productive farms were characterized by the presence of contagious pathogens likely because they failed to control the spread of the infection. The factor most associated with this unequal distribution of pathogens was the employment of specialized veterinarians to counsel and help managing the herds.

In conclusion, to improve the efficiency of farms located in marginal territories, it would be necessary to increase farmers' awareness regarding herd health problems. For this purpose, DHI and animal welfare assessment seemed a suited strategy.

Summary

List of abbreviations

A

AA = Assessment Area

ABM = Animal Based Measure

AIA = Associazione Italiana Allevatori

AIC = Akaike Information Criterion

AL = Alessandria province

AMC = Amoxicillin- Clavulanic acid combination

AMP = Ampicillin

AMR = Antimicrobial Resistance

ARA = Associazione Regionale Allevatori

AST = Antimicrobial Susceptibility Testing

ATI = Antimicrobial Treatment Incidence rate

ATIR = Antimicrobial Treatment Incidence rate Ratio

B

BHB = Beta-Hydroxy Butirate

$[BHBA_{blood}]$ = Beta-Hydroxy Butiric Acid concentration in blood

$[BHBA_{milk}]$ = Beta-Hydroxy Butiric Acid concentration in milk

BIC = Bayesian Information Criterion

BLV = Bovine Leucosis Virus

BPIV3 = Bovine Para-Influenza Virus-3

BRSV = Bovine Respiratory Syncytial Virus

BVDV = Bovine Viral Diarrhoea Virus

C

CASFM = Comitè de l'Antibiogramme de la Sociètè Francaise de Microbiologie

CEQ = Cefquinome

CEZ = Cephazolin

CFI = Days to first service

CFT = Ceftiofur

CFU = Colony Forming Units

CI = Confidence Interval

CLI = Days to conception

CLSI = Clinical and Laboratory Standard Institute

CM = Clinical Mastitis

CN = Gentamycin

CO_2e = Carbon dioxide equivalent

CReNBA = Centro di Referenza Nazionale per il Benessere Animale

D

DHI = Dairy Herd Improvement program

DIM = Days In Milk

E

E = Erythromycin

EBSR = Empirical Bayes Smoothed Rate

EEA= European Environmental Agency

EFSA = European Food Safety Agency

ENR = Enrofloxacin

F

FAO = Food and Agriculture Organization of the United Nations

FAWC = Farm Animal Welfare Council

FPCM = Fat and Protein Corrected Milk

FTIR = Fourier-Transformed Infra-Red spectroscopy

F:P = Fat to Protein ratio

G

GEE = Generalized Estimating Equation

GHG = Green House Gasses

GLEAM-2 =

GOF = Goodness Of Fit

GWP = Global Warming Potential

H

HI = Health Indicator

HK = Hyperketonemia

HL-CN = High-Level Gentamycin

I

I = susceptible to Increased dosage

IBRV = Infectious Bovine Rhinotracheitis Virus

ICAR = International Committee for Animal Recording

ICI = Inter-Calving Interval

ICL = Integrated Complete Likelihood

IMI = Intra-Mammary Infection

IP = Incidence proportion

IPCC =

IR = Incidence Rate

IRR = Incidence Rate Ratio

K

KN = Kanamycin

L

LCA = Life-Cycle Assessment

LDA = Linear Discriminant Analysis

LPA = Latent Profile Analysis

LU = Lucca province

M

MDR = Multi-Drug Resistant

MFA = Multiple Factor Analysis

MIC = Minimum Inhibitory Concentration

N

NASM = Non-Aureus Staphylococcal and Mammalicoccal species

N-ABM = Non-Animal Based Measure

O

OR = Odds Ratio

OXA = Oxacillin

P

PDO = Protected Designation of Origin

PEN = Penicillin

PIR = Pirlimycin

PM = Particulate Matter

PP = Prevalence Proportion

PWR = Poor Welfare Risk

R

R = resistant

RE = Reggio Emilia province

RF = Rifampicin

RR = Relative Risk

S

S = susceptible

SCC = Somatic Cells Count

SCM = Sub-Clinical Mastitis

SDR = Standard Disease Rate

SMR = Standard Mortality Rate

SXT = Trimethoprim - Sulphadiazine

T

TD = Test Day

V

VWP = Voluntary Waiting Period

W

WHO = World Health Organization

WOAH = World Organization for Animal Health

WPC = Welfare Promoter Characterization

WQ = Welfare Quality®

Foreword

This three-year PhD project originated from the observation that one of the most dairy-productive area in Europe, the Po Valley in northern Italy, is among the most polluted European areas (EEA, 2024). Livestock production has a relevant role in pollution, hence a change is needed approaching a more sustainable food production chain (FAO, 2023). The Food and Agriculture Organization of the United Nations (FAO) Strategy on Climate Change suggests that the livestock sector should challenge climate actions and simultaneously address the increasing demand for animal products (FAO, 2023). Among the five actions that FAO identified to meet the ambitious objective of providing food for all with a reduced impact on the environment, Action 1 states that it is necessary to enhance livestock production efficiency and resource use (FAO, 2019).

Boosting livestock production efficiency is an aim that can be addressed in several ways. In the dairy cattle sector, reducing the incidence of diseases that compromise the health of the cow will improve milk production efficiency. Indeed, FAO provides the GLEAM 2 simulation model, which evidences how cows raised in modern welfare-oriented farms tend to produce more and more efficiently, with a lower impact on the environment (FAO, 2024b). However, the FAO itself states that farming techniques should be tailored to the local needs and resources. Hence, at local scale, cow breeds that best valorize local resources should be preferred. In many livestock-dense areas of Europe, large portions of land are intended for feed production, with growing concerns for loss of biodiversity and food *vs* feed competition. Although only around 40% of grasslands are suitable for crop production because of water, terrain, and nutrient restrictions, the point raised by many environmental protection organizations remains of interest (Pullar et al., 2011). A possible answer to this issue is again suggested by FAO, as part of the Action 1 statement. The livestock sector should boost the use of local resources that are currently unexploited for other uses. In Italy, almost one-third of the territory is made up of mountains, but the livestock production is concentrated in plains. Mountain herds suffered abandonment like all other productive activities in remote mountain areas and they are almost disappearing to date (Bakudila, 2018)

In the past, productivity growth has mostly responded to increasing consumer demand, rather than to climate considerations. Up to date, current circumstances ask for strategies towards the dairy sector to become more sustainable, particularly where livestock serve social and economic purposes other than production.

In 1987, the Bruntland Report (United Nations World Commission on Environment & Development) defined sustainability as

> "meeting the needs of the present without compromising the ability of future generations to meet their own needs".

This definition has remained the most widely adopted and highlights the imperative of exploiting resources at a rate that does not exceed their natural replenishment, as a means to ensure food security. According to this interpretation, given the existence of millions of people experiencing hunger globally, food production could be considered unsustainable when evaluated against the first half of the definition (FAO, 2024c).

The Global Agenda for Sustainable Livestock provided a more livestock-centered definition of sustainability:

> “Livestock sustainability refers to production approaches that simultaneously meet long-term conditions to ensure society’s food and nutrition security, livelihoods and economic growth, animal health and animal welfare and stable climate and efficient resource use (the four livestock sustainability domains) in order to contribute to sustainable food systems." (FAO, 2024a)

Based on this definition, the sustainability of a food system does not simply depend on producing sufficient food without jeopardizing long-term resources but upon delivering food that responds to people's nutritional and socioeconomic needs, while respecting animals and the environment.

At a global scale, the sustainability of cattle farming is currently one of the most debated food production issues. Indeed, although it provides food of high nutritional value, generates economic gains, and socially benefits communities, a frequently posed question concerns whether the consumption of milk and meat is inherently unsustainable (Cockroft, 2015). A comprehensive answer to the question is beyond the purpose of this thesis, so the discussion focuses on the three pillars of sustainability, i.e., economic viability, social responsibility, and environmental stewardship, as they pertain to the dairy production system. Given the absence of a universal solution, individual production systems must be adapted to the specific context of available resources, climate, cultural practices, and market dynamics to achieve a balance among these pillars and ensure long-term sustainability. Moreover, no static solution exists either, as in a climate changing future, adverse meteorological events will challenge food production more often. Climate change has already challenged livestock production and will likely continue, driven by factors such as increased heat, drought, floods, pests, diseases, and declining soil health. Southern regions of Europe are expected to be disproportionately affected, primarily due to heightened temperatures and water scarcity. (EC, 2024a).

Hence, a sustainable dairy sector should meet people's nutritional needs, ensuring economic and social benefits to local communities, without jeopardizing natural resources or exploiting land useful for crop production, and possibly ameliorating the environmental conditions towards a more climate-resilient system. The only way to tackle such an ambitious goal is to break it down analytically into smaller research questions that can be answered. First, dairy herds benchmarking should be acknowledged. to permit the individuation of critical aspects in a certain area. Then, limits towards a more sustainable dairy production should be individuated.

In such a framework, the academia can play an important role, using scientific research as a driver of change. However, this project only aimed at assessing the state of the art of dairy herds, as a first step to evaluating the most critical point for improving dairy production efficiency and enhancing its sustainability. For this purpose, two case studies were evaluated.

The first case study regards the dairy herds of Piedmont region in the Po Valley. A retrospective longitudinal study was performed to benchmark herds and evaluate the most critical aspects and geographic areas in regards of several health and welfare indicators (Bellato et al., 2023).

The second case study is about mountain herds in marginal areas, as they might represent a viable solution for improving sustainability of dairy production (Bellato et al., under review). Animal husbandry, farm management, and antimicrobial use were evaluated by interviewing farmers. In addition, milk samples were collected and analyzed to determine the prevalence of intramammary infections in dairy cows.

Benchmarking dairy herds' health conditions: the Piedmont region case study

As seen previously, there is evidence that improving dairy herd's health would provide economical, nutritional, and environmental benefit (Özkan et al., 2022). The dairy herd's health is an umbrella definition that includes several aspects of cow's health. Although a comprehensive approach to animal health and welfare would provide a more precise picture of the herd's condition, a trade-off exists between measurement accuracy and cost-effectiveness of such investigation. The most accurate way of measuring the herd's health condition would be through an audit. However, for large scale studies, it would be unsustainable in terms of costs and workload. Fortunately, among the conditions that affect the most dairy cows' productivity, there are some that are more easily monitored even from remote, like mastitis, hyperketonemia and a prolonged inter-calving interval, and a practical approach is to gather available data, regularly collected by breeders' associations as part of Dairy Herd Improvement programs (DHI). They include milk yield volume, fat, protein and lactose percentage, and somatic cell count (SCC).

To benchmark dairy herds in the Piedmont region, four health indicators (HI) were estimated, namely (1) the incidence rate, prevalence, and probability of recovery of mastitis; (2) the incidence proportion of ketosis; (3) the duration of inter-calving interval; and (4) the rate of fresh cow removal. The estimation would consider both the cow-level and herd-level risk factors, providing risk estimates for the first and evaluating the weight of the second ones.

Introduction

Piedmont region

The Piedmont region is in the north-westernmost part of Italy, sharing its border with France on the west and Switzerland on the north. It has a population of approximately 4.4 million people (7.5% of the total), with an average population density of 170 inhabitants per km^2. It represents the second widest region of Italy, with a surface of 25,387 km^2 (8.4% of the whole country) (ISTAT, 2015). More than half of its territory is mountains (circa 51.5%), where there are approximately one quarter of all cattle herds of the region (ISTAT, 2010).

In Italy, the number of dairy cows in the last five years decreased from 1,643,117 to 1.574.406 but their percentage compared to the overall number of adult cattle remained the same throughout the entire period (60.2 ± 0.6%). The most part of dairy cows are raised in the Po Valley and, more in general, in the north part of the country. This proportion increased from 73.3% in 2019 to 77.2% in 2023. In the Piedmont region, in the last five years, the overall number of dairy cows oscillated between 136,186 in 2019 and 145,243 in 2021, being on average 8.8 ± 0.3% of the national dairy cows' population (ISTAT, 2024).

Table 1. *Number of cattle in Italy, north-Italy and Piedmont. Number of cattle older than two years in Italy, north-Italy and Piedmont in the last five years. Data from the last agriculture census (ISTAT, 2024)*

Area	**Production**	**2019**	**2020**	**2021**	**2022**	**2023**
Italy	All cattle (> 2 y. o.)	1,783,413	1,774,073	1,755,778	1,759,687	1,709,076
Italy	Dairy	1,643,117	1,638,382	1,609,948	1,631,128	1,574,406
North Italy	All cattle (> 2 y. o.)	1,783,413	1,774,073	1,755,778	1,759,687	1,709,076
North Italy	Dairy	1,204,760	1,204,048	1,192,054	1,264,355	1,215,675
Piedmont	All cattle (> 2 y. o.)	339,687	346,346	340,986	353,981	350,979
Piedmont	Dairy	136,186	143,284	145,243	143,414	140,971

Herd health monitoring

Mastitis

To date, mastitis remains the most common cause of morbidity in adult dairy cows (Halasa et al., 2007; Hogeveen et al., 2011). It causes economic losses due to therapy, reduction of milk production, milk discard, reproduction failure, and increased culling probability (Rollin et al., 2015). Mastitits cost varies around the world, but basically depends on the same direct and indirect components. Direct cost components include treatment, reduced milk sales due to discarded milk, labour, veterinary consultancy and intervention, loss of cow's value and potentially fatal outcome, lower milk price. Among the indirect cost, there are reduced milk yield, increase probability of repeated cases and of culling, spread to other cows of intramammary infection, and even loss of genetic potential due to forced culling. Preventative costs exist, too, including the maintaining of hygienic cow accommodation, appropriate milking routine, regular DHI or screening, potential segregation of cows based on udder health status (Cockroft, 2015).

Although clinical mastitis is immediately apparent, asking for prompt veterinary intervention and being easily evaluated in terms of incidence and prevalence, it is the subclinical mastitis that is considered to have a greater economic impact on dairy herds (Aghamohammadi et al., 2018; Busanello et al., 2017). Indeed, milk production decreases in cows with subclinical mastitis, as the loss is directly proportional to the inflammation. Subclinical mastitis represents the largest part of mastitis cases, being circa three times more prevalent than clinical mastitis (38-45% compared to 12-19%) (Krishnamoorthy et al., 2021). Therefore, for a comprehensive evaluation of udder health, it is essential to estimate subclinical mastitis incidence rate along with prevalence proportion, and the probability of recovery. (Busanello et al., 2017)

SCC in quarter, composite, and bulk tank milk, has been used from a long time to monitor subclinical mastitis both at the herd level (Bradley et al., 2007; Dohoo et al., 1984; Hiitiö et al., 2017; Østerås and Sølverød, 2009; Schukken et al., 2003) and represents an inexpensive yet useful method to estimate mastitis prevalence and incidence since it is positively correlated with inflammatory changes. Herd SCC, measured on bulk tank milk, is used as an epidemiologic and milk quality criterion. Herd SCC < 400 x 10^3cells/mL for raw cow's milk is the threshold set by Council Directive 92/46/EEC (EU, 1992) and Regulation (EC) 853/2004 of the European Parliament and of the Council (EU, 2004) for placing on the market of raw milk, heated milk and milk-based products. However, a herd SCC $< 200 \times 10^3$ cells/mL are considered desirable.

At individual level SCC is used for calculating performance indicators, based on a predefined SCC threshold and the comparison of the cows' SCC over the course of two subsequent tests. It still is a matter of debate which threshold is the most appropriate (Bradley and Green, 2005; Dohoo et al., 1981; Fauteux et al., 2014; Petzer et al., 2017a). Milk quality worsening has been observed with SCC as low as 100×10^3 cells/mL, but an SCC $\geq 200 \times 10^3$ cells/mL in a cow's composite milk is usually considered indicative of a high probability of infection. The sensitivity of this threshold ranges between 73 and 89% sensitivity, with 75 to 86% specificity (Dohoo and Leslie, 1991; Dohoo, 2001; McDermott et al., 1982). However, its accuracy varies based on individual characteristics, e.g., in primiparous cows 15.8% sensitivity and 84.4% specificity were recently reported (Lipkens et al., 2019). To calculate incidence rate, new cases should be distinguished from previously infected cows (i.e., chronic cases). However, SCC fluctuates over time, due to the inconstant excretion of some pathogens (e.g., *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium bovis*, etc.), and there is no consensus yet on the time that must elapse between two subsequent tests to consider an SCC increase from below 200×10^3 to above 200×10^3 cells/mL as a new case (Bradley et al., 2007; Petzer et al., 2017b).

Table 2. *Performance indicators calculated using somatic cells count (SCC) on individual cow's milk.*

Performance indicator	**Definition**
New infection	SCC $\geq 200 \times 10^3$ cells/mL at the most recent testing given that the SCC was $< 200 \times 10^3$ cells/mL at the previous testing
Percentage of new infections	The number of cows with a new infection divided by the number of cows in the lactating herd with at least two testings in the current lactation
Risk of new infection	the number of cows with a new infection divided by the number of cows that were not infected (SCC $< 200 \times 10^3$ cells/mL) at the previous testing

Performance indicator	Definition
Recovery	SCC $< 200 \times 10^3$ cells/mL at the most recent testing given that the SCC was $\geq 200 \times 10^3$ cells/mL at the previous testing
Recovery percentage	The number of cows that cured divided by the number of cows in the lactating herd with at least two testings in the current lactation
Recovery rate	The number of cows that cured divided by the cows that were infected (SCC $\geq$ 200,000 cells/mL) at the previous testing
Clean-cow percentage	The number of cows that had a low SCC ($< 200 \times 10^3$ cells/mL) at both the previous and the most recent testing
Chronic case percentage	The number of cows that were infected (SCC $\geq 200 \times 10^3$ cells/mL) at both the previous and the most recent testing
Fresh-cow mastitis percentage	The number of cows with a high SCC ($\geq 200 \times 10^3$ cells/mL) at the first testing after calving divided by the number of cows with a first test SCC after calving

Hyperketonemia

In dairy herds, hyperketonemia (HK), also known as ketosis, represents a common and costly problem occurring in early lactation phases (Carvalho et al., 2019; Tatone et al., 2016). Its onset depends on the transition period (3 weeks before and 3 weeks after calving), when requirements for milk synthesis rapidly increase (Caixeta and Omontese, 2021; Mezzetti et al., 2021; Tufrelli et al., 2024). During this period, when the fetus growth and the development of the mammary gland increase the cow's nutritional needs there is up to a 40% reduction in dry matter intake due to rumen's volume reduction (Ingvartsen, 2006; Mezzetti et al., 2021). Moreover, at the peak of lactation, within 6 weeks from calving, depending on the breed, the milk yield can quadruple in a few days. Given that milk contains an average of 4.1% fat, 3.4% protein, 4.6% lactose, and 0.7% ash, during the lactation peak, a cow producing 40 kg of milk a day secrete around 1.6 kg of fat, 1.3 kg of protein, 1.8 kg of lactose, and 0.3 kg of minerals (Borchardt et al., 2022). In summary, during this phase, dairy cows experience a negative energy balance which they cannot compensate increasing feed intake (Tamminga, 2006;

Buonaiuto et al., 2023; Gáspárdy et al., 2004). Therefore, cows need to mobilize body resources, i.e., fat and muscle tissue, losing up to 20 kg of muscular mass and 57 kg of fat during early lactation (Komaragiri et al., 1998).

As a result of the negative energy balance in the early lactation phases, the serum concentration of ketone bodies increases. Clinical HK, with blood beta-hydroxybutyrate (BHB) concentration equal or higher than 1.2 mmol/L, severe damage to the cow's health, metritis, displaced abomasum, and eventually culling can occurr (Duffield et al., 2009; McArt et al., 2012; McArt et al., 2015). The metabolic stress affects also the immune system and increase the inflammatory state. As a result, we might expect a possible relation between hyperketonemia and infectious diseases like mastitis (Arfuso et al., 2023). Subclinical HK has a negative effect on cow health, too. It determines economic losses due to treatment cost, reduced milk yield, increased risk of disease and increased risk of fresh-cow removal from the herd (McArt et al., 2015). Nonetheless, subclinical HK often remains unnoticed (Denis-Robichaud et al., 2014; Tatone et al., 2017).

The gold standard for HK diagnostics is the laboratory evaluation of serum or whole blood BHB concentration, but it is time-consuming, and other methods prevail in clinical practice, e.g., cow-side tests on blood, urine, or milk (Duffield et al., 2009). How closely the concentration of ketones in urine follows that in blood is not fully understood, and likely sensitivity and specificity vary reflecting the physiology of ketone production and elimination, among other factors. High-throughput infrared technology allows to measure milk BHB concentrations, hence it has been included in DHI (van der Drift et al., 2012). However, HK could be monitored through fat to protein ratio (F:P), too (Jenkins et al., 2015). This method, although being less accurate than BHB concentration, allows wide scale comparison and suits the purpose of epidemiological studies aimed at monitoring HK incidence, herd benchmarking, and assessing the risk in different geographic regions (van der Drift et al., 2012; Santschi et al., 2016; Tatone et al., 2017).

Inter-calving interval

For most dairy breeders, the reproductive-efficiency goal is to reach one calving per cow per year. Time interval measurements are commonly used as reproductive performance indicators, and many of them are calculated from the calving date, like inter-calving interval (ICI), days to first service (CFI), and days to conception or last insemination (CLI) (Hultgren and Svensson, 2010). Their main limitation is the introduction of a selection bias, since they can only be calculated for cows that have a subsequent calving or are either inseminated or checked for pregnancy. In fact, cows that are no longer inseminated, those that fail to conceive or to calve, and those culled because of fertility problems are excluded from the analysis. Therefore, such indicators are not representative of the herd's reproductive status and should be treated as a censored trait, considering the nonrandom scoring of the indicators (Olori et al., 2002). This issue can be overcome by measuring the proportion of pregnant cows at specific time intervals after calving and using survival analysis on the time-to-event data, where all the information is used, even that on animals not experiencing the event (Sheldon et al., 2006).

In addition, the reproductive performance indicators do not consider the different management strategies, such as the herd's voluntary waiting period (VWP) (Crowe et al., 2014; Remnant et al., 2018). The VWP is the time elapsing from calving to the decision that the cow is ready for breeding again. It necessarily gives the cow some time to resume ovarian cyclicity, but its length can be decided in advance by the breeder following farm management needs. It is suggested to span between 50-60 days, but it varies from 30 and 90 days (DeJarnette et al., 2007; Löf et al., 2012). Also, the VWP might vary within the herd according to cows' parity and milk yield (Petersson et al., 2008). Given the VWP's variability, the commonly used indicators reflect the reproductive performance of the cow as well as management strategies. Indeed, several factors jeopardizing the reproductive performance of the herd, e.g., infections, inadequate nutrition, ovarian cysts, endometritis, puerperal collapse, failure in estrus detection, do not depend on voluntary farmer's choices. Cows with adequate body conditions and efficient immune systems tend to become pregnant earlier (Berry et al., 2006; Remnant et al., 2018), while long ICI could be due to many reproductive disorders and has been associated to higher mortality rates (Crowe et al., 2014; Reimus et al., 2018). Therefore, depending on the target of the monitoring, different performance indicators might be suited, and the best indicator to measure reproductive performance is not necessarily the best indicator to evaluate reproductive management (Hultgren and Svensson, 2010; Löf et al., 2012).

Fresh-cow removal rate

The removal rate (i.e., the percentage of cows removed or culled) is important for evaluating dairy herd profitability. When dairy cows are removed too often or too quickly, replacement costs increase, while keeping cattle for too long might undermine milk production, reproduction, and genetic improvement (Hadley et al., 2006).

Culling is the act of identifying and removing a cow from a herd, and it is divided into voluntary and involuntary culling. Voluntary culling could represent a control or preventative strategy against undesired traits and defects or simply the strategic replacement of elder cows with heifers with superior potential (Dijkhuizen et al., 1985; Wakchaure et al., 2015) while maintaining herd size constant. At the herd level, voluntary culling is influenced by several management factors other than replacement plan, like milk quotas, and market prices of milk and beef (Beaudeau et al., 2000; Haine et al., 2017). At the cow level, the most common reasons for voluntary culling are old age, genetic amelioration, and low milk production (Admczyk et al., 2016; Weigel et al., 2003). On the other hand, involuntary culling happens when a cow is removed even though it was not planned to, and it accounts for most dairy cow removals (Compton et al., 2017; Wakchaure et al., 2015). The main reasons for involuntary culling are infertility, mastitis, lameness, but also metabolic diseases or disorders, injury, and accidents (Admczyk et al., 2016; Weigel et al., 2003, Yanga and Jaja, 2022).

Many aspects affect the survival probability of a dairy cow, both at cow and herd level. It varies greatly depending on the parity, being higher in first lactation cows and decreasing with age because of their productivity and market price (Langford and Stott, 2012). There is a trade-off between dairy cattle lifespan and herd's profitability, but it was observed that improving cow welfare over the long term increases the mean longevity of the herd and decreases the incidence of chronic diseases like mastitis, lameness, or infertility (Langford and Stott, 2012). The survival probability depends on lactation phase, too, as it exceeds 85% during the first 100 days after calving regardless of the parity, while it decreases below 75% in late lactation (Rilanto et al., 2020). At the herd scale, high producing herds are more prone to cull for reproductive issues but less for mastitis. On the other hand, low-producing herds have a lower overall culling rate (Hadley et al., 2006; Smith et al., 2000). Several studies have been conducted to determine the most relevant reasons for culling dairy cows, and most of them identified reproductive problems, low milk yield, and mastitis (Bascom and Young, 1998; Hadley et al., 2006; Milian-Suazo et al., 1989).

Also, the decision to cull a cow depends on many factors, e.g., the severity and the time of problem's occurrence (Carvalho et al., 2019). Indeed, replacing cows is a major cost of the dairy operation, which is even higher for fresh cows (i.e., within sixty days after calving) as the lactation peak is the most profitable phase (Heinrichs and Heinrichs, 2011; McArt et al., 2012; Vergara et al., 2014). Therefore, it is plausible to hypothesize that severe health issues (e.g., injury, reproductive problems, metabolic disorders, mastitis, etc.) should occur to induce the farmer to cull a fresh cow (Overton and Dhuyvetter, 2020; Rollin et al., 2015). For this reason, monitoring the removal rate of fresh cows in a herd could serve as an indirect indicator of the occurrence of unexpected (and likely unprevented) health problems.

Dairy Herd Improvement program

Dairy herd improvement program (DHI) is a widely diffused method to monitor dairy cows' performance and health, according to their lactation phase and age. DHI are usually provided by breeders' associations, private laboratories or dairies in the form of periodical milk analysis. The most common analyses regard milk composition (i.e., lactose, fat, and protein concentration), SCC, and total bacterial load. They are frequently made available through individual cow-based test day interval sampling, typically administered monthly. Based on DHI data, milk is priced, and dairy herds of a certain area are benchmarked. Since information about cow's lactation phase and age is collected, DHI data can serve as rough measure of reproduction efficiency, too, as ICI is easily calculated. However, from DHI data it is not always possible to determine the fate of individual cows, for example when a cow is moved to a farm not participating in the DHI program and goes missing.

Beside monitoring herd's productive performance and milk pricing, DHI data also represent an invaluable source of information for epidemiological purposes, as they provide information about the health condition of the whole herd as well as individual cows (Busanello et al., 2017; Dufour and Dohoo, 2013; Reyher et al., 2011; Torres et al., 2008). Among the health indicators (HI) that can be measured from DHI, there are: mastitis, hyperketonemia, and fresh-cow removal. Each of these indicators measures a different aspect of cow's health, although being related to each other in a complex intertwining of interactions. Some health conditions are easily spotted, as they are induced from a single parameter measurement (e.g., mastitis status is derived from SCC), while others are concealed and need some calculations prior to being identified. Although DHI data might appear a comprehensive monitoring tool for dairy herds since several cow-level risk factors (e.g., parity, days in milk, milk yield, breed, etc.) can be studied, many herd-level risk factors cannot, even if they contribute to a large extent to the cows' health. Still, these factors, which are mainly related to management and the breeder's expertise, need to be considered when estimating HI.

Animal welfare

In 1979, the Farm Animal Welfare Council (FAWC) of the British Government gave a definition of animal welfare that included both physical and mental state and stated that animals kept by humans must be protected from unnecessary suffering. To analytically assess animal welfare, the FAWC listed five freedoms (FAWC, 2009) originating from the Report of the Technical Committee to Enquire into the Welfare of Animals kept under Intensive Livestock Husbandry Systems, also known as the Brambell Report, published in 1965. The five freedoms were lately amended by the FAWC (FAWC, 1993), and today they stand as:

1. Freedom from thirst, hunger and malnutrition, by ready access to a diet to maintain full health and vigour.
2. Freedom from thermal and physical discomfort, by providing a suitable environment including shelter and a comfortable resting area.
3. Freedom from pain, injury and disease, by prevention or rapid diagnosis and treatment.
4. Freedom from fear and distress, by providing sufficient space, proper facilities and the company of the animal's own kind.
5. Freedom to express normal behaviour, by ensuring conditions which avoid mental suffering.

The main critics were the focus on poor welfare and suffering without embracing the concept of positive welfare (Mellor, 2016). However, in livestock production, some pain and distress are currently unavoidable, thus the long-term goal is to minimize them through skilled and conscientious husbandry, responsible animal management, appropriate living conditions, prevention of diseases, considerate handling and transport, and humane slaughter (Animal Welfare Act, 2006).

Up to date, there is no consensus yet over the animal welfare's definition (Dawkins, 2008; Webster, 2005). The description adopted by the WOAH (formerly Office International des Epizooties) in 2008 stated that "[…] good animal welfare requires disease prevention and veterinary treatment, appropriate shelter, management, nutrition, humane handling and humane slaughter/killing". It is in accordance with the definition of "a life worth living", which, for farm animals requires good husbandry, from handling to transport, merciful slaughter and skilled and conscientious approach (Animal Welfare Act, 2006).

However, to date, the most part of legislation is still based on the five freedoms, i.e., on avoidance of unnecessary suffering and the provision of needs, which reflects a negative consideration of farming and food production. In the European Union (EU), the regulation for protection of farm animals is considered general, vague and non-homogeneous throughout Europe (Broom, 2017). In particular, for dairy cow's welfare, there are currently no specific rules (EFSA, 2012b; EU, 1998). On the other hand, public concern over livestock production has been rising, and several surveys confirmed that EU citizens consider the animal welfare an important and established attribute of an overall food quality (EC, 2005; EC, 2007; EC, 2016; Kjaernes et al., 2007). To accommodate societal concerns about farm animal welfare, there is a pressing need for reliable science-based systems for assessing the animals' welfare status (Blokhuis et al., 2003; Walker et al., 2014). Therefore, in 2006, the European Commission adopted a Community Action Plan which outlines some measures to improve the protection and welfare of animals. It set minimum and higher standards for farming practices and recommended the implementation of standardized indicators and the adoption of an EU label for animal welfare (EC, 2006).

Several animal welfare assessment methods have been developed since. Most of them evaluate the animal's experience and its ability to cope with the environment through the assessment of animal-based measures (ABMs) (de Vries et al., 2013). ABMs (e.g., body condition, health aspects, injuries, behaviour, etc.) are more valid than management-based indicators (non-animal-based measures, N-ABMs; e.g., size of pen, flooring, etc.) to assess the actual welfare state and identify the most critical problems (Welfare Quality, 2009). However, ABMs could make farm audit complicated, less feasible, costly, and time-consuming (de Vries et al., 2013; Andreasen et al., 2014; Grandin, 2014). These limitations render ABM-based protocols difficult to apply on a regular basis (Heath et al., 2014). On the other hand, N-ABMs could be more efficient as they help identify welfare hazards (EFSA, 2012b; Lundmark et al., 2016). Therefore, the European Food Safety Agency (EFSA) recommended the implementation of protocols mixing both ABMs and N-ABMs for achieving an effective classification of animal welfare at farm level (EFSA, 2012b). Also, EFSA published guidelines adapting the well-established risk assessment methodologies for animal diseases and food safety to animal welfare (EFSA, 2012a), with the aim of assessing not only factors associated with negative welfare outcomes, but also the ones that positively influence the well-being of the animals (EFSA, 2012a).

Protocols for animal welfare assessment

Welfare Quality®

Through the collaboration of a large number of research groups and institutes, the European Welfare Quality® (WQ) project developed protocols for animal welfare assessment which assign farms and slaughterhouses a score from one (poor animal welfare) to four (good animal welfare). WQ combined consumer perceptions' analysis and scientific knowledge to identify 12 criteria that should be covered in the assessment systems. To address these areas of concern, it focusses on ABMs that reflect actual welfare state of the animals, e.g., behavior, fearfulness, health, and physical condition, including variations associated with the farming system and system-animal interactions. In addition, N-ABMs contribute to a welfare assessment and are used to identify risks and causes of poor welfare for implementing improvement strategies. Whence, an integrated, standardized and, wherever possible, animal-based methodology was developed (Keeling et al., 2012).

CReNBA protocol

In order to support the official controls, fulfill EU recommendations, foster animal welfare assessment, and provide consumers with information, the Italian National Reference Centre for Animal Welfare (CReNBA) was established. It carries out technical and scientific support activities for the Ministry of Health and the competent authority and promotes research and training in the field of animal welfare.

In this framework, a study was conducted to develop a simplified on-farm animal welfare assessment protocol for dairy cows in free-stable systems (Bertocchi et al., 2018). The animal welfare assessment procedure takes into account the Legislative Decree 146/2001 on the protection of farm animals, EFSA opinions on the welfare of dairy cattle, European Welfare Quality® publications, and scientific literature. It analyzes scientifically supported N-ABMs and ABMs that favor objective and easily measurable findings in almost all dairy cattle farms. An expert opinion elicitation served for hazard characterization and welfare promoter characterization, and experts were asked to weigh a list of N-ABM and ABM to be integrated in the protocol (items). The protocol is applied through an audit by a trained veterinarian who rates the items on a one to three scoring scale:

1. *Unacceptable:* conditions that may prevent many animals in the herd from meeting their biological needs and the five freedoms.
2. *Acceptable:* conditions that, with some exceptions, ensure the fulfillment of the five freedoms and psychophysical needs for (all) animals.

3. *Optimal:* conditions that allow (all) animals to fully meet physiological needs.

The indirect welfare measures are grouped into two risk areas, respectively: *Management business and personnel*; and *Facilities and equipment*. Direct measures of animal welfare and signs of adverse effects on their welfare are grouped into the third area *Animal based measures*. At the end, the welfare assessment procedure provides a list of legislative issues, along with a score for animal welfare and farm biosecurity obtained by weighing the field observations based on expert opinions for the three assessment areas.

Lately, CReNBA developed a specific system for assessing welfare of dairy cattle raised in tie-stall housing, too. The goal was to compare different herds ensuring objectivity. Many assessment parameters are similar or even identical for both free-stall and tie-stall housing farms, while others consider the structures and equipment that characterize the two of them.

The welfare assessment system was officially presented in 2014 to the Ministry of Health, the Ministry of Agricultural, Food and Forestry Policies, and trade unions.

Aim of the study

This retrospective longitudinal study aimed to estimate four HI (i.e., mastitis, ketosis, ICI, and fresh-cow removal) using test-day records (TD) obtained from DHI of a large cohort of dairy cows in Piedmont, northwestern Italy. The five-year study period, from 2015 to 2020, secures the estimates from spurious fluctuations. The geographic distribution of HI in Piedmont was explored, too. Finally, TD records were combined with animal welfare assessment data to assess whether HI can be used as early warning method for poor welfare risk (PWR) of dairy cows.

Materials and methods

Study population

The population under study included all lactating cows of dairy herds that participated in DHI programs, in Piedmont, from the 2nd of January 2015 to the 28th of April 2020. The Italian Breeders' Association (AIA) collected and analyzed individual milk samples every 20-40 days (median = 31 days, mean = 37.16 ± 30.97 days). Because of the reduced number of workdays due to national holidays, in August and December of each year, AIA analyzed a smaller amount of milk samples.

Milk sampling

During milking, the milk yield of each cow was measured, and a sample of composite milk was collected. Once taken to the regional laboratory (ARA Piemonte, Cuneo, IT), the milk is tested for the concentration of fat, protein, lactose, urea, somatic cells, and casein by Fourier-transformed infrared (FTIR) spectrometry using FOSS MilkoScan FT+ (various models depending on the laboratory; FOSS Analytical A/S, Hillerød, DK). All laboratory methods and techniques were certified by the national accreditation body designated by the Italian government (Accredia, https://www.accredia.it/) in compliance with the standards required by the UNI CEI EN ISO/IEC 1702. Also, AIA is certified by the International Committee for Animal Recording (ICAR).

Data management

The initial database contained 4,634,197 observations of 275,351 lactating cows from 1,408 different herds. Cows with only one TD were removed. Also, we excluded herds with less than five lactating cows. Out-of-range values of milk composition (i.e., fat < 1.5 g/100 g, fat > 10.0 g/100 g, protein < 1.5 g/100 g, protein > 7.0 g/100 g, lactose < 2.0 g/100 g, lactose > 6.0 g/ 100g) and SCC (SCC < 1,000 cells/mL, SCC > 15,000,000 cells/mL) were replaced with missing values, and observations with missing values of any relevant variable (herd ID, cow ID, breed, parity, date of birth, date of calving, SCC, fat, protein, lactose) were excluded. The remaining 4,483,057 observations formed the working database with 261,121 lactating cows and 1,315 herds (Figure 3).

Into the working database, the most represented breed was Holstein Friesian (n = 3,760,324 obs.), which was used as a reference for all breed comparisons (Supplementary Table 1).

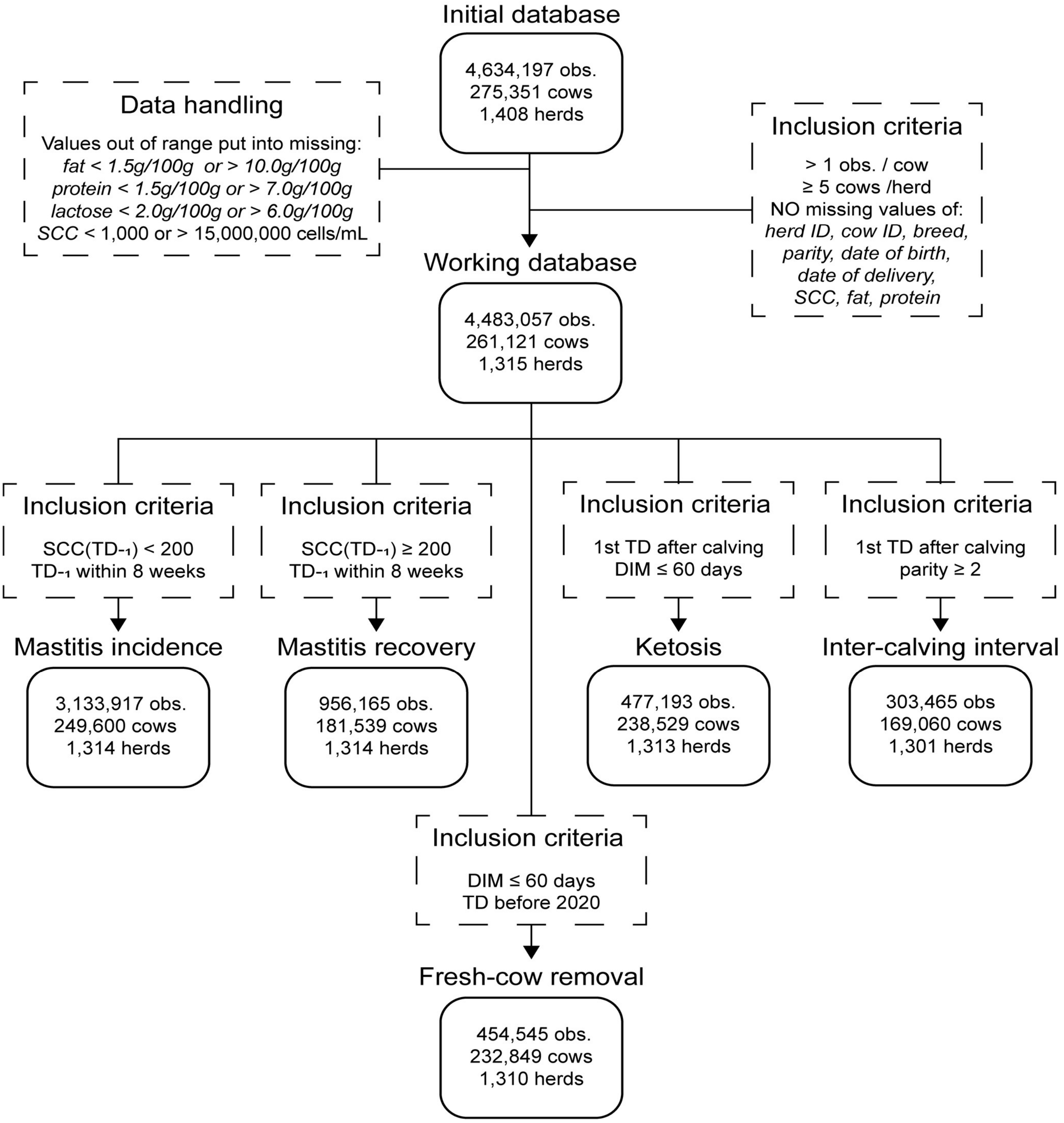


Figure 3. *Flowchart of database clean-up and data handling before analysis. Abbreviations: DIM: days in milk; obs.: observations; SCC: somatic cells count; TD: test-day; TD-1: previous test-day.*

AIA collected and managed all data used in this study and owns their property. Sensitive data were anonymized through the assignation of a dummy herd ID. Statistical analyses were performed using SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA). Graphs were made using the *ggplot2* package and base R language on R version 4.1.0 (R Core Team, 2023).

Herd health monitoring

Mastitis

SCC was reported as 10^3 cells/mL. SCC $\geq 200 \times 10^3$ cells/mL was considered indicative of subclinical mastitis (Dufour and Dohoo, 2013).

Mastitis prevalence. Mastitis prevalence proportion (PP) was calculated as the number of cows with SCC $\geq 200 \times 10^3$ cells/mL over the total number of cows.

Mastitis incidence. A new case of mastitis was observed when SCC increased from SCC $< 200 \times 10^3$ to SCC $\geq 200 \times 10^3$ cells/mL over two consecutive TD not more than 8 weeks apart (56 days). Cow-days at risk were calculated assuming that both new cases and recoveries occurred halfway between two consecutive TD, thus multiplying the days between TD for 0.5. Observations were excluded when the previous mastitis status was unknown, i.e., the first record of a cow or TD more than 56 days after the previous one. Chronic mastitis was defined as SCC $\geq 200 \times 10^3$ cells/mL for two consecutive TD, and censored. After data clean-up, the dataset for mastitis incidence contained 3,133,917 observations of 249,600 cows from 1,314 herds. The incidence rate (IR) of mastitis was calculated as the number of new cases divided by the sum of cow-days at risk. IR was reported as cases/cow-month, in reference to a standard month of 30.5 days (Dufour and Dohoo, 2013). When a new case occurred, the individual rate was calculated as 1 divided by the days elapsed from the previous negative TD.

Mastitis recovery. Mastitis recovery was observed when SCC went from $\geq 200 \times 10^3$ to $< 200 \times 10^3$ cells/mL over two consecutive TD within 56 days. The database for mastitis recovery was 956,165 observations of 181,539 cows from 1,314 herds. The recovery rate from mastitis was computed as the number of recoveries over the number of mastitis cases.

Hyperketonemia

HK was defined as F:P > 1.42 (Jenkins et al., 2015) at the first record within 60 days from calving. We did not differentiate between subclinical and clinical cases. The HK database included 477,193 observations of 238,529 cows from 1,313 herds. Incidence rate (IR) was calculated as the number of new HK cases over the total of cow-lactations at risk.

Inter-calving interval

The ICI was calculated as the difference between the date of calving and the date of the previous calving, whence it was measured from the second lactation. Since events occurred in the previous lactation are crucial for breeding and pregnancy success, some variables (i.e., milk yield, ketosis, and mastitis), were collected from the previous lactation. The ICI dataset included one record per cow per lactation, that were 303,465 observations of 169,060 cows from 1,301 herds.

Fresh-cow removal

Any removal of a cow from a herd within 60 days from the calving was considered regardless of the cow's fate (e.g., voluntary or unvoluntary culling, sale, etc.). Data from 2020 were excluded to avoid right censoring bias. The resulting fresh-cow removal dataset consisted of 454,545 observations of 232,849 cows from 1,310 herds. The IP was calculated as the number of fresh-cow removals over the number of cows within 60 days from calving per lactation.

Animal welfare

Animal welfare assessment was performed by trained veterinarians authorized to apply CReNBA protocol on dairy herds. All data collected in the Piedmont region between May 2017 and April 2020 were subsequently extracted from the national database, for a grand total of 849 animal welfare assessments performed on 357 herds (median = 2 evaluations / herd).

Data analysis

Analysis was performed on the working database unless otherwise specified. For each outcome, we evaluated several cow-level risk factors: breed, parity, days elapsed from calving (days in milk, DIM), milkings per day, daily milk production (milk yield), age at first calving, and other HI. Association between HI and milk yield was evaluated by Pearson's correlation (PROC CORR). AIA estimated milk yield (L/day) according to methods mentioned in ICAR's guidelines (ICAR, 2022) and it was reported as mean ± the standard deviation like other continuous variables. Estimates, odds, PP, IR, IP, and all measures of association, i.e., odds ratio (OR), relative risk (RR), and incidence rate ratio (IRR), are reported along with their 99 % confidence intervals (CI).

Milk components

The normality of milk components values was visually assessed, then univariate analysis (PROC UNIVARIATE) was performed to explore the consistency of data throughout the study period. The correlation among milk components was computed by Pearson's coefficient (PROC CORR).

Bivariate analysis

The annual and seasonal trends of each HI were studied. The association with cow-level risk factors were analyzed separately for each HI by the means of bivariate analysis (PROC FREQ, PROC MEANS), while regression models were used to estimate the unitary effect of risk factors.

Variables were handled to ease the analysis either aggregating or splitting values into categories. Local and/or less-represented breeds were grouped (Supplementary Table 1), as well as cows with five or more lactations were aggregated. DIM were categorized into four phases of the lactation curve, assuming a Legendre-shaped ideal curve (Macciotta et al., 2005): (1) early lactation, DIM ≤ 60; (2) production peak: $60 <$ DIM ≤ 120; (3) late lactation: $120 <$ DIM ≤ 305; (4) over-lactation: DIM > 305. However, for HK, DIM were divided by weeks. Milk yield and lactose were divided into quintiles.

Generalized regression model

Binary HI (i.e., mastitis, mastitis recovery, HK, fresh-cow removal) were modelled by logistic regression (PROC GLIMMIX, DIST=BINARY, LINK=LOGIT). Continuous outcome (i.e., ICI) was modelled by linear regression (PROC GLIMMIX, DIST=GAUSSIAN, LINK=IDENTITY). All biologically-relevant, cow-level risk factors were included in the model by manual stepwise forward selection. The gain in terms of goodness of fit (GOF) was assessed by comparing the log-likelihood of new models with that of the saturated model. To adjust for the unmeasured herd-level risk factors, random effect mixed models (PROC GLIMMIX) were fitted, with the herds as random intercepts. To mimic the seasonal variation, the calendar period was introduced into the model with an empirically produced sinusoid function. The function was designed to span from -1 to 1 following the trend of the certain HI. The final model was a generalized linear model, family and link function depending on the outcome, with a random intercept for each herd, fitted to estimate the effect of the selected covariates, as follows:

$$Y_{ij} = \beta_0 + \beta_1 X_{i,1} + \ldots + \beta_k X_{i,k} + \beta_S S(i) + \epsilon + u_j$$

where Y_{ij} is alternatively the outcome or its natural logarithm (depending on the outcome, binary or not), β_0 is the intercept, β_{1-k} are the k coefficients of each k^{th} covariate, $S(i)$ is the empirical function for calendar-period correction, ϵ is the random error, and u_j is the random intercept of each j^{th} herd with $u_j \sim N(0, \sigma^2)$.

Confidence intervals for proportions were calculated, using the formula proposed by Agresti and Coull (1998). OR were obtained exponentiating the model coefficients, while CI were calculated based on the observed frequency (Rothman et al., 2008). For incidence rates, the exact confidence interval was calculated from the χ^2 distribution (Garwood, 1936). We selected some of the estimates from the body of the text to be presented in the abstract. Since confidence intervals do not account for the additional uncertainty due to the selection process, we corrected the intervals as described by Benjamini and Yekutieli (2005).

Mastitis prevalence. In the final model, breed, parity, DIM, and the milkings per day were included along with the mastitis status at the previous TD. A sinusoid function with a six-month period, from March (-1) to August (1), was included in the model to adjust for seasonality. The estimated effect of each relevant variable was adjusted for unmeasured herd-level risk factors. The model formula was:

$$log(Y_{ij}) = \beta_0 + \beta_1 X_{i_{breed}} + \beta_2 X_{i_{parity}} + \beta_3 X_{i_{DIM}} + \beta_4 X_{i_{\frac{milkings}{day}}} + \beta_5 S(i) + \beta_6 X_{i,TD-1_{mastitis}} + \epsilon + u_j$$

where $log(Y_{ij})$ is the natural logarithm of mastitis prevalence, β_0 is the intercept, β_{1-6} are the coefficients of each covariate, $S(i)$ is the calendar-period correction, ϵ is the random error, and u_j is the random intercept of each j^{th} herd and $u_j \sim N(0, \sigma^2)$.

Mastitis incidence. Breed, parity, DIM, and milkings per day were included in the selected model, along with ketosis, and random herd-level effects. A six-month period function, spanning from -1 in January and 1 in July was included to adjust for the calendar period. The model formula was:

$$log(Y_{ij}) = \beta_0 + \beta_1 X_{i_{breed}} + \beta_2 X_{i_{parity}} + \beta_3 X_{i_{DIM}} + \beta_4 X_{i_{\frac{milkings}{day}}} + \beta_5 S(i) + \beta_6 X_{i_{ketosis}} + \epsilon + u_j$$

where $log(Y_{ij})$ is the natural logarithm of mastitis incidence, β_0 is the intercept, β_{1-6} are the coefficients of each covariate, $S(i)$ is the calendar-period correction, ϵ is the random error, and u_j is the random intercept of each j^{th} herd and $u_j \sim N(0, \sigma^2)$.

Mastitis recovery. Breed, parity, DIM, milkings per day, milk yield at TD-1, lactose at TD-1, and SCC at TD-1 were included as a covariate in the model for mastitis recovery, along with the season and herd-level random effects. The final model formula was:

$$log(Y_{ij}) = \beta_0 + \beta_1 X_{i_{breed}} + \beta_2 X_{i_{parity}} + \beta_3 X_{i_{DIM}} + \beta_4 X_{i_{\frac{milkings}{day}}} + \beta_5 S(i) + \beta_6 X_{i,TD-1_{milk\,yield}}$$
$$+ \beta_7 X_{1,TD-1_{[lactose]}} + \beta_8 X_{i,TD-1_{SCC}} + \epsilon + u_j$$

where $log(Y_{ij})$ is the natural logarithm of mastitis prevalence, β_0 is the intercept, β_{1-8} are the coefficients of each covariate, $S(i)$ is the calendar-period correction, ϵ is the random error, and u_j is the random intercept of each j^{th} herd and $u_j \sim N(0, \sigma^2)$. The sinusoid function for the season ranged from -1 in July to 1 in December.

Hyperketonemia. Breed, milk yield, parity, and DIM were included in the regression model for HK. Their effects were estimated by adjusting for herd-level risk factors and season. The season function followed the pattern of ketosis cases, with a six-month period from September (-1) to March (1). The model formula was:

$$log(Y_{ij}) = \beta_0 + \beta_1 X_{i_{breed}} + \beta_2 X_{i_{parity}} + \beta_3 X_{i_{DIM}} + \beta_4 X_{i_{milk\,yield}} + \beta_5 S(i) + \beta_6 X_{i_{mastitis}} + \epsilon + u_j$$

where $log(Y_{ij})$ is the natural logarithm of the ketosis incidence rate, β_0 is the intercept, $\beta 1 - 6$ are the coefficients of each covariate, $S(i)$ is the calendar-period correction, ϵ is the random error, and u_j is the random intercept of each j^{th} herd and $u_j \sim N(0, \sigma^2)$.

Inter-calving interval. Breed, parity, milk yield, age at first calving, mastitis, and ketosis were included in the final model, adjusted for unmeasured herd-level risk factors, too. The model formula was:

$$ICI = \beta_0 + \beta_1 X_{i_{breed}} + \beta_2 X_{i_{parity}} + \beta_3 X_{i_{milk\,yield}} + \beta_4 X_{i_{calving\,age}} + \beta_5 X_{i_{mastitis}} + \beta 6 X_{i_{ketosis}} + \epsilon + u_j$$

where ICI is the predicted inter-calving interval, β_0 is the intercept, β_{1-6} are the coefficients of each covariate, ϵ is the random error, and u_j is the random intercept of each j^{th} herd and $u_j \sim N(0, \sigma^2)$.

Fresh-cow removal. Based on the stepwise selection, breed, parity, milk yield, age at first calving, ketosis, mastitis and long-ICI (ICI ≥ 440 days) were included in the regression model. The estimates were adjusted for unmeasured herd-level risk factors including herds as random intercepts, and for the calendar period including a function that ranged from -1 in April to 1 in August. Estimates are reported in Table 4. The final model formula was:

$$log(Y_{ij}) = \beta_0 + \beta_1 X_{i_{breed}} + \beta_2 X_{i_{parity}} + \beta_3 X_{i_{milk\,yield}} + \beta_4 X_{i_{age\,at\,1^{st}\,calving}} + \beta_5 S(i) + \beta_6 X_{i_{mastitis}} + \beta_7 X_{i_{ketosis}} + \beta_8 X_{i_{ICI>440}} \epsilon + u_j$$

where $log(Y_{ij})$ is the natural logarithm of the incidence proportion of fresh-cow removal, β_0 is the intercept, β_{1-8} are the coefficients of each covariate, $S(i)$ is the calendar-period correction, ϵ is the random error, and u_j is the random intercept of each j^{th} herd and $u_j \sim N(0, \sigma^2)$.

Geographic distribution

The exact location of the herds was unknown; thus results of each HI were aggregated at municipal level. ICI was transformed into long ICI when ICI > 440 days. Since the way data are represented might conceal some aspects and reveal other, the spatial distribution of HI in the Piedmont region was investigated with several approaches. For each, choropleth maps were produced dividing municipalities by quartiles and by Fisher-Jenks natural breaks' intervals (Fisher and Jenks, 1971), which display homogeneous groups minimizing difference withing groups while maximizing that between. Also, local aggregation of disease was assessed. Geographic representation was performed using QGIS version 3.16.2 (QGIS.org, 2024).

Raw HI. Raw values do not consider the population size nor the population rate, whence the HI of different municipalities were hardly comparable.

Standard disease rate. Standard disease rates (SDR) of each HI were calculated using indirect standardization, i.e., the number of observed cases of each HI was compared to the number of events that would be expected had the Piedmont average rate been applied. The average rate ($\overline{p}$) is expressed as:

$$\overline{p} = \frac{\sum_{i=1}^{k} O_i}{\sum_{i=1}^{k} N_i}$$

where O_i and N_i represent the number of observed cases and population size of the i^{th} geographic portion, out of the k considered. The average rate is calculated from all observations and not the average of local rates. It yields the expected number of events for each i^{th} area:

$$E_i = \overline{p} \times N_i$$

Then, standard disease rate was calculated as the ratio of observed events over E_i, in a certain area:

$$SDR_i = \frac{O_i}{E_i}$$

The main disadvantage of SDR is an intrinsic variance instability, meaning that small populations can have spuriously high or low values because estimate precision is inversely proportional to the population at risk (N_i).

Empirical Bayes smoothed rate. Essentially, the empirical Bayes' smoothed rate (EBSR) is a weighted average between the raw HI rate for each geographic portion and the overall area's average, with weights proportional to the population at risk. It is a shrinkage estimator borrowing strength from other observations to adjust estimates and improve precision.

In the Bayesian framework, the knowledge about the distribution of a variable is updated after observing data. Formally, this concept is resumed into the Bayes Law that decomposes a joint probability into two conditional probabilities:

$$P[AB] = P[A|B] \times P[B] = P[B|A] \times P[A]$$

where A and B are random events, and | stands for the conditional probability of one event, given the value of the other. It follows that:

$$P[A|B] = \frac{P[B|A] \times P[A]}{P[B]}$$

$$P[A|B] \propto P[B|A] \times P[A]$$

The *a priori* knowledge about the distribution of the parameter A ($P[A]$), is updated after observing the observed data B into the posterior distribution ($P[A|B]$). The prior and the posterior distribution are linked through the likelihood of having observed data B given the distribution of the parameter A ($P[B|A]$). Using a notation where π stands for the parameter and y for the observations, this gives:

$$P[\pi|y] \propto P[y|\pi] \times P[\pi]$$

The usual approach in rate estimation is the Poisson-Gamma model, which specifies a Poisson distribution for y, and a Gamma distribution for π (Clayton and Kaldor, 1987; Marshall, 1991). It follows that mean and variance of the prior distribution are calculated as:

$$E[\pi] = \alpha/\beta$$

$$Var[\pi] = \alpha/\beta^2$$

where α and β are the shape and scale parameters of the Gamma distribution, respectively, which are estimated from the observed data and reflect the confidence on prior information. The Gamma distribution for the *a priori* risk parameter (π) with a Poisson distribution for the observed number of events (y) combined into the $Gamma(y + \alpha, \pi + \beta)$ posterior distribution.

The smoothed rate is expressed as a weighted average of the crude HI rate (p) and the prior estimate ($\overline{\pi}$, i.e., the reference rate), thus the portions of territory with a small population at risk have their rates adjusted considerably, whereas for larger portions the rates barely change. The ESBR in the i^{th} area is:

$$\pi_i = w_i p_i + (1 - w_i)\overline{p}$$

and the weights correspond to:

$$w_i = \frac{\sigma^2}{\sigma^2 + \mu/N_i}$$

where N_i is the population at risk in the i^{th} area, and μ and σ^2 are the prior distribution's mean and variance, estimated from the observed data and determining shape and scale of the Gamma distribution. Therefore, in this case μ is the reference rate of disease in the whole Piedmont population (as it was used for SDR):

$$\mu = \overline{p} = \frac{\sum_{i=1}^{k} O_i}{\sum_{i=1}^{k} N_i}$$

while its variance is:

$$\sigma^2 = \frac{\sum_{i=1}^{k} N_i\,(p_i - \overline{p})^2}{\sum_{i=1}^{k} N_i} - \frac{\overline{p}}{\sum_{i=1}^{k} N_i\,/k}$$

Since by convention $\sigma^2 \geq 0$, negative variance values are set to 0. As a result, when $\sigma^2 = 0$, then $w_i = 0$, whence the ESBR equates the reference population's rate (Calyton and Kaldor, 1987).

Local aggregation. Spatial autocorrelation is defined as a correlation in signal among nearby locations. It was assessed through the Moran's index (*I*):

$$I = \frac{N}{\sum_{i=1}^{k}\sum_{j=1}^{k} w_{ij}} \frac{\sum_{i=1}^{k}\sum_{j=1}^{k} w_{ij}\,(p_i - \bar{p})(p_j - \bar{p})}{\sum_{i=1}^{k}(p_i - \bar{p})^2}$$

where N is the overall population size, and $\sum_i \sum_j w_{ij}$ the sum of all spatial weights. *I* ranges between -1 and 1. If similar HI values, regardless of whether they are high or low, are spatially localized ($I \cong 1$), there is a positive spatial autocorrelation of the data, i.e. spatial homogeneity. On the contrary, a spatial proximity of dissimilar values ($I \cong -1$) indicates a negative spatial autocorrelation, i.e., spatial heterogeneity. A spatial pattern that is no different from a random phenomenon yields $I \cong 0$ (Fotheringham et al., 2000). In the equation, the weight (w_{ij}) represents the strength of the spatial relationship between i and j, which can be measured as either a binary adjacency ([0,1]) or a continuous distance-decay measure between centroids.

The calculation was made through the manual definition of the *I* statistic function in R environment (R version 4.3.2), then results were geographically represented using QGIS version 3.16.2 (QGIS.org, 2024).

Animal welfare

The CReNBA evaluation of animal welfare consists of five areas of assessment (AA), whose weighted contribution concur in the *Total* score. The five AA are: ABMs, Management, Structures, Great risks, and Biosecurity (Bertocchi et al., 2018).

Of the collected CReNBA evaluations, only the most recent evaluation of each herd was included. The difference between herds participating in DHI and the others was calculated for each AA and *Total* score then evaluated by Wilcoxon's rank sum test. Similarly, the difference between herds with and without CReNBA evaluation for each HI was calculated and evaluated by Wilcoxon's rank sum test.

We identify clusters of herds with similar HI pattern by cluster analysis, using Bayesian information criterion (BIC), integrated complete likelihood (ICL), and Akaike information criterion (AIC) to evaluate model GOF. Then, herds were assigned to patterns based on VEE (i.e., variable volume, equal shape and orientation, ellipsoidal shape of clusters on two-dimension plot) latent profile analysis

(LPA). Eventually we estimated the posterior marginal probability of each herd belonging to each pattern.

We employed linear discriminant analysis (LDA) to evaluate HI patterns as an early warning for poor welfare risk (PWR), where PWR was defined as scoring the worst values of *Total* score, i.e., values falling in the 4th quartile. The LDA was trained bootstrapping a subset of herds having both DHI and CReNBA information.

Analysis was performed using R version 4.3.2 (R Core Team, 2023), with *mclust 5* and *tidyLPA* packages (Rosenberg et al., 2018; Scrucca et al., 2016).

Results

Study population

On average, each herd had an average of 7.53 ± 3.18 TD per year. The size of the herds ranged from 5 to 2,143 lactating cows (mean = 195.93 ± 207.53). Each cow was sampled 17.16 ± 11.12 times during the study period, having an average of 7.94 ± 3.89 TD per lactation.

Within the study period, more than one-third of the cows (35.26%) were observed for one lactation only, 32.01% for two, and 32.73% for three or more lactations. The 37.28% of observations were of primiparous cows, 27.25% of 2nd lactation, 17.11% of 3rd lactation, and 9.54% of the 4th one; 8.82% were from the 5th lactation.

Since complete data were not available for all the 1,315 herds, some HIs were estimated only for a fraction of the herds. A sensitivity analysis was performed to compare milk parameters between herds with (n = 82) and without (n = 1,233) any missing HI (Table 3).

Table 3. *Comparison between herds with and without missing DHI records.*

Parameter	**Herds w/out missing values (mean ± s.d.)**	**Herds with missing values (mean ± s.d.)**	**p-value (Wilcoxon's)**
Herd size	71.9 ± 68.3	34.3 ± 61.2	<0.0001
Milk yield	23.7 ± 8.9	16.0 ± 7.9	<0.0001
Milk fat	3.92 ± 0.29	3.81 ± 0.40	0.0226
Milk protein	3.47 ± 0.12	3.38 ± 0.19	0.0002
Milk lactose	4.79 ± 0.07	4.72 ± 0.14	<0.0001
Linear score (SCC)	3.27 ± 0.65	3.82 ± 0.90	<0.0001
Parity	2.3 ± 0.7	2.5 ± 1.1	0.0524

Fewer DHI information was obtained from smaller herds with lower milk yield, worse milk parameters, and increased SCC. Correlation analysis showed that that bigger herds tend to have increased milk yield, with more lactose, fat (p<0.0001) but not protein (p=0.2354), whereas they had lower parity and linear score (p<0.0001).

The incidence of mastitis remained constant or slightly increased with size in herds smaller than 20 lactating cows. It decreased significantly (p<0.0001) by 0.4% per unit increase in the herds between 21 and 100 lactating cows, while it remained constant in herds with more than one hundred lactating cows.

The incidence of HK increased by 0.5% per unit increase of size in herds smaller than 50 lactating cows. From 50 to 200 lactating cows, the increment reduced to 0.3%, then HK reached a plateau over the 200 lactating cows.

Milk components

All milk components were quite normally distributed (Figure 4).

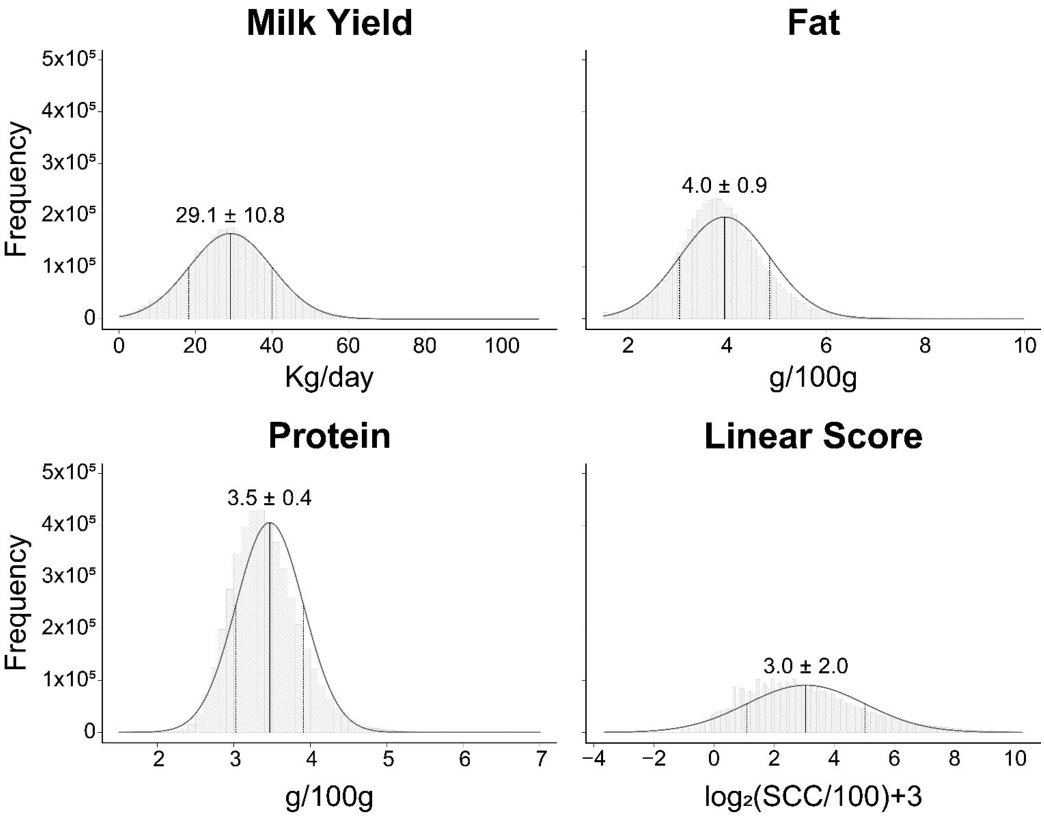


Figure 4. *Histograms of milk yield and milk component values. Milk yield, lactose, fat, and protein are reported as raw values, with mean and standard deviation (solid and dashed line, respectively).*

Somatic cells count (SCC) is reported as the linear score [LS=$\log_2(SCC/100)+3$*] to improve data visualization.*

Milk yield was positively correlated with lactose (ρ = 0.24), while negatively with fat (ρ = -0.21, protein (ρ = -0.42), and SCC (ρ = -0.12). Accordingly, a negative correlation was observed between lactose and fat (ρ = -0.22), protein (ρ = -0.24), and SCC (ρ = -0.26), whereas a positive one was observed between fat and protein (ρ = 0.42). Their correlation with SCC was very low (ρ = 0.04 and ρ = 0.05, respectively).

Milk yield varied by breed, and Holstein-Friesian was the only breed whose daily production exceeded 30 Kg (30.80 ± 10.08 Kg). It increased monotonically from below 21 Kg/day in 2015 to over 31 Kg/day in 2020. During the year, it recorded minimum values in summer (July to September) and maximums in spring (February to April). Also, fat and protein increased during the years of study, both peaking in autumn (November and December, respectively) and reaching minimum concentrations in July. Lactose did not show a seasonal pattern or a monotonic trend during the study period. The average SCC decreased during the study period by more than 50,000 cells/mL. Annually, it ranged between a minimum in March and a maximum in August. The average SCC for cows with and without mastitis remained consistent throughout the study period, suggesting that the SCC decrease from 2015 to 2020 was due to the reduction of mastitis cases.

Herd health monitoring

Mastitis

Mastitis prevalence. Out of 261,121 cows, 23% (n = 60,937) never had SCC ≥ 200 during the study period. The average prevalence proportion of mastitis was 0.29 (CI: 0.28 – 0.29). It decreased linearly from 0.30 in 2015 to 0.27 in 2020. The seasonal pattern repeated every year and ranged from 0.26 in March to 0.32 in August. Mastitis prevalence increased by 22% (RR = 1.22, CI: 1.22 – 1.23) between early and late lactation (Figure 5A). Among breeds, only Holstein Friesians have a prevalence lower than the population average (data not shown).

From the bivariate analysis, we observed that the prevalence of mastitis increased quite linearly by 27% after each calving, ranging from 0.19 in primiparous cows to 0.47 over the 5th delivery (Figure 5B).

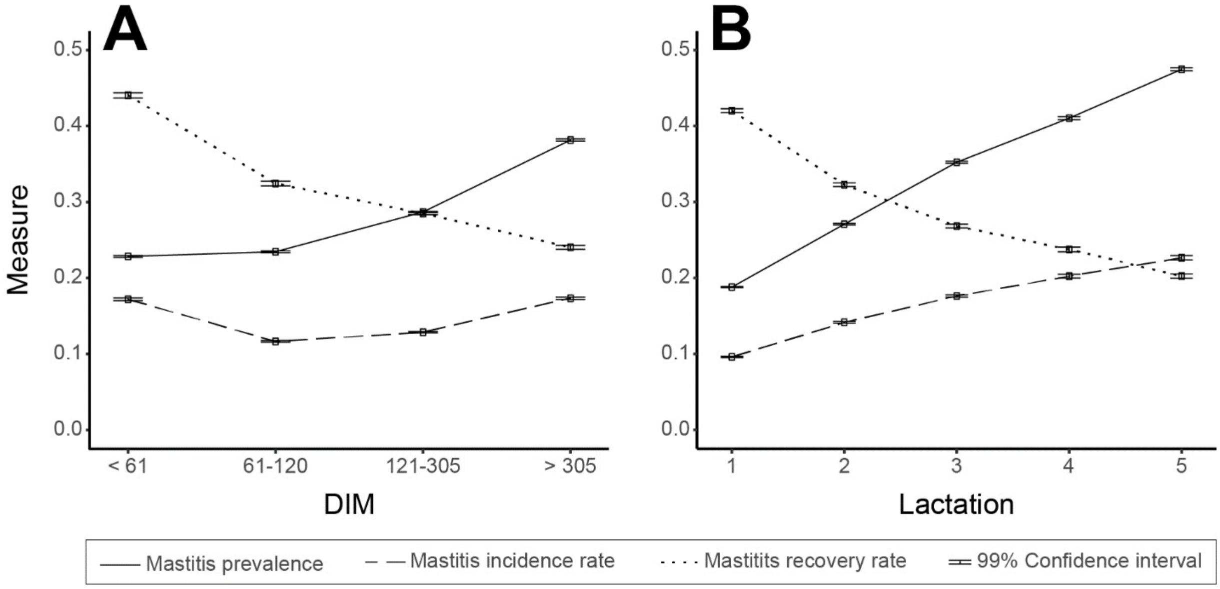


Figure 5A-B. *Mastitis prevalence according to days in milk and parity. A: prevalence of mastitis, its incidence rate, and the recovery proportion by days in milk; lactation phases were divided based on a Legendre-shaped lactation curve. B: prevalence of mastitis, its incidence rate, and the recovery proportion by parity; observations over the 4th lactation were aggregated.*

Only 9% (n = 392,488) of the observations were from cows milked three times a day, which had 22% less risk of mastitis (RR = 0.78, CI: 0.77 – 0.79). HK increased the risk of mastitis (RR = 1.03, CI: 1.03 – 1.04) by 3%. On average, the cows with mastitis had an average 4.81 Kg lower milk yield (25.66 ± 11.12 Kg) than healthy ones (30.46 ± 10.40 Kg).

The results of the regression model for mastitis prevalence are reported in Table 4.

Table 4. *Estimates of odds ratio and their 99% confidence intervals adjusted for other covariates and herd-level unmeasured risk factors. The effect of each relevant variable is reported for mastitis prevalence, mastitis incidence rate, mastitis recovery, ketosis prevalence, the prevalence of reproductive disorders, and fresh-cow removal prevalence.*

		Mastitis prevalence		**Mastitis incidence**		**Mastitis recovery**		**Ketosis incidence**		**Inter-calving interval**		**Fresh-cow removal**	
	Effect	**OR**	**99 % C. I.**	**OR**	**99 % C. I.**	**OR**	**99 % C. I.**	**OR**	**99 % C. I.**	**β**	**99 % C. I.**	**OR**	**99 % C. I.**
	Intercept	0.09	0.08 – 0.09	0.07	0.07 – 0.08	0.17	0.14 – 0.20	0.30	0.28 – 0.32	424.44	419.56 – 429.32	0.10	0.08 – 0.13
Breed	Holstein Friesian	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-	1 (ref.)		1 (ref.)	-	1 (ref.)	-
	Cross-bred	0.99	0.97 – 1.00	1.00	0.98 – 1.03	0.98	0.95 – 1.01	**0.80**	**0.76 – 0.84**	**-18.71**	**-21.28 – -16.14**	**0.58**	**0.50 – 0.67**
	Brown Swiss	0.97	0.93 – 1.01	0.99	0.93 – 1.05	0.95	0.88 – 1.03	**0.70**	**0.61 – 0.79**	-3.48	-10.58 – 3.61	**0.29**	**0.19 – 0.43**
	Italian red roan	**0.88**	**0.85 – 0.90**	**0.92**	**0.89 – 0.96**	**1.06**	**1.01 – 1.12**	**0.63**	**0.58 – 0.68**	**-19.53**	**-23.87 – -15.18**	**0.36**	**0.29 – 0.45**
	Piedmontese	**0.88**	**0.77 – 0.99**	1.15	0.99 – 1.34	1.11	0.88 – 1.39	**0.34**	**0.24 – 0.49**	**-29.36**	**-50.44 – -8.29**	**0.19**	**0.08 – 0.44**
	Oropa red roan	**1.12**	**1.02 – 1.22**	**1.30**	**1.18 – 1.45**	**0.87**	**0.77 – 0.99**	**0.24**	**0.19 – 0.30**	**-12.18**	**-23.14 – -1.21**	**0.11**	**0.07 – 0.16**
	Grauvieh	1.07	0.97 – 1.18	1.09	0.96 – 1.24	**0.69**	**0.58 – 0.82**	**0.45**	**0.34 – 0.61**	**-41.74**	**-57.82 – -25.66**	**0.17**	**0.08 – 0.38**
	Jersey	1.02	0.94 – 1.10	1.02	0.92 – 1.12	1.01	0.87 – 1.16	1.02	0.83 – 1.25	**-17.68**	**-29.63 – -5.73**	**0.53**	**0.28 – 0.99**
	Abondance	0.91	0.79 – 1.06	1.00	0.82 – 1.23	0.87	0.64 – 1.19	**0.62**	**0.39 – 0.99**	27.58	-5.00 – 60.16	1.92	0.67 – 5.49
	Brown	1.05	0.91 – 1.22	**1.37**	**1.14 – 1.65**	0.98	0.74 – 1.29	**0.34**	**0.22 – 0.53**	**-36.79**	**-58.2 – -15.39**	**0.13**	**0.05 – 0.39**
	Pustertaler	0.94	0.86 – 1.02	1.06	0.94 – 1.18	1.04	0.89 – 1.21	**0.44**	**0.33 – 0.58**	**-21.75**	**-34.74 – -8.76**	**0.15**	**0.07 – 0.30**
	Valdostana	0.96	0.90 – 1.02	**1.10**	**1.02 – 1.19**	1.02	0.91 – 1.13	**0.31**	**0.26 – 0.38**	**-35.56**	**-44.14 – -26.98**	**0.16**	**0.11 – 0.24**
	Other	**1.17**	**1.04 – 1.30**	1.15	0.98 – 1.35	**0.73**	**0.58 – 0.91**	1.05	0.79 – 1.41	-16.98	-37.05 – 3.09	0.44	0.16 – 1.23
Parity	1st lactation	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-
	2nd lactation	**1.56**	**1.55 – 1.58**	**1.53**	**1.51 – 1.55**	**0.68**	**0.67 – 0.69**	**1.21**	**1.18 – 1.25**	**1.70**	**0.49 – 2.90**	**6.36**	**5.83 – 6.93**
	3rd lactation	**2.08**	**2.06 – 2.10**	**1.94**	**1.91 – 1.96**	**0.52**	**0.51 – 0.53**	**1.76**	**1.71 – 1.81**	-1.56	-3.27 – 0.14	**10.15**	**9.22 – 11.18**
	4th lactation	**2.47**	**2.44 – 2.50**	**2.24**	**2.20 – 2.27**	**0.44**	**0.43 – 0.45**	**1.85**	**1.78 – 1.91**	**-8.87**	**-11.73 – -6.02**	**12.22**	**10.78 – 13.84**
	5th lactation	**2.86**	**2.82 – 2.90**	**2.45**	**2.40 – 2.49**	**0.37**	**0.36 – 0.38**	**1.70**	**1.64 – 1.76**	**-24.76**	**-31.75 – -17.78**	**13.99**	**11.13 – 17.59**
Days since calving	DIM (30.5 days)	**1.07**	**1.07 – 1.07**	**1.05**	**1.04 – 1.05**	**0.94**	**0.93 – 0.94**	-	-	-	-	-	-
	1st week after calving	-	-	-	-	-	-	1 (ref.)	-	-	-	-	-
	2nd week after calving	-	-	-	-	-	-	0.99	0.95 – 1.02	-	-	-	-
	3rd week after calving	-	-	-	-	-	-	0.96	0.93 – 1.00	-	-	-	-
	4th week after calving	-	-	-	-	-	-	0.97	0.93 – 1.00	-	-	-	-
	5th week after calving	-	-	-	-	-	-	**0.84**	**0.81 – 0.88**	-	-	-	-
	6th week after calving	-	-	-	-	-	-	**0.74**	**0.71 – 0.78**	-	-	-	-
	7th week after calving	-	-	-	-	-	-	**0.67**	**0.63 – 0.71**	-	-	-	-
	8th week after calving	-	-	-	-	-	-	**0.62**	**0.58 – 0.66**	-	-	-	-
	9th week after calving	-	-	-	-	-	-	**0.54**	**0.49 – 0.59**	-	-	-	-
Milkings per day	2 milkings/day	1 (ref.)	-	1 (ref.)	-	1 (ref.)	-	-	-	-	-	-	-
	3 milkings/day	**0.88**	**0.85 – 0.91**	**0.88**	**0.82 – 0.94**	**1.12**	**1.18 – 1.06**	-	-	-	-	-	-
	Milk yield (5 Kg)	-	-	-	-	-	-	**0.95**	**0.94 – 0.95**	**-0.69**	**-0.76 – -0.62**	**0.59**	**0.58 – 0.60**
	Age at first calving	-	-	-	-	-	-	-	-	**0.76**	**0.64 – 0.89**	1.00	0.99 – 1.01
Contingent problems	Ketosis	**1.22**	**1.20 – 1.23**	**1.43**	**1.41 – 1.45**	-	-	-	-	**7.27**	**5.93 – 8.61**	**1.75**	**1.61 – 1.90**
	Mastitis	-	-	-	-	-	-	**1.16**	**1.13 – 1.18**	**7.09**	**5.72 – 8.46**	**1.31**	**1.21 – 1.42**
	Long ICI (> 440 days)	-	-	-	-	-	-	-	-	-	-	**1.34**	**1.24 – 1.45**
Previous parameters	Previous mastitis	**8.76**	**8.70 – 8.82**	-	-	-	-	-	-	-	-	-	-
	Previous milk yield	-	-	-	-	1.00	0.99 – 1.00	-	-	-	-	-	-
	Previous lactose	-	-	-	-	**1.59**	**1.54 – 1.65**	-	-	-	-	-	-
	Previous SCC	-	-	-	-	1.00	1.00 – 1.00	-	-	-	-	-	-

We observed a covariance of 0.20 ± 0.01 within herds. After adjusting for other risk factors, Holstein Friesian was no longer the breed with the lowest prevalence of mastitis, while the effect of other risk factors was confirmed. The effect of ICI was removed by the herd-level risk factors.

Mastitis incidence. The overall IR was 0.14 new cases per cow-month at risk (CI: 0.14 – 0.14), while the monthly individual rate was 0.27. The IR decreased from 2015 (IR = 0.15, CI: 0.15 – 0.15) to 2020 (IR = 0.13, CI: 0.12 – 0.13). The burden of new cases reached the maximum in July (IR = 0.17, CI: 0.17 – 0.17) and the minimum in January (IR = 0.12, CI: 0.11 – 0.12), with an annual oscillation of 47.2%. The individual rate peaked in September (0.41) and reached minimum values in January (0.23). The incidence rate varied among breeds, from Holstein Friesian (IR = 0.13, CI: 0.13 – 0.13) to Brown cows (IR = 0.23, CI: 0.20 – 0.27). It increased from the first lactation (IR = 0.10, CI: 0.09 – 0.10), reaching the highest values after the fourth lactation (IR =0.23, CI: 0.22 – 0.23). The incidence rate of mastitis was high in the first 60 days after calving (IR = 0.17, CI: 0.17 – 0.17), then it decreased in mid-lactation (IR = 0.12, CI: 0.11 – 0.12) to increment again towards the end of the lactation (IR = 0.13, CI: 0.13 – 0.13). Over 305 DIM, the incidence rate reached the same values observed in fresh cows (IR = 0.17, CI: 0.17 – 0.17). Milking three times a day decreased the risk of new mastitis cases by 15%, while ketosis at the beginning of the lactation increased the risk of new cases by 38% (IRR = 1.38).

Like the regression model for prevalece, the model for mastitis incidence showed that Holstein Friesian had no longer the lowest mastitis incidence rate, while all other risk factors were confirmed (Table 4). The average covariance among observations of the same herd was lower than for mastitis prevalence (0.18 ± 0.01).

Recovery from mastitis. On average, 31% of mastitis cases recovered. The recovery proportion followed a seasonal pattern opposite to mastitis incidence, from 0.28 in July to 0.33 in December. Jersey and Holstein Friesian cows had the best chance of recovery (0.33 and 0.32, respectively) and were the sole breeds whose recovery proportion exceeded 30%. The probability of cure was the highest in primiparous (0.44) and fresh (0.44) cows. It decreased with parity, reaching 0.20 after the fourth lactation, and with DIM, to 0.29 in late lactation and even lower beyond 305 DIM (0.24). Milking three times a day ameliorated the probability of recovery by 20% (RR = 1.20, CI: 1.19 – 1.21).

Some variables measured at the previous TD were useful for predicting the outcome of mastitis (Figure 6A-B).

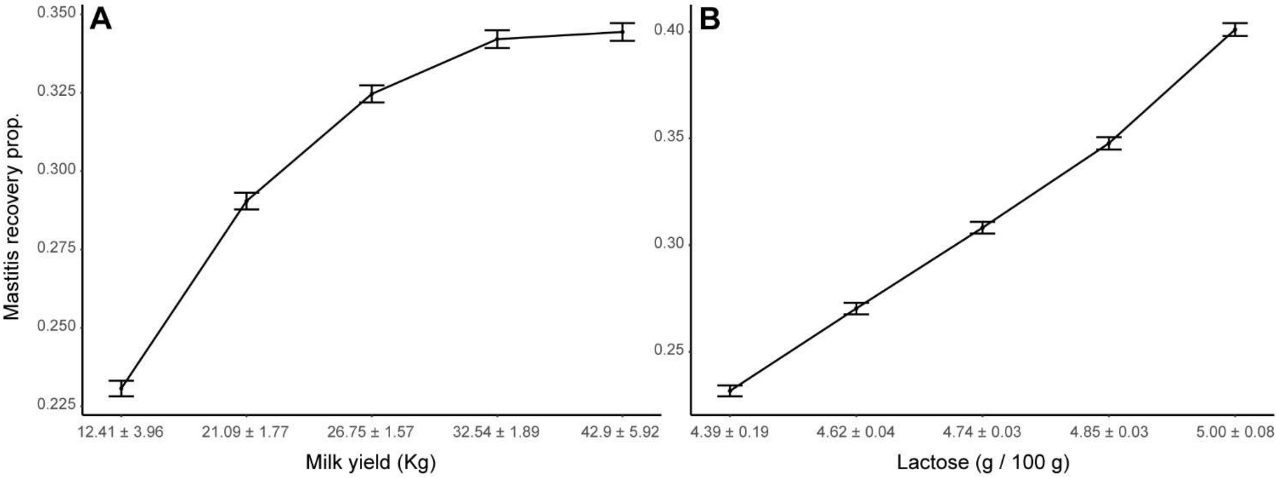


***Figure 6A-B**. Most important predictors of mastitis recovery. The figure reports the observed recovery proportion with 99% confidence intervals, by milk yield (A), and lactose concentration (B).*

The chance of recovery increased by 9% per 5 kg increase in milk production (RR = 1.09, CI: 1.09 – 1.09). Lactose was the best predictor of recovery, as a 0.25 g / 100 g increment in its concentration led to a 29% increase in the chances of recovery (RR = 1.29, CI: 1.27 – 1.31), while SCC was not a predictor at all (RR = 1.00, CI: 1.00 – 1.00).

The regression model evidenced a 0.21 ± 0.01 covariance among observations of the same herd.

Geographic distribution

For the entire Piedmont region, the reference rate of mastitis incidence was 13.3 cases / 100 cow-months (99%CI: 13.2-13.4). Out of the 34 herds with high mastitis incidence: none had high HK incidence; four had long inter-calving intervals; two had high rates of fresh-cow removal. The distribution of incident mastitis cases and that of SDR of mastitis are displayed in Figure 7. The SDR of mastitis did not strongly differ from the raw incidence rate (Figure 7). With SDR it is possible to observe municipalities with mastitis incidence lower than or equal to population average are displayed in the brightest color. A similar pattern is observed using the EBSR of mastitis incidence (Figure 8), although in this case the rate of municipalities with a small population was smoothed towards the average.

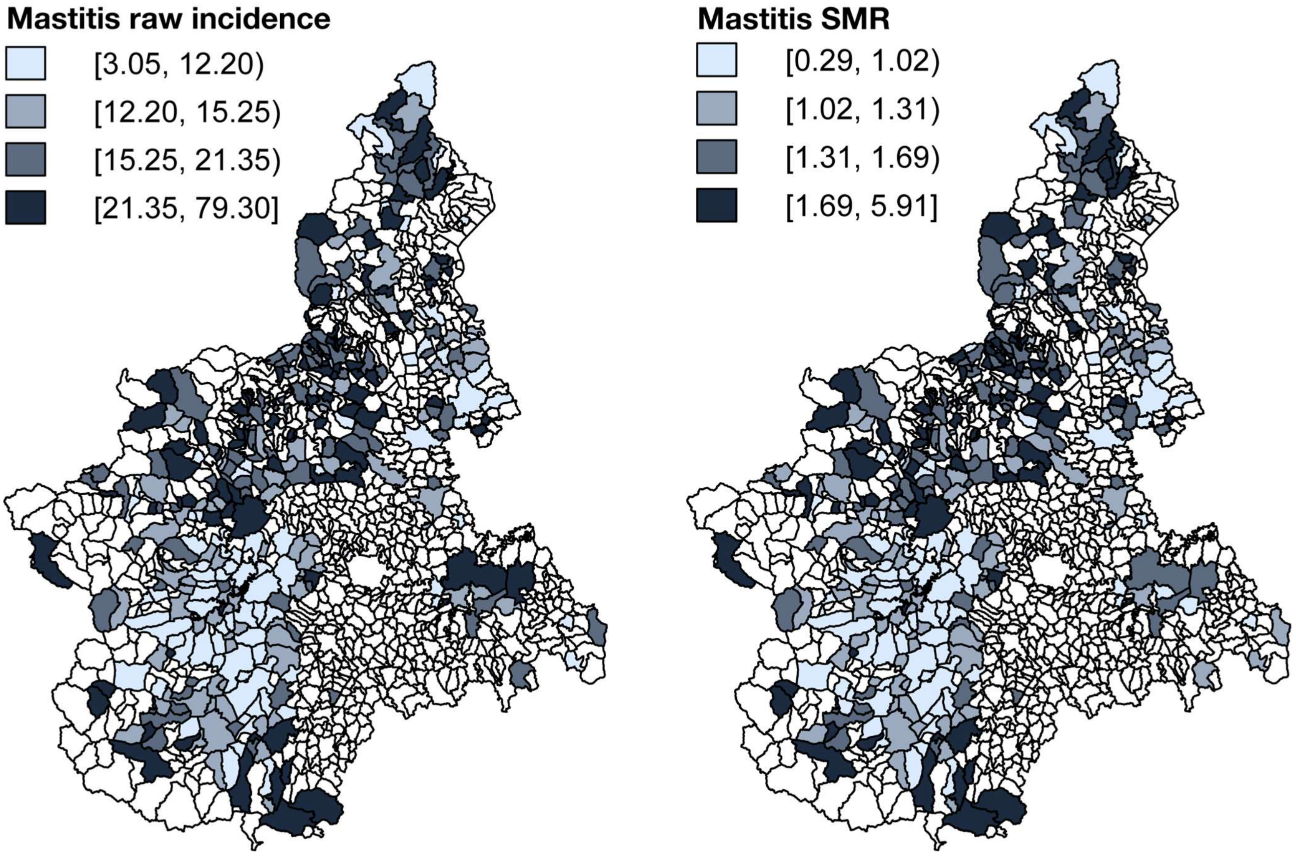


Figure 7. *Distribution of incident mastitis cases (left) and standard mastitis rate (right) in dairy herds in the Piedmont region. Data are aggregated at municipality level. Municipalities are divided in quartiles, from the lowest incidence (brightest color) to the highest (darkest color).*

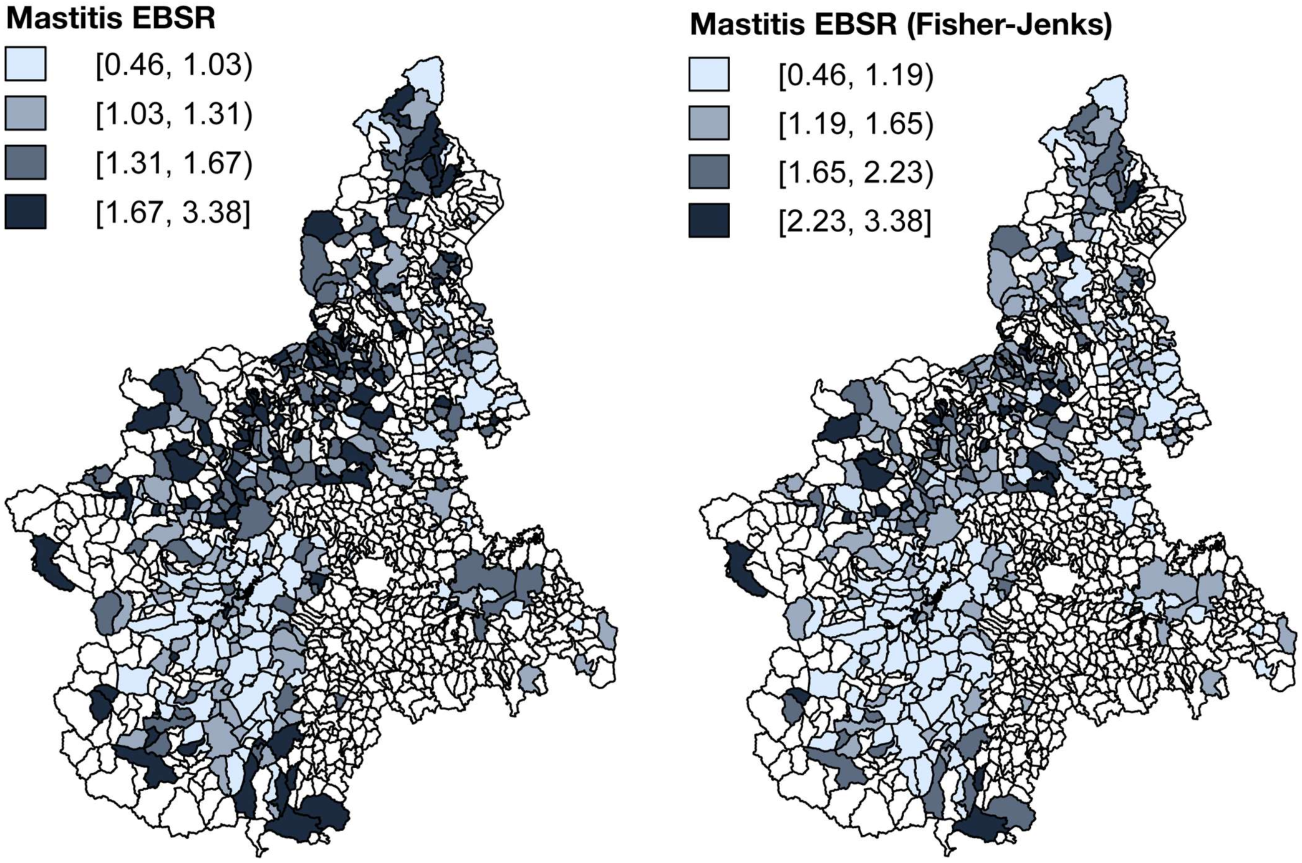


Figure 8. *Distribution of empirical Bayes smoothed rate of mastitis incidence in dairy herds in the Piedmont region. Data are aggregated at municipality level. Municipalities are divided in quartiles (left) and by Fisher-Jenks natural breaks intervals (right), from the lowest incidence (brightest color) to the highest (darkest color).*

Comparing quartile-colored map with the one using Fisher-Jenks natural breaks, it appeared evident that a limited number of municipalities with mastitis incidence significantly higher than the population average existed (Figure 8).

The local aggregation of mastitis cases, both calculated on the basis of adjacency and distance, showed that mastitis cases tend to be more frequent in dairy herds in marginal areas and in the mountains (Figure 9).

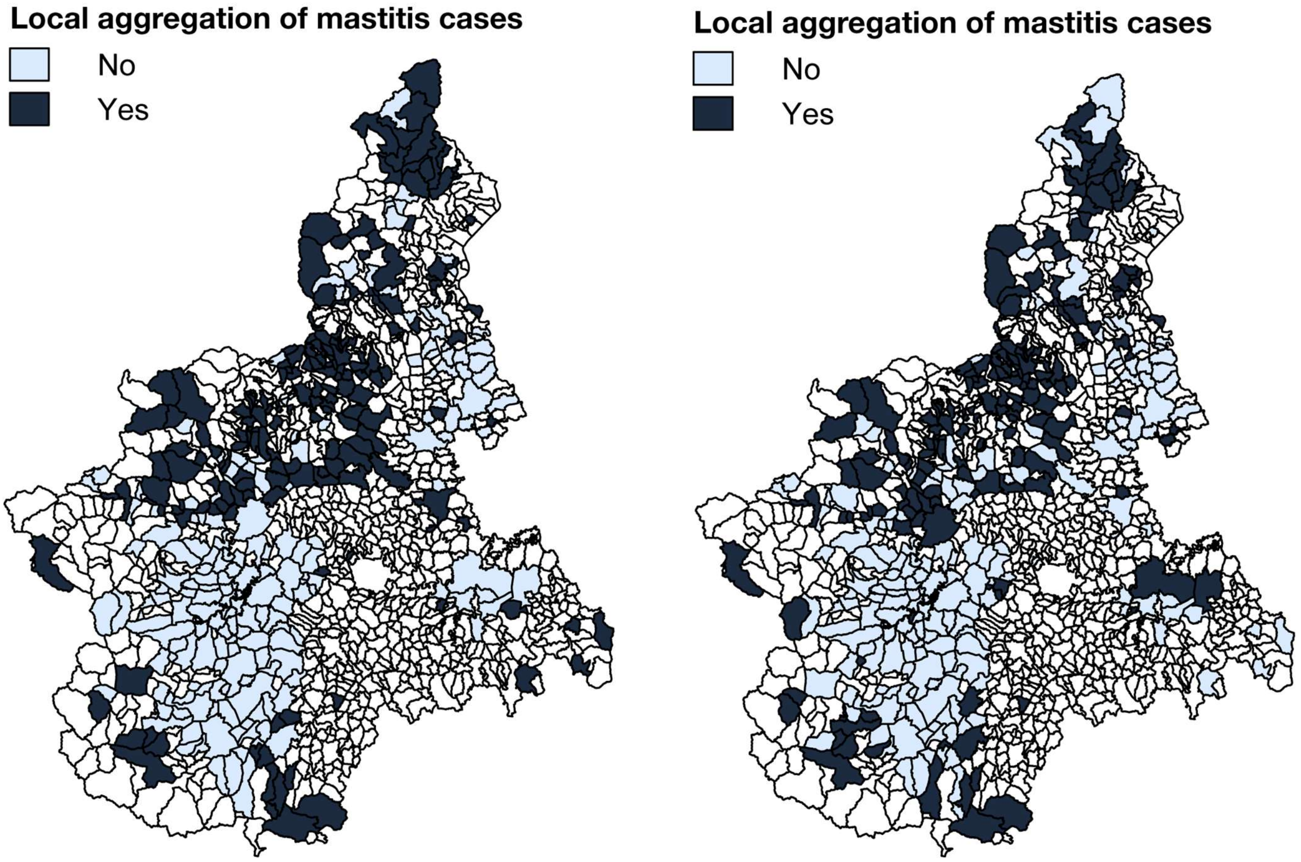


Figure 9. *Adjacency- (left) and distance-based (right) local aggregation of mastitis incidence in dairy herds in the Piedmont region. Data are aggregated at municipality level. Municipalities are divided on the basis of the I statistics being positive (aggregation) or negative (no aggregation).*

Hyperketonemia

The first TD occurred on average 22.91 ± 12.84 days after calving. The mean F:P was 1.25 ± 0.34, and the overall incidence rate was 0.23 HK / cow-lactation. We did not observe an annual trend, but a seasonal pattern opposite to mastitis repeated every year, with the minimum number of cases in September (IR = 0.19, CI: 0.18 – 0.19) and the maximum in March (IR = 0.26, CI: 0.26 – 0.27). HK varied with the breed, reaching the highest values in Jersey, and Holstein Friesian cows. Based on bivariate analysis, the risk of HK increased from the first (IR = 0.20, CI: 0.20 – 0.21) to the fourth lactation (IR = 0.28, CI: 0.27 – 0.28), then decreased over the fifth lactation (IR = 0.23, CI: 0.23 – 0.24).

In the first four weeks after calving, it remained permanently above 0.24 then declined linearly until the ninth week (IR = 0.14, CI: 0.13 – 0.15). The risk of ketosis increased by 5% for every 5 kg increase in milk yield (IRR = 1.05, CI: 1.05 – 1.05), and by 15% with mastitis (IRR = 1.15, CI: 1.13 – 1.17), while milking the cows three times a day did not ameliorate nor worsen their metabolic condition (IRR = 0.99, CI: 0.96 – 1.01).

In the regression model, the covariance for observations within the same herd was 0.52 ± 0.02. All the estimates are reported in Table 4.

Geographic distribution

The reference population rate of hyperketonemia was 23.0 cases / 100 cow-lactations (99%CI: 22.8-23.1). Out of the 40 herds with the highest ketosis incidence: three had high rate of fresh-cow removal; and none had long ICI or high mastitis incidence. The distribution of SDR showed higher values mainly in lowland areas, while values below the average were observed in the other areas (Figure 10).

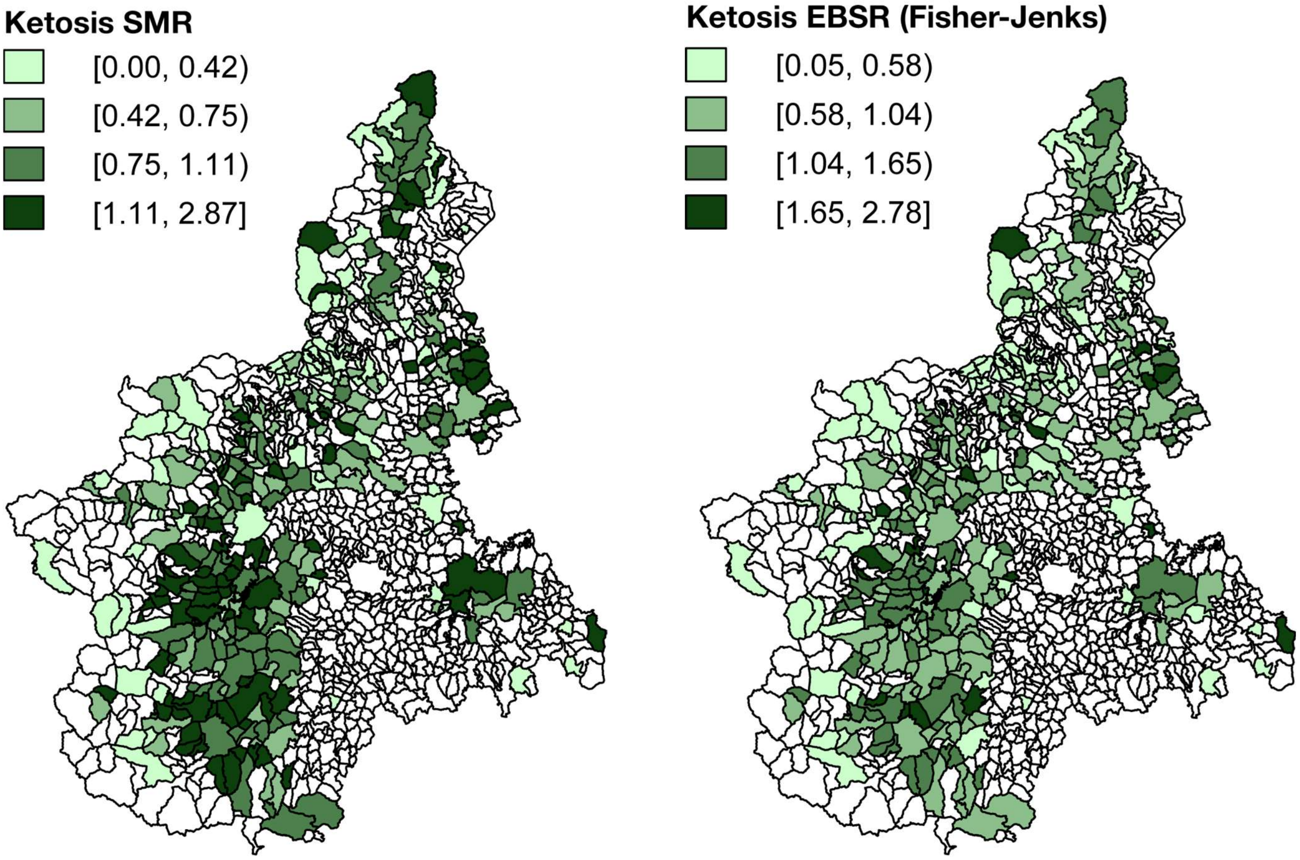


Figure 10. *Distribution of standard rate (left) and empirical Bayes smoothed rate (right) of hyperketonemia in dairy herds in the Piedmont region. Data are aggregated at municipality level. Municipalities are divided in quartiles (left) and by Fisher-Jenks natural breaks intervals (right), from the lowest incidence (brightest color) to the highest (darkest color).*

The ESBR, and the use of Fisher Jenks' intervals highlighted the presence of a few municipalities where HK cases were above the population mean. The lowest values were observed in mountain and marginal areas, while the highest were disseminated throughout the region.

The local aggregation analysis showed that HK tend to be more frequent in lowland dairy herds with few exceptions for the northernmost part of the region (Figure 11).

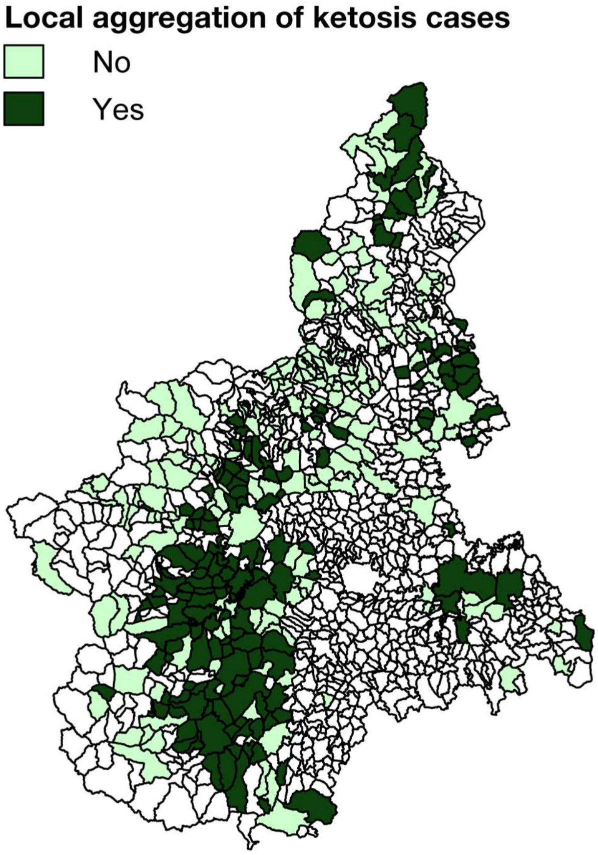


Figure 11. *Distance-based local aggregation of hyperketonemia in dairy herds in the Piedmont region. Data are aggregated at municipality level. Municipalities are divided on the basis of the I statistics being positive (aggregation) or negative (no aggregation).*

Inter-calving interval

The mean inter-calving interval was 428.92 ± 104.18 days, and it was longer than 500 days in more than 10% of the cows. The average duration of ICI decreased during the study period from 448.19 ± 129.09 in 2015 to 409.06 ± 76.02 in 2019. The mean ICI of 2020 (ICI = 367.03 ± 39.81) could be underestimated due to the study end. The ICI of Holstein Friesians was 429.70 ± 101.07 days long, a middle ground between 411.91 ± 115.96 days of Piedmontese and 473.87 ± 181.97 days of Abondance cows. The ICI decreased from the first (429.33 ± 109.89) to the second calving (427.71 ± 98.37), then increased monotonously to the fifth one (431.13 ± 104.53). Also, the higher the milk yield the shorter the ICI, and the decrement was monotonous. The ICI duration slightly decreased in cows milked three times a day (424.07 ± 92.07) in reference to those milked twice (429.36 ±105.20), while it increased with the age at first calving. We considered ages at first calving that ranged from 20 to 37 months. Ketosis and mastitis during pregnancy determined an extension of ICI, which came from 419.75 ± 93.01 to 427.31 ± 94.61 in the case of ketosis, and from 424.50 ± 100.23 to 443.64 ± 115.19 in the case of mastitis.

The regression model showed that nine breeds had ICI shorter than Holstein Friesian. All other estimates confirmed what was observed by bivariate analysis.

Geographic distribution

The prevalence of long-ICI (ICI > 440 days) was 33.3% (99%CI: 33.1-33.4) in Piedmont. Out of the 34 herds with the highest long-ICI prevalence: four had high mastitis incidence; one had high fresh-cow removal rate; and none had high hyperketonemia incidence. The distribution of long-ICI in the Piedmont region was scattered with no clearly recognizable pattern. Several areas where long-ICI rate was more frequent than the average were identified. The scattered distribution of long-ICI was observed even by EBSR, although less extreme values were reported (Figure 12).

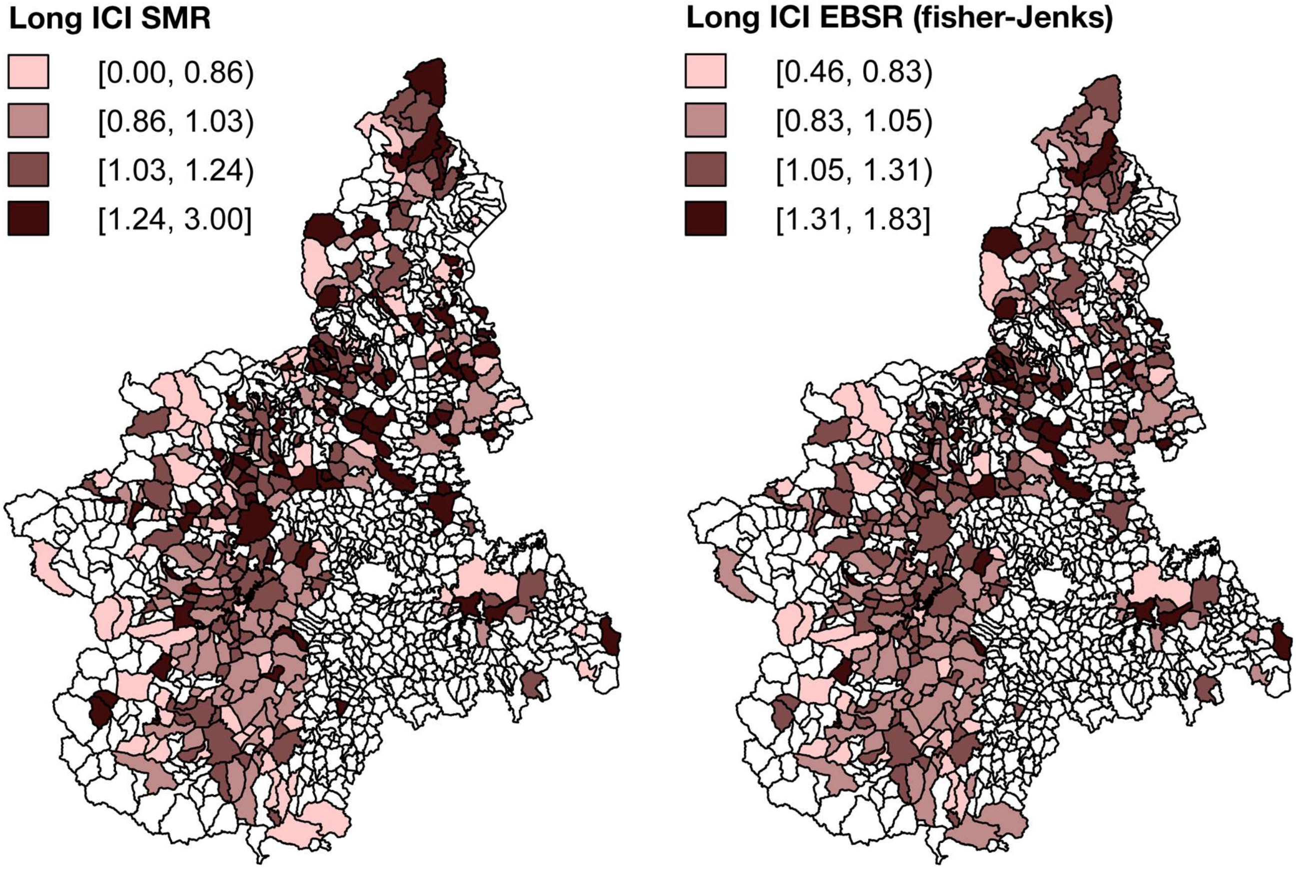


Figure 12. *Distribution of standard rate (left) and empirical Bayes smoothed rate (right) of long-ICI in dairy herds in the Piedmont region. Data are aggregated at municipality level. Municipalities are divided in quartiles (left) and by Fisher-Jenks natural breaks intervals (right), from the lowest incidence (brightest color) to the highest (darkest color).*

Based on the *I* statistics calculated on distance, the local aggregation of long-ICI was estimated. The figures shows that the scattered distribution is mirrored by a local aggregation at municipality level, with no recognizable pattern at a wider scale nor throughout the region (Figure 13).

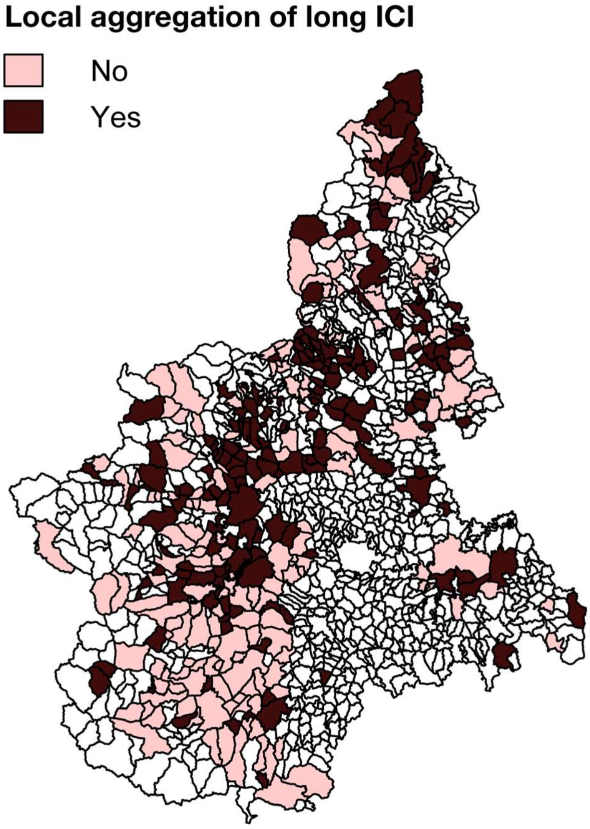


Figure 13. *Distance-based local aggregation of long-ICI in dairy herds in the Piedmont region. Data are aggregated at municipality level. Municipalities are divided on the basis of the I statistics being positive (aggregation) or negative (no aggregation).*

Fresh-cow removal

The overall proportion of fresh cows removed was 3%, and no trend was observed over years. Fresh-cow removal followed a seasonal pattern which peaked in August (IP = 0.05, CI: 0.04 – 0.05) and reached the minimum in April (IP = 0.03, CI: 0.02 – 0.03).

The bivariate analysis showed that the risk of removal increased with parity, from about 1% in primiparous cows to 7% (0.06 – 0.07) in the fifth lactation. Holstein Friesians had the fourth to lowest incidence proportion (IP = 0.03); lower values were recorded for Brown Swiss, Italian red roan, and other breeds

For each 5 kg increment in milk production, the risk of fresh cow removal decreased by 24% (RR = 0.76, CI: 0.76 – 0.76). The risk of being removed raised by 37% if the cow had an ICI $\geq$ 440 days (RR = 1.37, CI: 1.32 – 1.41), almost doubled in the case of ketosis (RR = 1.96, CI: 1.91 – 2.00), and it grew even higher in case of mastitis (RR = 2.36, CI: 2.32 – 2.40).

Based on the results of the model, most breeds had a lower prevalence than Holstein Friesian, which recorded the second-highest value. Adjusting for herd-level risk factors, the age at first calving was no longer significant; however, keeping it into the model ensures a better fit. The covariance among observations of the same herd was 0.56 ± 0.04.

Geographic distribution

The reference population rate was 3.3% (99%CI: 3.2-3.3%). Out of the 45 herds with the highest fresh-cow removal rate: three had high ketosis incidence; two had high mastitis incidence; and one had high fresh-cow removal rate. The distribution of fresh cow removal rate is presented in Figure 14. Most municipalities showed values around the average, but some had extremely high values, up to ten fold the population mean. However, smoothed rates in combination with Fisher Jenks natural breaks intervals depicted a neatly different image, with high values in a few areas, and homogeneous values in most of the region (Figure 14).

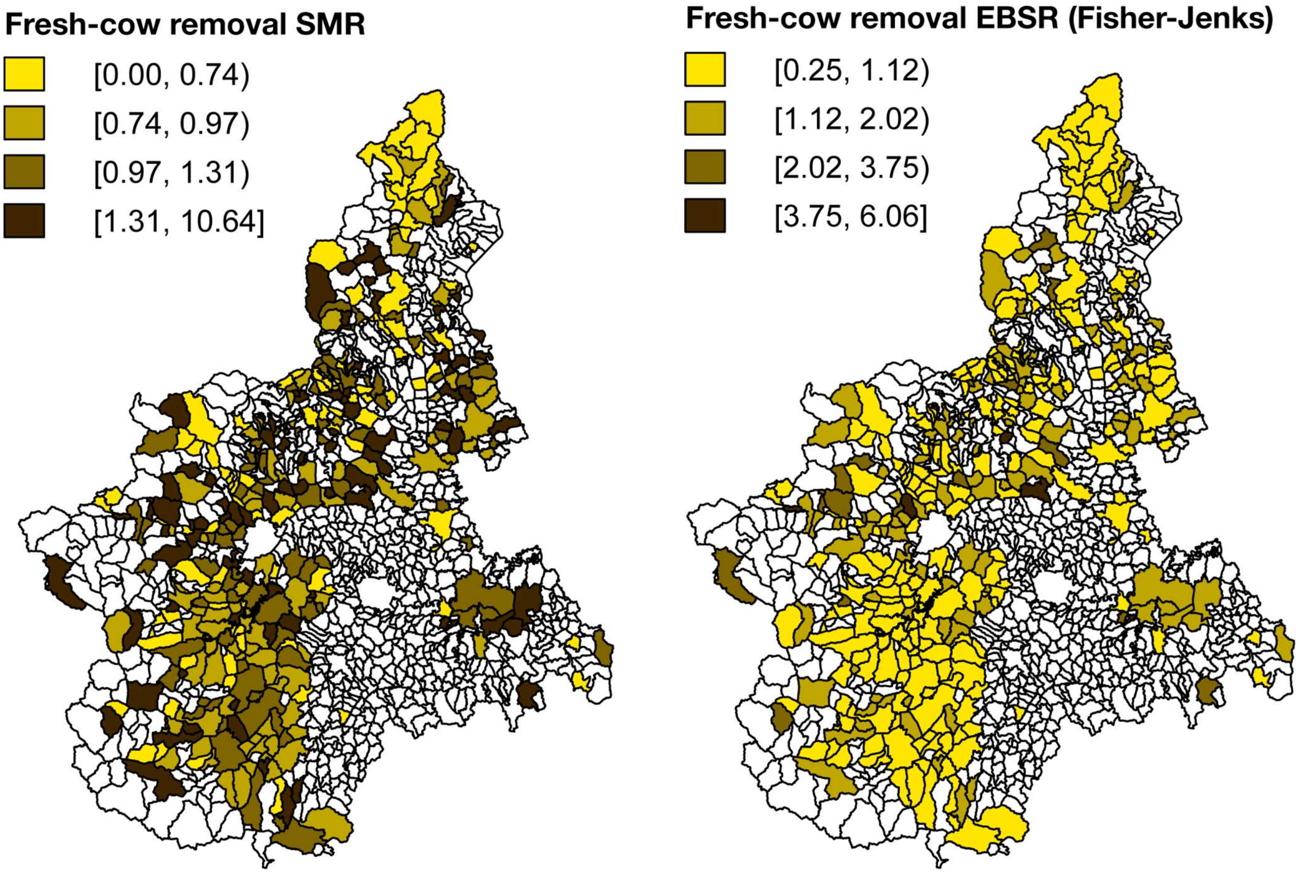


Figure 14. *Distribution of standard rate (left) and empirical Bayes smoothed rate (right) of fresh-cow removal in dairy herds in the Piedmont region. Data are aggregated at municipality level. Municipalities are divided in quartiles (left) and Fisher-Jenks natural breaks intervals (right), from the lowest incidence (brightest color) to the highest (darkest color).*

As evidenced by the SDR and EBSR, also local aggregation based on distance was observed without a recognizable pattern. There were several *foci* of aggregation both in lowland and in marginal and mountain areas (Figure 15).

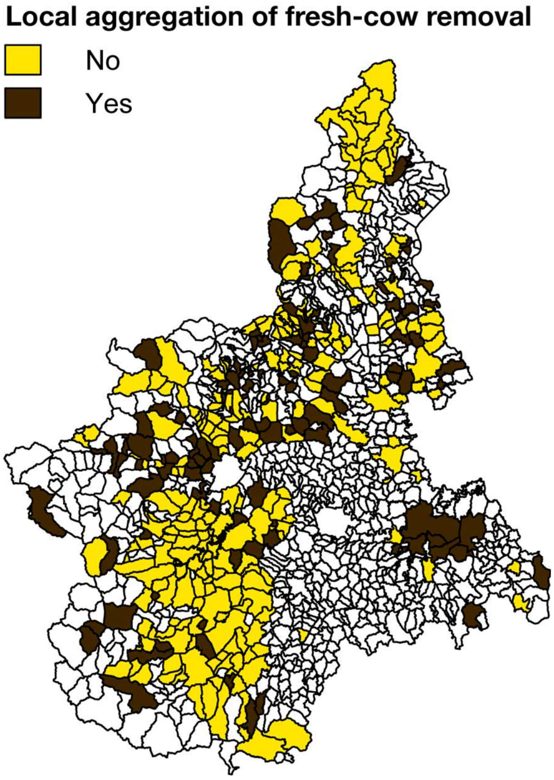


Figure 15. *Distance-based local aggregation of fresh-cow removal in dairy herds in the Piedmont region. Data are aggregated at municipality level. Municipalities are divided on the basis of the I statistics being positive (aggregation) or negative (no aggregation).*

Animal welfare

Out of 849 animal welfare evaluations, only the most recent for each herd (n = 357) were analyzed. Out of the 357 herds, 277 (77.6%) also participated in DHI, thus information about HI were available. The herds which underwent welfare evaluation and were also involved in DHI had better scores for AA (Table 5).

Table 5. *Results of the most recent animal welfare assessment with the CReNBA protocol for dairy herds in the Piedmont region between 2017 and 2020. Mean (± standard deviation) of the five assessment areas and Total scores are displayed for herds participating and non-participating in dairy herd improvement program (DHI).*

DHI	**ABM**	**Management**	**Structures**	**Great risks**	**Biosecurity**	**Total**
no	0.83 ± 0.11	0.74 ± 0.13	0.69 ± 0.12	0.59 ± 0.17	0.56 ± 0.14	0.77 ± 0.09
yes	0.84 ± 0.09	0.80 ± 0.11	0.71 ± 0.11	0.63 ± 0.16	0.62 ± 0.13	0.80 ± 0.08
p-value	0.412	0.000	0.021	0.018	0.000	0.008

On the other hand, herds undergoing welfare assessment showed a lower risk of mastitis and a shorter ICI, but higher risk of hyperketonemia and fresh cow removal rate (Table 6).

Table 6. *Mean (± standard deviation) of the results of health indicators analysis (from DHI data) for dairy herds undergoing and non-undergoing animal welfare assessment.*

Welfare assessment	**Mastitis**	**Hyperketonemia**	**ICI**	**Fresh cow removal**
no	0.53 ± 0.17	0.47 ± 0.15	0.17 ± 0.07	0.45 ± 0.12
yes	0.43 ± 0.16	0.51 ± 0.16	0.16 ± 0.05	0.50 ± 0.11
p-value	0.000	0.000	0.003	0.000

Based on LPA, four herd profiles (A, B, C, D) were identified. Compared with C, the most frequent profile (n = 1043), herds in A (n = 11) had higher mastitis risk ($p = 0.020$) and fresh-cow removal rate ($p < 0.001$), and longer ICI ($p < 0.001$); herds in B (n=76) had shorter ICI ($p = 0.004$) but higher fresh-cow removal rate ($p < 0.001$); those in D (n = 111) had higher risk of hyperketonemia ($p < 0.001$) and fresh cow removal rate ($p < 0.001$) but shorter ICI ($p < 0.001$) and lower risk of mastitis ($p < 0.001$).

Table 7. *Mean (± standard deviation) of the results of health indicators analysis (from DHI data) for dairy herds in the four profiles.*

Profile	Mastitis	Ketosis	ICI	Removal
A	0.64 ± 0.21	0.48 ± 0.14	0.50 ± 0.20	0.56 ± 0.23
B	0.53 ± 0.19	0.46 ± 0.12	0.15 ± 0.04	0.71 ± 0.08
C	0.52 ± 0.17	0.45 ± 0.13	0.17 ± 0.06	0.43 ± 0.10
D	0.40 ± 0.14	0.75 ± 0.09	0.13 ± 0.04	0.49 ± 0.09

Posterior marginal probabilities of a herd showing one of the four profiles were: A) 0.9%; B) 9.1%; C) 79.5%; and D) 10.5%.

Table 8. *Mean (± standard deviation) of the animal welfare assessment scores for dairy herds in the four profiles.*

Profile	ABM	Management	Structures	Great risks	Biosecurity
A	-	-	-	-	
B	0.85 ± 0.09	0.84 ± 0.12	0.76 ± 0.11	0.67 ± 0.12	0.68 ± 0.13
C	0.83 ± 0.10	0.78 ± 0.10	0.70 ± 0.11	0.61 ± 0.17	0.61 ± 0.13
D	0.86 ± 0.08	0.85 ± 0.08	0.74 ± 0.09	0.69 ± 0.11	0.66 ± 0.10

Since no welfare data was available for herds with A profile, the LDA was performed on the others. Sensitivity in identifying herds with welfare scores in the lowest quartile was 12%. However, it confirmed negative values with 96% specificity. Using this dataset, the LDA analysis had a positive predictive value equal to 50%, and a 78% negative predictive value.

Discussion

Benchmarking dairy cattle herds through DHI data is pivotal for enhancing herd efficiency and productivity. DHI programs provide comprehensive and systematic records of milk production, reproductive performance, and health status of individual animals within a herd. By leveraging this data, dairy producers can identify performance gaps, optimize management practices, and implement targeted interventions to address specific issues such as low milk yield or poor reproductive performance. Studies have shown that herds participating in DHI programs consistently exhibit superior performance compared to non-participating herds. For instance, research by de Vries et al. (2011) demonstrated that herds utilizing DHI data had higher milk yields, better reproductive efficiency, and lower somatic cell counts, indicative of improved udder health. Similar results have been obtained in this study, where it was observed that herds with complete DHI data perform better in terms of milk yield, composition and SCC. Benchmarking against industry standards and peer herds allows for the objective assessment of herd performance, facilitating data-driven decision-making. This process not only aids in improving the economic viability of dairy operations but also contributes to sustainable dairy farming by promoting best practices in animal health and welfare. Indeed, best performing herds also tend to have better animal welfare assessment scores. Additionally, continuous monitoring and evaluation through DHI data enable producers to track progress over time, ensuring long-term improvements and resilience in the face of changing environmental and market conditions (Weigel, 2001). Thus, the strategic use of DHI data is indispensable for achieving operational excellence and sustainability in dairy farming.

Herd health indicators

Four herd HI were estimated from DHI for a large cohort of dairy cows and herds in the Piedmont region. The estimates were calculated considering cow-level risk factors, as previously described, and herd-level risk factors. The latter were accounted for as random effects, whose covariance was the measure of the variability that occurred within the same herd. To reduce the computational load, we chose to limit the hierarchy of the model at the herd level, and no auto-correlation factor was included for the cows, although some authors suggested that the herd performance depends on a single cow’s resilience (Poppe et al., 2021).

Mastitis

The mastitis prevalence observed in Piedmont was 29%. It was consistent with the prevalence reported in other European countries (Krishnamoorthy et al., 2021) and fell inside the interval estimation by Shook et al. (2017) for intramammary infections, but it was lower than what was reported in Italy by Ceniti et al. in 2017, and in other geographical regions (Fesseha et al., 2021; Krishnamoorthy et al., 2021) In this work, the underestimation of mastitis prevalence could be due to the choice of excluding infection occurred during the dry period and to the chosen threshold of 200,000 cells/mL. In fact, it has been reported that 44% of the cows with at least one infected quarter in composite milk do not exceed this threshold in composite milk (Petzer et al., 2017). On the other hand, the mastitis incidence rate was similar to that reported by Busanello et al. (2017), where the same threshold was used, but lower than in other studies (Aghamohammadi et al., 2018; Olde Riekerink et al., 2008; Reyher et al., 2011). More in general, the lack of recent studies in the same geographical area and the difference in the methods, threshold selection, and definition of incident cases make results difficult to compare. The optimal SCC threshold for mastitis diagnosis is still under debate (Bradley and Green, 2005; Dohoo et al., 1981, 1984; Fauteux et al., 2014; Petzer et al., 2017b). It was reported that the 200,000 cells/mL threshold has a sensitivity between 73 % and 89 %, and a specificity between 75 % and 86 % (Dohoo and Leslie, 1991; McDermott et al., 1982), but it predicts poorly in primiparous cows, where 15.8 % sensitivity and 84.4 % specificity were recently reported (Lipkens et al., 2019). This could represent a limitation of this study since more than one-third of the cows were primiparous which can result in an underestimation of mastitis cases. Regarding the distinction between an incident and a recurrent case, cows with SCC persistently over the threshold should be excluded from the incidence rate calculation. However, in chronic infections, the SCC fluctuates over time, and there is no consensus yet about the time that must elapse before a new case can be defined (Bradley et al., 2007; Petzer et al., 2017b). The choice of an eight-week timespan instead of a shorter one can partially explain the underestimation of the incidence rate of mastitis in this study.

To provide the state of the art of mastitis in the Piedmont region, both prevalence proportion and incidence rate were estimated since they measure different aspects of udder health. High incidence rates could be the result of transient infections as well as recurrent infections, while high prevalence proportions are likely caused by chronic infections. The simultaneous evaluation of prevalence, incidence rate, and recovery rate suggested that prevalent cases are the results of incident cases which failed to recover. Across the study period, the decreasing trend of both prevalent and incident mastitis cases could be explained by the rewarding systems for milk quality and increased awareness thanks to DHI programs (Barkema et al., 1998; Østerås and Sølverød, 2009). The burden of cases and the recovery rate followed a seasonal pattern typical of temperate areas, where during summer, the high temperatures stress the immune system and promote bacterial growth (Vitali et al., 2020).

On average, 30% of cows recovered from mastitis. However, as mentioned above, some of them might have experienced a temporary decrease in SCC and not a complete recovery. This limitation is hardly avoidable using only DHI data, and a more detailed analysis should comprehend a bacteriologic examination. The chance of recovering decreases as the cow grows older since the probability of developing a chronic infection increases while decreasing the possibility of being treated. As cows grow old, their market value decreases, leading to a lower cost-efficacy ratio of the therapy (De Vries and Marcondes, 2020). Also, although mastitis incidence was higher in the first lactation stage, the chance of recovering decreased as lactation progressed since a cow at the end of the lactation had more probability of developing a chronic infection. Eventually, our results suggested that milking three times a day reduced the risk of mastitis and increased the chance of recovery, in contrast to what was previously reported (Allen et al., 1986). However, the number of farms where cows were milked three times a day was considerably lower than those where they were milked twice.

Our findings confirmed that milk production and lactose concentration could serve as prognostic indicators of mastitis recovery. In vitro studies suggested that the lactose concentration has an association with the number of living bacteria, being the primary energy source for many mastitis pathogens (Stürmlin et al., 2021). On the other hand, an association has been reported between high-yielding cows, which are more prone to mastitis, and lactose concentration and between the latter and subclinical mastitis (Antanaitis et al., 2021). Indeed, lactose concentration depends on many factors, e.g., energy balance, inflammation and infection occurrence, and amount of water activity, thus suggesting that a more complex relationship exists (Antanaitis et al., 2021).

Italian red roan cows had a lower prevalence and incidence of mastitis as well as a higher chance of recovery than Holstein Friesian. On the contrary, Oropa red roan cows had all three mastitis indicators worse than Holstein Friesian. Some rustic breeds are linked to defined geographical areas, like in some valleys of Piedmont, with breeding traditions and animal husbandry techniques. This may have biased estimates related to these breeds, as breeding methods and environmental conditions were not randomly distributed, although the large sample size and inclusion of the herd-effect as random effects in the estimation process should have mitigated the effect of this potential bias.

Cow-level risk factors explained most of the risk of mastitis (Hogeveen et al., 2010; Steeneveld et al., 2008), but herd-level risk factors exerted an effect which was responsible for part of the variability we observed within herds for all three mastitis indicators. However, herd-level variables would require a dedicated research since this information is not available from DHI records.

Hyperketonemia

The beta-hydroxybutyric acid concentration in blood ([$BHBA_{blood}$]) is described as the best diagnostic tool for hyperketonemia (Duffield et al., 2009; McArt et al., 2015; Ospina et al., 2010), while different thresholds for its concentration in milk ([$BHBA_{milk}$]) have been studied, yet no universal consensus has been achieved (de Roos et al., 2007; Denis-Robichaud et al., 2014; Ježek et al., 2017; Santschi et al., 2016; Tatone et al., 2017). Regarding HK, the major limitation of this study is that [$BHBA_{milk}$] was not consistently provided throughout the Piedmont region at the time of the study, whence the use the fat-to-protein ratio was the only available indicator of HK. It has been reported to have worse accuracy and precision, although the cut-off of 1.42 is reported to predict [$BHBA_{blood}$] ≥ 1.25 mmol/mL with a 92 % sensitivity and 65 % specificity (Jenkins et al., 2015). Other thresholds have been suggested for the same or different blood concentrations, and the choice of F:P and [$BHBA_{blood}$] cut-offs could result in over- or underestimation of HK. Also, the choice of a time frame of 60 days after calving during which ketosis can occur is questionable. It was selected to match the definition of fresh cow, even though the first TD after calving usually occurred earlier.

The 23% incidence rate of HK is consistent with previous reports (McArt et al., 2012; Tatone et al., 2017), but lower than what was estimated using [$BHBA_{blood}$] (Berge and Vertenten, 2014; Vanholder et al., 2015). Breeds were affected depending on their productivity since the risk increased with milk yield. The risk of HK remained high as late as sixty days postpartum which could be an overestimation due to the chosen threshold or the effect of undiagnosed HK in the earlier phase of the lactation. Ketosis incidence increased until the fourth lactation but decreased afterwards, probably because of the milk yield reduction in older cows. Like it was previously described (Tatone et al., 2017), HK peaked during winter and spring, possibly due to the peak of milk production and the poorer feed quality, as worse quality forages are available in the cold season. Our findings confirmed previously reported risk factors for ketosis, e.g., milk yield, stage of lactation, and parity (Tatone et al., 2017). The high covariance we observed within herds suggested that the risk of ketosis depended on herd-level as much as on cow-level risk factors.

Inter-calving interval

In dairy herds in Piedmont, the observed mean inter-calving interval was longer than 420 days and exceeded 500 days in more than 10 % of the cows. This average was inflated by extreme values which are mainly observed in rustic breeds. The difference among breeds likely reflected the herd management more than breeds diversity, as well as the age at first calving which proved to be a good predictor for long ICI and is strictly related to the breeder's choice. The ICI decreased significantly from 2015 to 2020, suggesting that DHI programs are increasing awareness of cows' reproductive health and efficiency, too. This trend is consistent with the general improvement observed for other health indicators like mastitis. Except for the first lactation, the ICI increased with parity, probably because reproductive problems arose progressively during the lifespan of the cow. The enlargement of ICI has many risk factors that occur during the milking and the dry period (Carvalho et al., 2019; McDougall, 2006), but the main limitation of ICI is that it is observed only once per lactation leaving all those risk factors occult. Also, in this research, it was measured only in parturient cows, thus issues of survival bias arise (Fetrow et al., 2007; Olori et al., 2002). Our findings confirmed that diseases like ketosis and mastitis heavily affected the reproductive performance of the cow (Carvalho et al., 2019; McArt et al., 2012), resulting in an increment in the duration of the subsequent ICI. Also, our results corroborated the hypothesis that productive and reproductive performances are associated (De Vries and Marcondes, 2020), since we observed that the ICI decreased as milk yield increased. On the other hand, we can partially exclude that longer ICI was due to the breeder's choice to postpone the breeding of high-yielding cows. Regarding the reproductive performance estimation, the main limitation of this study is the choice of the inter-calving interval as an indicator, since other reproductive indicators are better suited for this purpose, e.g., the calving-conception interval or the pregnancy rate. However, those are not available from DHI data.

Fresh-cow removal

The peak of milk yield occurs in the first two months after calving when the expenses for cow maintenance during pregnancy are paid back by the production. Therefore, a cow is most valuable during the first lactation phase, and severe problems should occur to make the breeder decide to remove a fresh-cow from the herd (Carvalho et al., 2019). However, our results were based on the previous assumption and could overestimate the occurrence of such severe problems, since from TD data it was not possible to distinguish health issues from voluntary sales.

We observed a seasonal pattern in the fresh-cow removal, which peaked during the summer months. It suggested that heat stress could aggravate the situation and jeopardise the ability of the cow to recover (Polsky and von Keyserlingk, 2017; Vitali et al., 2020). HK and mastitis affected the removal of a fresh cow from the herd as they likely influenced the farmer's decision to cull the cow. Also, the risk of being removed in the first 60 days increased with parity, possibly because older cows are more prone to health problems and less valuable on the market (De Vries and Marcondes, 2020). Similarly, high-producing cows had a lower risk of being reformed, although it is difficult to assess the direction of this association since healthier cows tend to live longer and produce more (De Vries and Marcondes, 2020). Eventually, cows with ICI longer than 440 days had more chance of being removed within 60 days from calving. This could depend on reproductive issues, e.g., the cow not becoming pregnant. Nonetheless, it was not possible to exclude that it was a planned decision of the breeder who voluntarily decided to cull the cow.

Geographic distribution of herd health indicators

The geographic representation suggested that the distribution of HI follows different patterns. For mastitis incidence, higher values were observed in mountain and marginal areas, where the access to veterinary services is limited. Limited access to veterinary services in mountain and marginal regions poses significant challenges to livestock health and productivity as it often results in higher incidences of diseases and lower overall herd performance. Indeed, the scarcity of veterinary services in remote regions can lead to delayed disease diagnosis and treatment, increasing mortality rates and reducing reproductive success (Pasteur et al., 2024).

An opposite pattern was observed for hyperketonemia, that reached the highest values in lowland areas, where the largest and most productive herds were located. High-producing herds are most exposed to HK due to the negative energy balance the cows experience in early lactation. Moreover, in large herds, the prevalence of hyperketonemia can be particularly problematic due to the challenges in individual animal monitoring and timely intervention (McArt et al., 2012).

For ICI and fresh cow removal, no geographic pattern was observed. On the contrary, they seemed scattered throughout the region. Their distribution suggested that several factors act at herd level and their prediction was likely more complex than that of mastitis and hyperketonemia.

The analysis of co-morbidity showed that herds with the highest mastitis incidence had also long ICI and sometimes high fresh-cow removal rate, but not high hyperketonemia incidence. This, along with geographic distribution, suggested the existence of two different kind of herds: high-producing herds that were affected mainly by nutritional diseases due to the high productive performance; non high-producing herds, where several health issues, like mastitis and reproductive disorders, jeopardize profitability and reduce sustainability. Fresh cow removal rate was associated with all the three other HI, confirming that cows were culled due to multiple reasons. No herd was observed to have detrimental values for all HI.

Animal welfare

The results of the sensitivity analysis performed comparing herds with and without a complete set of DHI data were consistent with the results of animal welfare assessment. Indeed, smaller and less controlled herds had worse productive performance likely due to reduced awareness of the ongoing problems, The comparison of CReNBA assessment between herd participating and not participating in DHI suggested that the participation exerts a positive effect on welfare, again, possibly related to farmer's awareness and confirming the importance of monitoring not only for performance purposes. LPA results highlighted the existence of few herds having significantly worse HI than the rest (A profile). Unfortunately, for none of them welfare evaluation was available, and this lack of information about the most informative herds limited the representativeness of the dataset. Among the other herds, three different profiles could be identified with different assessment areas' scores of welfare evaluation. Profiles B and D had similar welfare results, both better than profile C. However, they represented two different types of herds based on HI. Arguably, profile D included the most productive herds, as it is demonstrated by the high incidence of hyperketonemia. It also included herds with the lowest risk of mastitis and ICI duration. Compared to B profile, D profile herds have a significantly lower mastitis risk and fresh cow removal rate, thus identifying the 9.8% of herds with the best performance.

However, these differences are not sufficient to train a LDA able to identify the herds of the worst welfare assessment scores as LDA showed poor performance in terms of sensitivity and positive predictive value. This was probably due to the lack of the most important information, that about the worst performing herds. On the other hand, LDA gave good results in terms of specificity and negative predictive value, suggesting that observations were consistent. Therefore, the findings of this analysis prevent from the use of HI as early warning tools of poor welfare risk in dairy cow herds, but, based on the high specificity, it can confirm the absence of PWR. On the long run, given that welfare assessment is costly and time-consuming, the implementation of a confirmation system based on LDA and DHI data would help reduce the workload for the veterinarians, as they could screen herds from remote and evaluate best performing herds with lower frequency.

Conclusion

At the end of June 2021, in Piedmont, there were 298,023 cows in 2,125 dairy herds (ISTAT, 2024). In this regard, the estimates provided by this study are deemed generalizable to the dairy cattle population since most of the cows in Piedmont and about 9% of all Italian dairy cows were evaluated for five years. The estimates of four HI in Piedmont showed that there has been a constant improvement in dairy cows' health and performance during the last years, and they could be useful for the benchmarking of dairy herds in the next future.

The results confirmed the importance of previously described cow-level risk factors, but also suggested that they are not enough to explain the whole risk of mastitis, ketosis, long ICI, and fresh cow removal alone. Therefore, a detailed analysis of the herd-level risk factors is needed. Although this level of detail is not achievable with DHI data, they serve as a useful monitoring tool at a regional scale, and geographic representation could help identifying critical areas since each HI had its own distribution pattern.

The combination of DHI data and on-premises animal welfare assessment could lead to an early alarm method for poor welfare risk. However, to date, the lack of data for the herds in most critical conditions limits the implementation of such analysis and DHI can only confirm animal welfare assessment results. On the other hand, this can be of great interest in welfare risk analysis, since herds with good welfare conditions showing also good HI need to be monitored less frequently than the others. From this point of view, DHI data can serve as confirmation of the absence of PWR. Identifying low-risk herds, thus reducing the workload for the public health system, can help focus on herds whose welfare status is uncertain.

Mountain dairy herds: the northern Apennines case study

Animal production emits significant amounts of green-house gasses (Gerber et al., 2010). To continue feeding a rapidly increasing global population while reducing emissions (Gerber et al., 2013), a structural change will be necessary. Another way to improve the sustainability of the dairy cattle sector, apart from improving productivity, is to use local feed resources, which is paramount in mitigating the food versus feed competition, a significant concern in global food security (FAO, 2024a). Indeed, exploitation of indigenous forage species, agricultural by-products, and crop residues can enhance the sustainability and economic viability of dairy operations while alleviating pressure on human-edible grain supplies (FAO, 2023). Strategic use of local feeds can reduce reliance on imported feeds, thus lowering production costs and fostering resilience against market fluctuations (Makkar, 2016). In addition, by prioritizing the use of locally available resources, dairy farmers can support a more circular agricultural economy, thereby promoting environmental sustainability and food security (Gill et al., 2010; Montrasio et al., 2020; Salvador et al., 2016).

Italian mountains, mostly characterized by steep valleys where mechanized agriculture is not feasible, would be suitable for cattle farming, which would use an otherwise unexploited resource. However, most Italian mountains have been experiencing a progressive abandonment of dairy herds, with consequences for the ecosystem and the society (Bakudila, 2018). In fact, less demand of services because of companies moving away leads to the disappearing of service providers from the mountains, which negatively impact on the remaining companies, creating a vicious cycle (McDonald et al., 2000). This study aimed at assessing the current situation and health status of dairy herds in marginal areas of the northern Apennines. The results of the first part of this research confirmed that mastitis was the primary concern in dairy herds, regardless of the size and location. Since in marginal areas, mastitis could have an even higher impact on herd profitability, due to the lack of veterinary care, in this study, mastitis was studied in detail.

Introduction

The role of livestock production in marginal areas

Marginal areas refer to regions that are geographically and socio-economically peripheral, often characterized by their remoteness, limited accessibility, and lower levels of economic development compared to core regions. These areas typically exhibit a range of environmental, demographic, and economic challenges, including depopulation, aging populations, and reduced public and private investment (ESPON, 2017). The marginality of these areas is not solely defined by their physical remoteness but also by socio-economic factors, such as limited employment opportunities, lower income levels, and inadequate infrastructure and services (Slee et al, 2022). Consequently, these areas face significant barriers to development and integration within broader economic systems, often resulting in social exclusion and persistent poverty (Bartolini et al., 2014). However, marginal areas also possess unique cultural and natural resources that, if effectively leveraged, can contribute to sustainable development and regional resilience (Ali et al., 2024). This necessitates tailored policy interventions that address the specific needs and potentials of these regions to foster inclusive growth and territorial cohesion. The marginal areas have often been depicted through representation by subtraction: everything that remains once the coastal areas, the fertile plains, the cities have been removed (Bottazzi, 2015). Several studies focus retrospectively on productive and social rarefaction, the decline in activities and employment, the lack of essential services, abandonment of the land, environmental degradation, landscape modifications, decrease in cultivated area, grazing and forestry practices, loss of importance of territorial heritage accumulated over history (Cersosimo and Donzelli, 2020). A prospective interpretation instead focuses on territorial resources, with attention to the local socio-economic systems, new agricultural entrepreneurship, multifunctionality of the territory (Ploeg et al., 2019).

The agricultural sector can benefit the social and economic well-being of the community through the production of food goods and raw materials, supply of various types of services, e.g., the protection, management and valorization of the rural landscape, protection of the environment, social and cultural services, valorization of the cultural peculiarities of the territory, forms of solidarity between citizens and producers (Idda et al., 2005). Comparative research on specific trends currently underway in agricultural and food systems in Europe identifies a process of re-peasantisation, a silent transition towards proto-agroecological systems, as an expression of innovative resistance (Ploeg et al., 2019). Indeed, agriculture is inherently multifunctional because it produces simultaneously goods and other services. Among them, some have a market (e.g., agritourism, social agriculture), others do not and generate collective, public goods such as landscape, water quality, biodiversity, culture, etc. (Cavazzani, 2006; OECD, 2001; Polman et al., 2010). When agriculture is supported, through specific policies, it is also supporting the production of a series of public goods that cannot be reproduced in a specialized and intensive context (van der Ploeg, 2008).

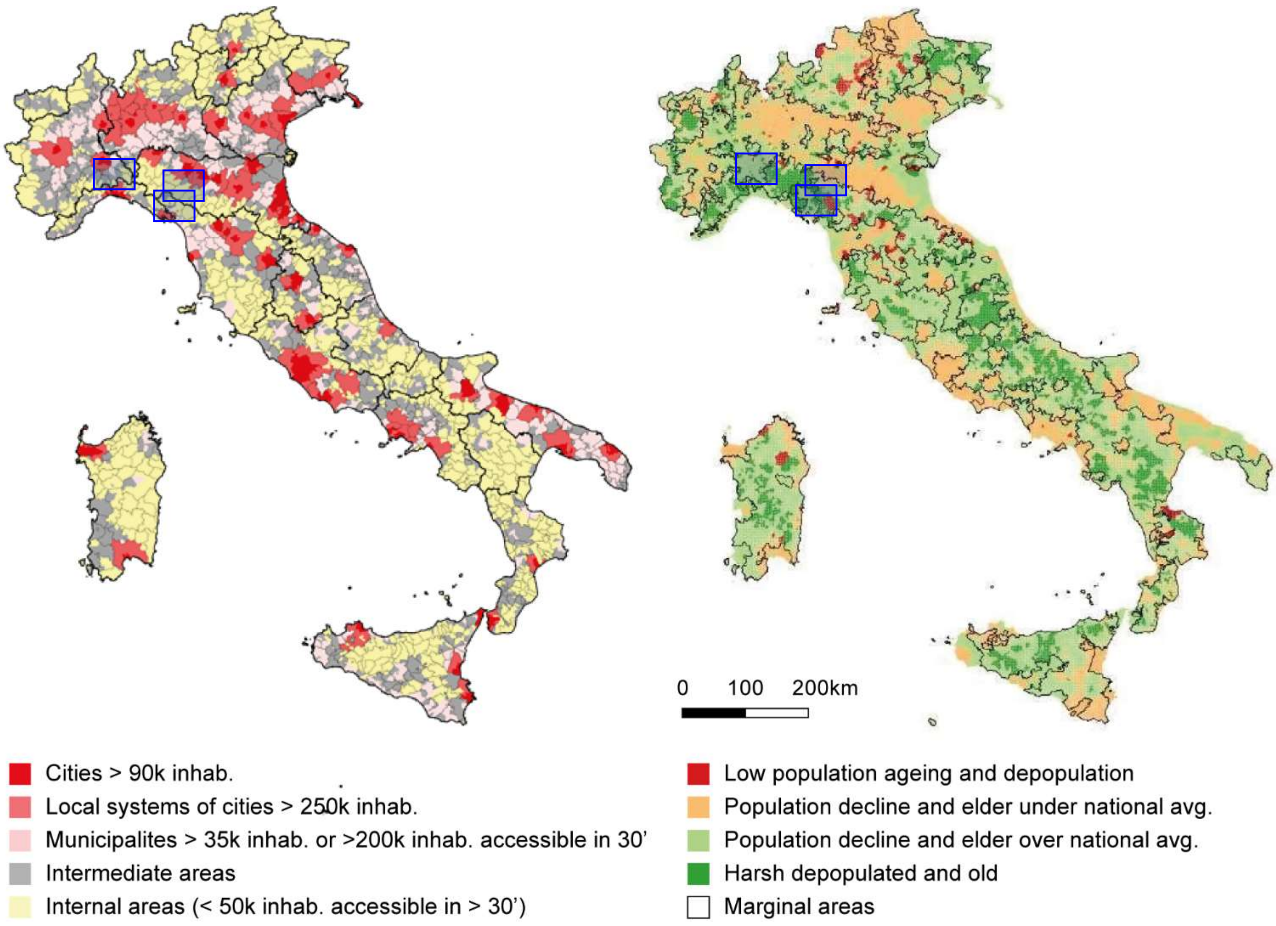


Figure 16. *Population distribution in Italy and indication of internal (marginal) areas. The blue squares identify the three areas objective of this study. Maps were taken from MIPAAF (2010).*

Livestock production in marginal areas still has improving opportunities to be exploited, although production methods and technologies used by intensive farms are seldom applicable in marginal areas (Bernués et al., 2011). On a local scale, even a small production system could be important to ensure the food supply. In this regard, the low-input farming system in mountainous areas could represent a viable solution to sustainability in many European areas, but it is facing the low production efficiency. Moreover, mountain areas are recognized as having productive, environmental, and societal functions, thus the loss of meadows and pastures due to global warming, abandonment, and the resulting reforestation is of concern (Laurent et al., 2003; Gibon, 2005). In this context, livestock production plays an essential role in the development and conservation of mountain areas (Boulangeat et al., 2014; Koczura et al., 2019; McDonald et al., 2000; Montrasio et al., 2020). The traditional grazing practices in mountainous regions can enhance carbon sequestration in soils, as the managed grasslands typically have higher carbon storage compared to degraded lands (Conant et al., 2017). This contributes to mitigating climate change by reducing atmospheric CO_2 levels. Furthermore, these farming practices support biodiversity; the heterogeneity of mountain landscapes fosters diverse plant species, which in turn supports various insect and bird populations, maintaining ecological balance (Tälle et al., 2016).

Socially, mountain dairy farming sustains rural communities by providing livelihoods and preserving cultural heritage. Overall, mountain dairy cattle farming offers a sustainable agricultural model that aligns environmental benefits with socio-economic stability. Nonetheless, there is little research on dairy farming systems in mountain setting (Fuerst-Waltl et al., 2019) as most existing studies focus on the beneficial effects of mountain pastures on cows' health, welfare, and the quality of dairy products (Alsaaod et al., 2022; Manzocchi et al., 2022), while the actual state of the art of dairy farming in several European mountainous areas is neglected.

The marginality concerns the largest part of the Italian territory, essentially rural areas distant from the agglomeration and service centers, which have been marginalized by the urbanization process, but endowed with resources that the central areas lack (Barca, 2013). In a positive sense, internal areas are less subject to anthropic pressures, they have specific yet undervalued productive and environmental resources (Barca, 2013). In Italy, approximately one third of the territory is covered by mountains. Stretching from north to south throughout the entire peninsula, the Apennines host a variety of different environments. In the last decades, certain areas of the Apennines have experienced agricultural abandonment, which has led to reforestation, land re-wilding, and an increase in wildlife populations

(Palli et al., 2022). Data on dairy cattle farms in mountain areas are fragmented and hardly retrievable but, based on the sixth farm structures' census of 2015 and on personal communication from public veterinarians, there is noticeable diversity in the number of dairy herds across areas of the northern Apennines. In the provinces of Modena, Parma, Reggio Emilia and in some municipalities of the province of Bologna, the PDO cheese Parmigiano-Reggiano is produced (EC, 2011; Consorzio Parmigiano Reggiano, 2018). In these areas, since the establishment of the Mountain Parmigiano Reggiano PDO assignation, the number of active dairy farms has steadily increased, mostly because PDO cheeses fetch a significantly higher price than drinking milk.

Only a short distance away, the scenario is different. Dairy farming on the south-west-facing slope of the Apennines in the province of Lucca, Tuscany, has been largely abandoned. The same trend has been seen in the province of Alessandria, Piedmont region (Bakudila et al., 2018). Both these areas have experienced among the most severe demographical abandonment in northern Italy, with dairy farms having almost entirely disappeared (veterinarians of the local veterinary services, personal communication). In these areas, there are no PDO or other systems for local dairy product protection capable of preserving the economic profitability of dairy farms in the face of low prices for milk and intense competition from large companies. Consequently, the number of dairy farms has been in decline for decades (Bakudila et al., 2018) with socio-economic consequences (Laurent et al., 2003) and repercussions for agricultural services (e.g., specialized veterinarians, veterinary laboratories, diagnostic services, etc.).

Mastitis

Literally, the term mastitis describes an inflammation of the mammary gland, due to various etiology. However, with few exceptions, mastitis occurs when microorganisms enter the udder via the teat canal and determining intramammary infection (IMI). IMI can determine clinical and subclinical mastitis.

Clinical mastitis (CM) is the result of the inflammatory response to infection, which causes milk alteration (e.g., color, fibrin clots), and changes in the udder (e.g., swelling, heat, pain, redness). CM cases are subdivided into mild, moderate and severe depending on the systemic involvement (e.g., fever, anorexia, shock) (Wenz et al., 2006). Coliforms (lactose-fermenting gram-negative rods of the family *Enterobacteriaceae*) are the most common cause of severe clinical mastitis, which occur when bacterial concentrations in milk increase enough to stimulate a marked immune response (Schukken et al., 2011). CM onset is usually rapid, whence it is termed acute mastitis and is severe in most cases (Hogan et al., 1999).

Although less apparent, the largest part of mastitis cases is represented by subclinical mastitis (SCM), which is almost three times more prevalent than CM (38-45% compared to 12-19%) (Krishnamoorthy et al., 2021). SCM is the presence of infection with the absence of any local or systemic clinical signs, although milk alterations might transiently appear. When the infection lasts over two months, it is termed chronic. Many chronic SCM persist for the entire lactation or the whole life of the cow, but it depends on the causative agent (Nyman et al., 2007).

While almost any microorganism can opportunistically determine a mastitis under certain circumstances, most infections are caused by a limited number of bacteria belonging to few groups, e.g., streptococci, staphylococci and coliforms. Therefore, historically, mastitis causative agents have been divided between major and minor pathogens, based on their frequency and importance (Table 9)

Table 9. *Classification of mastitis causing pathogens. Modified from Cobirka et al., 2020.*

Classification	**Contagious**	**Environmental**
Major pathogen	*Mycoplasma bovis* *Staphylococcus aureus* *Streptococcus agalactiae*, *Streptococcus dysgalactiae*	Coliforms: *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae* *Enterococcus* spp.: *E. faecalis*, *E. faecium*, *E. durans* Non-coliforms: *Proteus* spp., *Serratia* spp., *Yersinia* spp. *Streptococcus* spp.: *S. bovis*, (*S. dysgalactiae*), *S. equinus*, *S. uberis* Others: *Acinetobacter* spp., (*Trueperella pyogenes*), *Pseudomonas aeruginosa*
Minor pathogen	Non-aureus staphylococci and	Fungi

Classification	Contagious	Environmental
	mammalicocci: *S. chromogenes*, *S. epidermidis*, *S. simulans*, *M. sciuri* *Corynebacterium bovis*	Yeasts: *Candida* spp., *Pichia* spp.

Both CM and SCM have a profound impact on dairy herd profitability, affecting cows' health and milk quality (Rollin et al., 2015). A practical way to differentiate mastitis causative agents is based on their epidemiology and the reservoir of infection (Riekerink et al., 2020). In most cases, the recognition of a pattern permits to identify the potential source and reservoir of mastitis pathogens in the herd. However, this classification might be inappropriate for some pathogens, and a third class of opportunistic pathogen is conveniently adopted in some situations (NMC, 2023a, 2023b). Historically, subclinical mastitis control focused on pathogens like *Streptococcus agalactiae*, *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, enterococci, and numerous other non-aureus staphylococci, e.g., *Staphylococcus hyicus*, *Staphylococcus epidermidis*, *Staphylococcus xylosus*, and *Staphylococcus intermedius*. However, *Mycoplasma bovis*, several gram-negative rods like *Klebsiella* spp., *Serratia marcescens*, *Pseudomonas aeruginosa*, and other non-bacteria, e.g., *Candida* spp. and *Prototheca zopfii*, are now targeted (Ruegg, 2017; Zadoks and Fitzpatrick, 2009).

Contagious pathogens

Contagious infections usually determine long-duration intramammary infections (IMI), high average SCC, and a strong link between the prevalence of chronic cases and new ones. In addition, numerous mild clinical mastitis cases are temporary relapses in the balance between contagious pathogens and host defenses that occur in chronic mastitis. For contagious pathogens (e.g., *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium bovis*, etc.), the primary reservoir of infection is the mammary gland, and transmission occurs at milking with either milkers' hands or milking equipment acting as fomites (Cheng & Han, 2020; Cobirka et al., 2020; Fox and Gay, 1993). Also, the horn fly, *Haematobia irritans*, causing teat-end dermatitis, can spread *S aureus* to heifers (Owens et al., 1998; de Vliegher et al., 2012). Non-photosynthetic algae of the *Prototheca* genus (*P. zopfii* genotype 1 and 2, recently renamed *P. ciferri* and P. *bovis*, *P. wickerhamii*, *P. cutis*, *P. blaschkeae*, etc.) use contagious infection route, too (Buzzini et al., 2004; Möller et al., 2007; Huilca-Ibarra et al., 2022; Jagielski et al., 2019; Park et al., 2019). *Prototheca* spp. are unicellular algae commonly found in wet environment with organic matter in the soil like wet outdoor resting areas, and humid pastures in dairy farms (Buzzini et al., 2004). Protothecal mastitis is usually chronic, without clinical signs, but with increased SCC. Although infections may spontaneously resolve, long-term carriage with intermittent shedding is common. The primary causative agent of protothecal mastitis is *Prototheca zopfii*, although, on a practical basis, cows identified as having mastitis caused by *Prototheca* spp are managed regardless of species or genotype (Pore et al., 1987).

An exception to contagious transmission pattern is *Mycoplasma* spp., which infects the cow via aerosol transmission and invade the udder after bacteremia (Fox et al., 2005; Gonzales and Wilson, 2003).

For contagious pathogens, the single most important management practice to prevent transmission of new infections is the use of an effective germicide as a post-milking teat dip (Barkema et al., 1999; Breen, 2019). Other practices that augment teat hygiene include the use of individual towels, gloves, a pre-milking germicide, the respect of a proper stimulation time before units' attachment, the avoidance of overmilking, and dedicated cleaning of milking equipment after use. Also, routine milking equipment evaluation should be conducted to ensure teat-end vacuum is operating at a proper level and remains stable during milking.

Environmental pathogens

Environmental pathogens cause most CM cases (Wenz et al., 2006; Roberson, 2003). Environmental infections usually determine short-duration IMI, with lower average SCC, high incidence of IMI in the first phases of lactation and none to negative correlation between chronic-case prevalence and new ones. Environmental pathogens include a wide range of species, namely *Aerococcus* spp., *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas* spp., etc. (Bradley, 2002; Smith and Hogan, 1993). Environmental pathogens have the bedding as the primary source, but infection can occur through various routes, like contaminated teat dips or intramammary infusions, cleaning water, mud, but also skin lesions, and flies (Smith et al., 1985; Hogan et al., 2003). Environmental pathogens like *Trueperella pyogenes*, *Lactococcus* spp, and pathogenic *Escherichia coli* might spread in a contagious-like manner under exceptional circumstances (Cobirka et al., 2020).

To prevent environmental mastitis, cows should be provided dry, clean bedding, heat stress should be reduced, and a healthy teat condition maintained. Also, the use of barrier teat dip after milking can reduce the incidence of IMI by environmental pathogens (Smith and Hogan, 1993).

Other mastitis pathogens

Some mastitis agents act as opportunistic pathogens. They inhabit healthy cows' teat skin but can infect the udder when the host's immune response is reduced (Taponen and Pyörälä, 2009). These include the non-aureus staphylococcal and mammalicoccal species (**NASM**; *Staphylococcus arlettae*, *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus hycus*, *Staphylococcus schleiferi*, *Macrococcus caseolyticus* -formerly *Staphylococcus caseolyticus*-, *Mammalicoccus sciuri* -formerly *Staphylococcus sciuri*-, etc.). Other than NASM, many bacterial species (e.g., *Bacillus* spp., *Lactococcus* spp., etc.) can occasionally cause mastitis (Riekerink et al., 2020).

Antimicrobial use for mastitis treatment

Since each pathogen type has its own different epidemiology and risk factors, effective mastitis control requires an appropriate strategy based on the epidemiology of the involved pathogen (Roberson, 2003). Therefore, it is essential to identify the causative agent since a poor or absent diagnosis induces the adoption of empirical and possibly ineffective treatment and renders the cure more difficult. In addition, antimicrobial susceptibility testing (AST) should follow mastitis diagnosis to recommend an adequate treatment, reduce antimicrobial misuse, and limit antimicrobial resistance (AMR).

Subclinical mastitis treatment is indicated when treatment costs are expected to be outweighed by production gains after elimination of infection. Prevention is always preferred to treatment, and the need to treat subclinical mastitis should be carefully considered based on the causative agent and duration of infection. Therefore, mastitis treatment should always include two distinct phases: (1) the determination of the causative agent; and (2) the selection of the best suited antimicrobial therapy.

In the case of contagious pathogens, elimination may result in a decrease of the reservoir of infection for previously noninfected cows. No noteworthy economic losses will occur because of delaying treatment until bacterial culture can be completed. However, many subclinical mastitis cases are chronic, and, particularly in the case of *S. aureus*, in vitro susceptibility does not assure the efficacy of the treatment. This is due to *S aureus*' ability to produce deep-seated abscesses and to survive intracellularly after phagocytosis. Also. drug distribution after intramammary administration may not be adequate because of extensive fibrosis and abscesses in the mammary gland (Barkema et al., 2006). Moreover, *S. aureus* resistance to antimicrobials is more common than with streptococcal infection. As a result, intramammary infusions cure less than 20%–40% of chronic infections.

On the contrary, prevalence of *Streptococcus agalactiae* IMI is successfully reduced by treating all the infected cows with antimicrobials. All infected quarters should be treated to ensure elimination of the pathogen to prevent possible cross-infection. Cure rates often range from 75% to 90% (Dingwell et l., 2004). Most other streptococci also display in vitro susceptibility to numerous antimicrobials, especially beta-lactam antimicrobials. Despite this apparent susceptibility, many streptococcal infections are not as easily cured as those caused by *S. agalactiae*. However, increased cure rates are obtained treating *S. uberis* and *S. dysgalactiae* IMI at the end of lactation (Kabelitz et al., 2021). Indeed, the dry period of the lactation cycle is a critical time for the udder health of dairy cows. During the first involution phase, the gland is particularly vulnerable to new infections (Bradley et al., 2000). However, once involuted, mammary gland offers the most hostile immune environment for bacterial pathogens. Consequently, the dry period is an ideal time to attain synergy between antimicrobial treatment and immune function. (Halasa et al., 2009). Therefore, the most effective strategy would be dry cow therapy instead of lactating cow therapy

Other streptococcal-like organisms such as *Lactococcus* spp and *Enterococcus* spp are often refractory to treatment (Cheng et al., 2020). Culling may be a practical option for cows that do not recover. Alternatively, it is common to dry off the infected quarter and continue to milk the cow, thus decreasing the risk of infection for other cows. However, drying off quarters, culling, or treatment is a palliative approach to mastitis control, which is better addressed by the prevention of infections.

Aim of the study

This study aimed at exploring the conditions of the dairy cattle herds in the northern Apennines, comparing thriving areas, where the number of dairy farms increased steadily in the last decades, with valleys facing zootechnical abandonment, where the number of dairy farms and, consequently, of agricultural service has reduced. The assessment of the to-date situation included the animal husbandry practices, farm management strategies, prevalence of IMI of different etiology (contagious, environmental, and opportunistic), antimicrobial resistance of mastitis pathogens, and the antimicrobial use. This information was collected through interviews with the farmers, milk analysis, and the scrutiny of farm's treatment registries.

Material and methods

Study area

Three study areas were selected to represent different geographic areas of the northern Apennines: the valleys within Erro and Borbera rivers in the province of Alessandria (Piedmont region, AL); the Garfagnana, which embraces the high valley of the Serchio river, in the Lucca province (Tuscany region, LU); and the mountains surrounding Castelnovo 'ne Monti in the valleys of Secchia and Enza rivers in the Reggio Emilia province (Emilia Romagna region, RE).

Eighteen dairy herds located at (mean ± standard deviation) 555 ± 181m a.s.l were selected by convenience. In AL, six dairy herds remained but four accepted to participate in the study. They were situated in the natural reserve of Capanne di Marcarolo and Borbera valley (643 ± 286m a.s.l.). In Garfagnana (LU) all the six remaining dairy herds (435 ± 95m a.s.l.) were enrolled. In RE, six herds were selected by the local public veterinary services officer (657 ± 66m a.s.l.). All surveys were performed between April 23rd and the September 1st, 2021.

In AL and LU, the number of dairy herds has been in decline for decades (Bakudila et al., 2018), thus the access to agricultural and veterinary services was limited or completely absent. In these areas, there are no PDO or other systems for local dairy product protection capable of preserving the economic profitability of dairy herds in the face of low prices for milk and intense competition from large companies. A short distance away, in RE the PDO cheese Parmigiano-Reggiano is produced. Since the establishment of the Mountain Parmigiano Reggiano PDO assignation, the number of active dairy herds has steadily increased (Lupatelli, 2020), thanks to a product that fetches a significantly higher price than drinking milk.

Herd characterization

Questionnaire

After asking for informed consent, farmers were interviewed employing an 86-question questionnaire. The questionnaire was divided into 11 sections, regarding general information (questions = 9), farmer's education (q = 7), litter management and structures (q = 6), milk production (q = 5), cows' feeding and diet (q = 4), reproduction (q = 18), perceived health problems (q = 12), biosecurity (q = 15), cultivated land (q = 2), waste management (q = 3), and additional questions (q = 5). Farmers were asked to answer questions referring to the previous year. The complete questionnaire is available at https://zenodo.org/record/7067099 (last access: 21st February 2023). Surveys were anonymized before being processed by three different researchers.

Data analysis

Categorical variables are reported as frequencies and compared among areas using contingency tables and Fisher's exact test. Quantitative variables are reported as median (minimum-maximum) and compared by Kruskal-Wallis's test and Wilcoxon's rank sum test for paired comparisons.

Multidimensional scaling

To synthesize farms' variability, multiple factor analysis (MFA) was performed. Variables were grouped into 11 categories, namely structures, external biosecurity, internal biosecurity, awareness, farmer's education, animals, milk production, diet, reproduction, nursery, and perceived health problems (Supplementary table 2). When necessary, values were normalized between 0 and 1.

MFA was performed on qualitative and quantitative variables separately. Then, it was performed on variables altogether. For each group, the single-variable average contribution ($\overline{c}_{gk}$) to the first five dimensions was evaluated as:

$$\overline{c}_{gk} = \frac{\sum_{k=1}^{5} c_{gk} * s_k^2}{n_g}$$

where c_{gk} is the g^{th} group contribution to the k^{th} dimension times the k^{th} dimension's aliquot of explained variance (s_k^2), and n_g is the number of variables in the g^{th} group. MFA was performed using R version 4.2.1 (R Core Team, 2023) and R packages: *cluster*, *factoextra*, and *factoMineR*.

Also, linear discriminant analysis (LDA) was performed as a supervised clustering method, to highlight the strongest differences among the areas. LDA was performed using R version 4.2.1 (R Core Team, 2023), and R package *MASS*

Intramammary infection

The handling of the animals and the collection of samples were carried out as for routine milking, so no approval from the ethics committee was required.

Based on herd size, up to 23 lactating cows per herd were randomly selected. Cows undergoing antibacterial treatment for mastitis at the time of sampling were excluded. After removing visible dirt with a dry cloth, the teat was first cleaned with gauze soaked in benzalkonium-chloride solution then with a cotton ball soaked in alcohol and let dry for a minute. The first milk streams were discarded, then up to 15 mL of milk was collected, mixing milk from each quarter into a single vial. Milk samples were kept at refrigeration temperature (≤ 8°C) during transportation, then stored at -20°C until analysis.

Sheep blood (5%) Columbia agar plates (Oxoid Ltd., Basingstoke, UK) were inoculated with 10uL of milk and incubated overnight at 36 ± 1°C. Morphology-based bacterial identification was confirmed by MALDI-ToF mass spectrometry (Bruker Daltonics GmBH, Bremen, DE). A score of 2.0 or higher was considered indicative of species identification. Following the National Mastitis Council guidelines (Riekerink et al. 2020), when at least a colony of *S. aureus*, *S. agalactiae*, or *C. bovis* was observed, the sample was considered positive for contagious IMI. When three or more colonies of an environmental pathogen (e.g., *Aerococcus viridans*, *Enterococcus* spp., *Lactococcus* spp., *Streptococcus dysgalactiae*, *Streptococcus uberis*) were observed, the sample was deemed positive for environmental IMI. If three r more colonies of NASM were observed, the sample was considered positive for opportunistic IMI. When there were three or more colonies of a pathogen described as occasionally causing mastitis (e.g., *Bacillus* spp.), the sample was considered positive for other IMI.

To test *Prototheca* spp. infection, 10 uL of milk was streaked onto Prototheca Isolation Medium agar and incubated aerobically up to 72 hours at 37°C ± 1°C (Pore, 1973). Colony and cellular morphology of suspected samples were inspected by stereomicroscope and optical microscope after lactophenol cotton blue staining. Molecular identification was performed by 16S sequencing.

The odds of (contagious, environmental, opportunistic, and other) IMI were estimated adjusting for individual cow risk factors (i.e., DIM, and lactation). To account for repeated measures within herds, a generalized estimating equation (**GEE**) was implemented using *xtgee* command in STATA 15.0 (STATA Corp., 2017). Exchangeable correlation structure was assumed since any pair of cows within a herd were assumed to be equally correlated. To overcome the effect of potential outliers, robust 95% confidence intervals (**CI**) for each area were estimated. The formal expression of the GEE, binomial family, logit link, and exchangeable correlation structure, was:

$$ln\left(\frac{\pi_{ij}}{1-\pi_{ij}}\right) = \beta_0 + \beta_1 DIM_{ij} + \beta_2 lact_{ij} + \beta_{area} + \varepsilon$$

where $\left(\frac{\pi_{ij}}{1-\pi_{ij}}\right)$ was the log odds of IMI, β_0 was the constant intercept, DIM_{ij} and $lact_{ij}$ were the number of days in milk and the number of lactations of the i^{th} cow of the j^{th} herd, respectively, β_{area} was the coefficient for AL and LU areas compared to RE, and ε was the overall error term. Since RE was set as reference, IMI odds ratio (OR) for AL or LU were obtained as $exp(\beta_{area})$. For overall IMI estimation, β_{area} was omitted. Using *margins* command (StataCorp., 2017), the marginal prevalence was estimated. To avoid interpretative issues with the OR (Altman et al., 1998), the relative risk (RR) was calculated from the estimated OR, as follows (Grant, 2014):

$$RR = \frac{OR}{[1 - prev(IMI_{RE})] + [prev(IMI_{RE} * OR)]}$$

Antimicrobial resistance

Minimum inhibitory concentration (MIC) of *S. aureus* and *Streptococcus* spp. isolates was tested following the Clinical and Laboratory Standards Institute (CLSI) guidelines VET01 15th edition (CLSI 2018a). Commercially prepared Sensititre™ microtiter plates (Thermo Fisher Scientific, Cleveland, OH, USA) included a panel of 15 commonly used antibacterials (abbreviation; concentration range in ug/mL): penicillin (PEN; 0.03-16), ampicillin (AMP; 0.03-16), amoxicillin/clavulanic acid combination (AMC; 0.125/0.0625-16/8), oxacillin + 2% NaCl (OXA; 0.125-4), cephazolin (CEZ; 0.125-8), ceftiofur (CFT; 0.125-32), cefquinome (CEQ; 0.125-8), enrofloxacin (ENR; 0.125-4), erythromycin (E; 0.125-8), pirlimycin (PIR; 2-4), gentamicin (CN; 2-32), gentamicin High Level (HL-CN; 250-500), kanamycin(KN; 4-32), rifampicin (RF; 0.0625-2), and trimethoprim + sulphadiazine (SXT; 0.125/2.375-4/76). Results were interpreted using available CLSI breakpoints according to VET08 4th edition guidelines (CLSI, 2018b), the Comitè de l'Antibiogramme de la Sociètè Française de Microbiologie guidelines (CASFM, 2019), and the breakpoints reported in the literature (Feβler et al., 2012; Simjee et al., 2011), when specific standards were not established by any international recognized guidelines. Breakpoint selection was based on breakpoint availability in cattle, human, and then other animal species. Based on clinical breakpoints, isolates were considered susceptible (S), susceptible to increased dosage / intermediate (I), or resistant (R) to a certain antibacterial. For further analysis, ‘I’ results were counted as ‘S’. Isolates resistant to three or more antimicrobial classes were considered as multi-drug resistant (MDR).

Antimicrobial use

Information on antibacterial use was collected from each herd’s treatment registry. Treatment date, reason for treatment, number of treated cows, and prescribed drug were recorded. The reason for treatment was classified into nine categories, namely: gastrointestinal infections, mastitis, metabolic disorder, musculoskeletal disease, periodic parasitic treatment, respiratory infections, systemic or multi-organ disease, skin and foot infections, and urogenital disease. Antibacterial treatments were divided into 13 chemical classes (aminoglycosides, beta-lactams, 1st to 4th generation cephalosporins, lincosamides, macrolides, rifamycins, sulfonamides, sulfonamides with diaminopyrimidines, and tetracyclines). When a combination of multiple antibacterials was used as a single treatment, each antimicrobial class was counted. To compare treatment frequency, the antimicrobial treatment incidence rate (ATI) was estimated based on the information available from the treatment registry, modifying the formula proposed by Stevens et al. (2016):

$$ATI = \frac{Total\ amount\ of\ treated\ cows}{Number\ of\ cows * \left(\sum T_j + 1\right)}$$

The number of treated cows was divided by the cow-days at risk, which were calculated as the total of cows in the herd times the days the j^{th} herd was observed ($\sum T_j$) plus one day, added to avoid zeros.

To account for overdispersion, ATI and 95% confidence intervals were estimated for each area by random-effects negative binomial regression model (*xtnbreg* command) with herd-level random effects, areas as fixed effects, and cow-days as offset:

$$ln\left(treated\ cows_j\right) = \beta_0 + \beta_{area} + ln\left(cow\ days_j\right) + u_j + \varepsilon$$

Where $ln\left(treated\ cows_j\right)$ was the natural logarithm of the number of treated cows of the j^{th} herd, β_0 was the constant intercept, β_{area} the coefficient of AL and LU compared to RE, u_j the within-herd error term, and ε the overall error term. By construction, $u_j \sim Beta(r, s)$ varied randomly across herds but remained constant within. To estimate coefficients’ standard errors, we preferred the more conservative Jackknife method instead of likelihood estimators. Since RE was set as reference, the antimicrobial treatment incidence rate ratio (ATIR) of AL or LU compared to RE were obtained as

$exp(\beta_{area})$. For overall ATI estimation, β_{area} was omitted. Using *margins* command (StataCorp., 2017), the marginal ATI was estimated for each area. The analysis was performed with STATA 15.0 (STATA Corp, 2017).

Results

Herds characterization

Questionnaire

Destination of products. Seven farmers (AL = 3/4, LU = 3/6, RE = 1/6) sold raw milk or dairy products directly to the consumers. Among the others, the five RE farmers delivered the milk to a cooperative dairy, of which they are co-owners, and the remaining two farmers delivered the milk to the food industry. For three farmers (AL = 1/4, LU = 2/6), dairy production was not the primary income source.

Breed and housing method. Ten farmers (AL = 3/4, LU = 3/6, RE = 4/6) raised one single breed among Friesian Holstein and Brown Swiss. The other breeds raised were Italian red pied, Reggiana, and Piedmontese. On average, RE herds were larger than LU (p = 0.065), while AL herds were alternatively medium or small sized (Table 10).

Table 10. *Animals by production category. Median (min.-max.) number of animals for each production category by area.*

Area	Cows (Min.-Max.)	Calves (Min.-Max.)	Heifers (Min.-Max.)	Fattening (Min.-Max.)	Bulls (Min.-Max.)	Overall (Min.-Max.)
AL	24.5 (14-44)	7.5 (0-14)	6.5 (1-17)	4 (0-12)	0 (0-5)	47 (18-80)
LU	18 (14-67)	3.5 (1-16)	4.5 (1-20)	0.5 (0-2)	0 (0-2)	26 (22-120)
RE	39.5 (24-120)	9 (2-39	18 (0-30)	0.5 (0-40)	0 (0-1)	67 (26-230)

Cows were housed in tie-stall in 75% of the farms (Supplementary Table 3). However, four farmers sent the entire herd to pasture throughout the year or for part of it (AL = 2/4, LU = 2/6), while three let only heifers graze free range (AL = 1/4, RE = 2/6). Overall, the animals spent a median of 180 days per year at pasture, from spring to fall (Supplementary table 4). The median frequency of bedding material changes was higher in LU farms than in AL (p = 0.050) and RE (p = 0.054).

Farmer's experience and education. Farmers worked a median of 7 hours and 20 minutes a day. Their median age was 49.5 years, with an age range from 32 to 68 years old. All but one farmer (RE) had a family history of cattle breeding. Years of experience raising cattle ranged from six to 53 years (median = 31.5). Eleven farmers (AL = 4/4, LU = 2/6, RE = 5/6) had received specific education on cattle breeding and herd husbandry. Three studied agricultures in high school, and all attended courses on animal welfare, genetic selection, nutrition, etc. (Supplementary Table 5).

Other workers. Up to seven people (median = 2.5) worked per farm. On eleven farms (AL = 4/4, LU = 5/6, RE = 2/6), one veterinarian was responsible for cow health. On the others, different veterinarians were consulted depending on the health problem (Table 11).

Table 11. *Other workers operating on the farms. Median (min-max) number of workers and veterinarians operating on the farms by area. Median (min-max) frequency of veterinary and feeder visits per month, by area.*

Area	**Workers (Min.-Max.)**	**Veterinarians (Min.-Max.)**	**Veterinarian visits/month (Min.-Max.)**	**Feeder visits/month (Min.-Max.)**
AL	3 (2-5)	1 (1-1)	1.5 (1-10)	0.1 (0-0.2)
LU	2 (1-5)	1 (1-2)	2 (0.2-3)	0.1 (0-1)
RE	2.5 (2-7)	2 (1-3)	2 (1-12)	0.3 (0.3-0.3)

Structures. Five farmers did not have a proper infirmary (AL = 2/4, LU = 1/6, RE = 3/6), five could arrange one (AL = 1/4, LU = 2/6, RE = 2/6), if necessary, five had a proper infirmary (AL = 1/4, LU = 3/6, RE = 1/6) but only three (LU) of these used it exclusively for that purpose (Supplementary Table 6). All but one farm had a manure storage area, which was not covered in 88% of cases, where manure remained up to 255 days (median = 112.5). Five farms had a sewage tank, not covered in four out of five cases, where sewage remained stored a median of 90 days. Farms had up to 210 ha (median = 30 ha) of usable agricultural land. One RE farm had a pasture plan made by an expert and two (LU = 1/6, RE = 1/6) had a soil fertilization plan prepared by an agronomist (Supplementary Table 7).

Dairy production. The farmers reported a median of 5.25 lactations per cow. They milked the cows a median of 265 days per lactation then -all but one LU farmer- dried them off at least 60 days before calving. Three farms had a milking parlor, eight a milking pipeline, and five used a milking cart. When asked to estimate the average milk yield per cow per day (median = 22.0 kg, min = 15.0, max = 31.0), RE farmers reported higher values than LU (p = 0.013) and AL (p = 0.028) farmers (Table 12).

Table 12. *Dairy production and lactation. Median (min.-max.) milk yield, lactation length, number of lactations, and dry-off length by area. Frequency of farms having milking parlour, pipeline, or cart by area.*

Area	Milk yield (Min.-Max.)	Lactation length (Min.-Max.)	Dry-off length (Min.-Max.)	Lactations (Min.-Max.)	Milking parlour (%)	Milking pipeline (%)	Milking cart (%)
AL	18 (17-22)	205 (260-280)	60 (60-90)	5.5 (5-6)	2 (50.0)	0 (0.0)	2 (50.0)
LU	20.1 (15-27.5)	330 (210-360)	60 (50-90)	6 (3-8)	0 (0.0)	3 (50.0)	3 (50.0)
RE	30.25 (25-31)	247.5 (176-360)	60 (60-65)	4.5(2.5-6.5)	1 (16.7)	5 (83.3)	0 (0.0)

Feeding. On median, cows were fed twice a day (Supplementary Table 8). The diet was prepared by a veterinarian or nutritionist on three farms (AL = 2/4, RE = 1/6), by a feed-industry technician on seven (LU = 4/6, RE = 3/6), and by the farmer on the others. All diets were based on hay, produced on site in 15 out of 16 farms. The cows' diet was integrated with cereals or concentrate in all but three farms (AL = 4/4, LU 5/6, RE 4/6), and half of the herds (AL = 2/4, LU = 2/6, RE = 4/6) received fresh grass from alfalfa or polyphite fields.

Reproduction. Two farmers (AL = 1/4, LU = 1/6) always employed bulls for natural mating and one (RE) only when artificial insemination did not work. Bulls were never rented to other herds neither borrowed. Seven farmers (AL = 2/4, LU = 1/6, RE = 4/6) synchronized cows' estrus. The median age at first insemination was 17.75 months, and it was higher in AL than in LU (p = 0.053) (Supplementary Table 9). The median proportion of cows giving birth once a year was 87.5%. Five farms had a calving room (AL = 2/4, LU = 3/6), while two RE farmers moved parturient cows to pasture. Cows were moved into the calving room a median of 60 days before calving and kept there for ten days after. Farmers reported a 3% median abortion proportion, which was lower in AL than RE herds (p = 0.051). Four farmers (LU = 1/4, RE = 3/6) always had a necroscopy performed on aborted fetuses, six decided depending on the circumstances (AL = 3/4, LU = 1/6, RE = 1/6), and six never had one (AL = 1/4, LU = 4/6, RE = 1/6).

Nursery. Twelve farmers reported that no calves died during the previous year (AL = 3/4, LU = 6/6, RE = 3/6), the others recorded a mortality from 2.4% to 19.2% (median = 10.5%). Calves' mortality was lower in LU than RE farmers (p = 0.074) (Supplementary Table 10). Ten farmers (AL = 4/4, LU = 2/6, RE = 4/6) evaluated colostrum quality based on color and texture, but none used a refractometer. Eight farmers (AL = 3/4, LU = 3/6) stored colostrum for long periods at -20°C.

Biosecurity. Seven farmers (AL = 1/4, LU = 3/6, RE = 3/6) replaced up to 28.6% of the herd in the previous year. Three farmers (AL = 1/4, LU = 1/6, RE = 1/6) introduced all the animals at the same time, while four others (LU = 2/6, RE = 2/6) had multiple introduction events. Three bought new cows from a single herd within 50 km, one from a single herd located more than 50 km away, and three bought cows from several herds or at exhibitions (Supplementary Table 11). When buying, four farmers (AL = 1/4, LU = 2/6, RE = 1/6) asked for cows certified to be free from *Brucella* spp., *Staphylococcus aureus*, *Corynebacterium paratuberculosis*, infectious rhinotracheitis virus (IBRV), leucosis virus (BLV), and parainfluenza virus (BPIV3). Two farmers quarantined new animals for 30 days in a separate place with dedicated instruments (LU), or for 15 days in a partially separated space (RE).

Five farmers (AL = 1/4, RE = 4/6) had a vaccination plan which included IBRV and bovine viral diarrhea virus (BVDV). Two of them (AL, RE) also had bovine respiratory syncytial virus (BRSV) vaccination, while two others (RE) had BPIV3 vaccination. Three farmers (AL = 2/4, RE = 1/6) implemented a prevention and control plan for parasites.

In three farms (AL = 1/4, LU = 1/6, RE = 1/6), other domestic species were raised in contact with the cows, and nine herds (AL = 3/4, LU = 4/6, RE = 2/6) encountered wildlife (Supplementary Table 12). In 11 farms (AL = 3/4, LU = 4/6, RE = 4/6), biosecurity measures were adopted, namely five farmers required visitors to use shoe covers, five required full disposable clothing (Supplementary Table 13), and one (LU) sanitized the tyres of vehicles, too, while another one (RE) fortnightly performed a sanitization of the stable. Four farmers gathered the carcasses outside where the disposal-service truck stops (AL = 1/4, LU = 2/6, RE = 1/6), nine kept them inside but away from other animals (AL = 2/4, LU = 4/6, RE = 3/6), the others left dead animals in a range of possible contact with the living ones.

Awareness and perceived health problems.

LU farmers reported a 0.2% mortality rate, lower ($p = 0.064$) than reported by AL (2.0%) and RE (1.9%). Overall, health problems in order of relevance for the farmers were orthopaedic (43.8%), followed by reproductive issues (37.5%) and mastitis (37.5%), gastrointestinal infections or infestations (25.0%), metabolic disorders (18.8%), and respiratory diseases (18.8%). The perceived health problems differed by study area (Table 13).

Table 13. *Perceived health problems. The table presents the proportion of farmers reporting different types of health problems occurred in their herd during the previous year, by study area.*

Area	Respiratory	Gastrointestinal	Orthopaedic	Reproductive	Metabolic	Mastitis
AL	0 (0.0)	0 (0.0)	2 (50.0)	2 (50.0)	0 (0.0)	2 (50.0)
LU	1 (16.7)	2 (33.3)	1 (16.7)	3 (50.0)	1 (16.7)	0 (0.0)
RE	2 (33.3)	2 (33.3)	4 (66.7)	1 (16.7)	2 (33.3)	4 (66.7)

One AL farmer reported no health problems at all. A RE farmer reported no reproductive problems, although the herd experienced cluster abortions due to *Coxiella burnettii* in the previous year.

Bulk-tank milk somatic cells count (SCC), and colony-forming units (CFU) were measured in all herds, from 11 to 26 times per year (Supplementary Table 13). In addition, the national breeders' association provided herd-performance monitoring service and individual-cow milk analysis (DHI) to 13 farmers (AL = 3/4, LU = 4/6, RE = 4/6). Farmers were asked to recall the SCC and CFU results of the previous three controls, and they remembered a median of one previous SCC of CFU result. Four (AL = 2/4, LU = 1/6, RE = 1/6) did not remember any, one (RE) remembered only last-month's SCC, one (AL) only the CFU, six (AL = 1/4, LU = 3/6, RE = 2/6) remembered both last-month's SCC and CFU. The others could recall the SCC (LU = 1/6, RE = 2/RE) and CFU (LU) from more than one month previous.

Given the questionable information source, no statistical comparison was performed. However, SCC values they reported differed between AL (100×10^3 cells/mL), LU (median = 216×10^3 cells/mL, min-max: 70-315 $\times 10^3$ cells/mL), and RE (median = 127.5×10^3 cells/mL. min-max: 60-400 $\times 10^3$ cells/mL). Also, CFU varied among geographic areas, with lower values reported by AL farmers (median = 16 x 10^3 cfu/mL, min-max: 7-25 x 10^3 cfu/mL) than LU (median = 28.5 x 10^3 cfu/mL, min-max: 16-50 x 10^3 cfu/mL) and RE (median = 22.5 x 10^3 cfu/mL, min-max: 4-36 x 10^3 cfu/mL).

Multidimensional scaling

The MFA was unsuccessfully able to reduce data complexity. Indeed, up to eleven dimensions were necessary to summarize 90% of the herds variability, with the first three dimensions counting for 15.7%, 13.5%, and 12.9% of the total variance, respectively. The group of variables that contributed the most to herds' diversity was the external biosecurity (number of employees and veterinarians, new introductions, contact with wild and other domestic species, and biosecurity measures) (Figure 17).

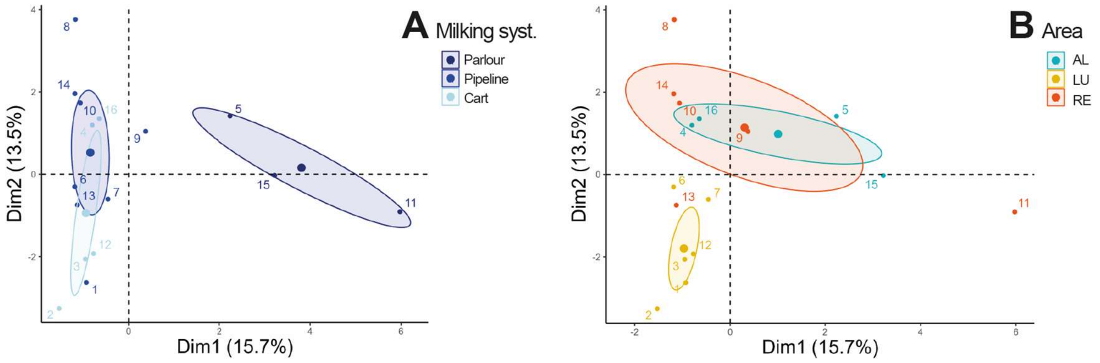


Figure 17. *Results of multi-factorial analysis (MFA) grouped by milking system (A, left) and geographic area (B, right). The first two dimensions are represented. The ellipses represent the 95% confidence interval.*

However, different groups of variables contributed the most to the first five dimensions, namely structures, internal and external biosecurity, farmer education and disease perception. Although the questionnaire results, through MFA, could not distinguish the geographic area, it was possible to differentiate free-stall farms with from the others based on their characteristics other than milking parlor and the housing method (Figure 18).

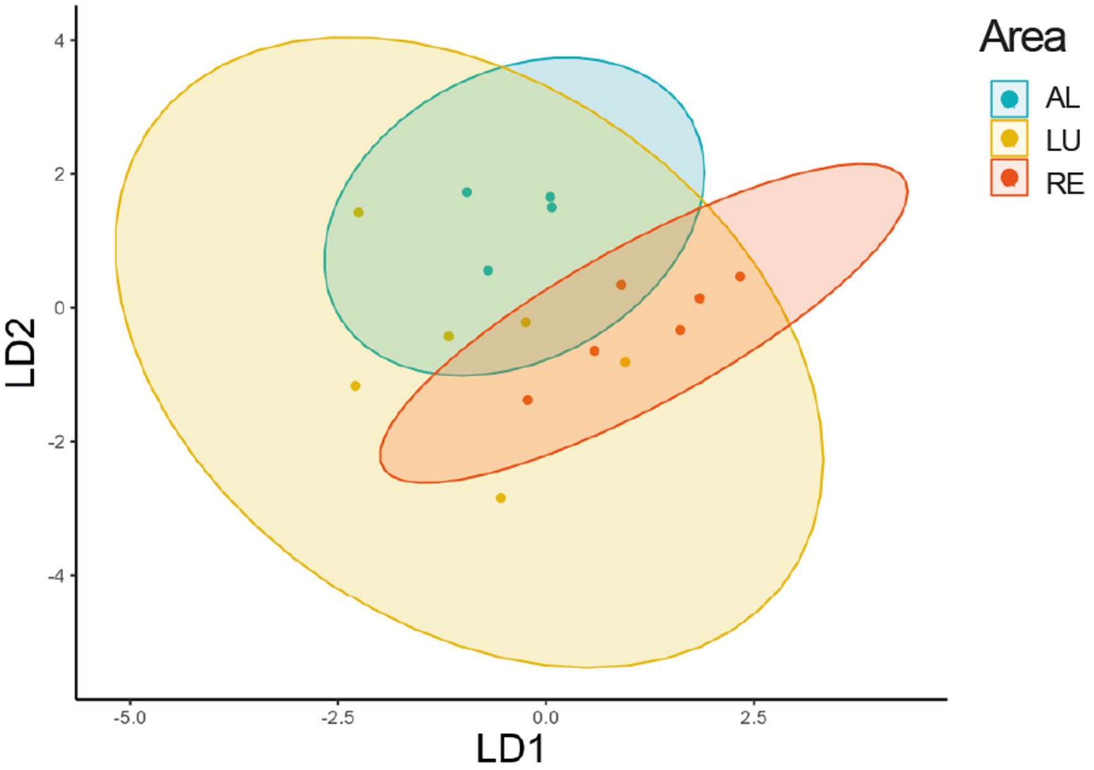


Figure 18. *Result of linear discriminant analysis (LDA) grouped by geographic area. The ellipses represent the 95% confidence interval.*

In addition, LDA had 81.3% accuracy in classifying the herds into geographic areas. Based on the multivariate linear model it was observed that the number of employees and veterinarians, as well as the frequency of vet visits was positively associated with milk yield (Figure 19A-B). The working hours were positively associated with milk yield, too (Figure 19D), while the number of employes was not associated with an increase in milk yield.

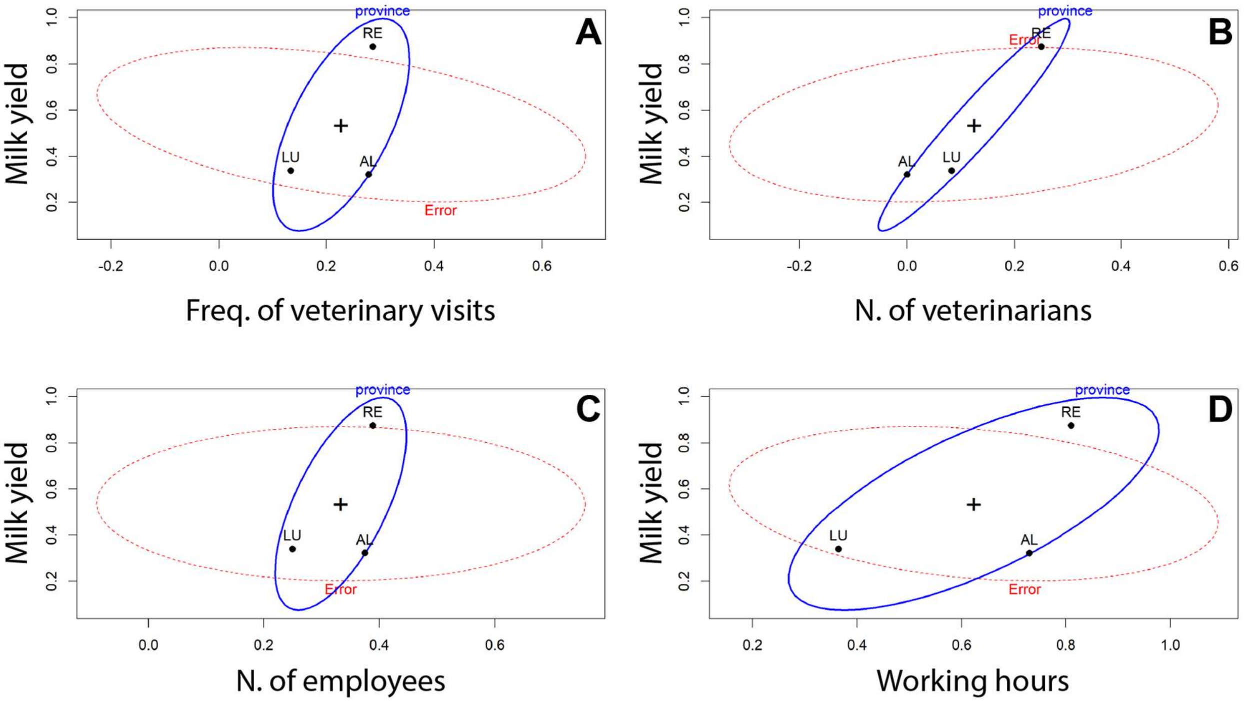


Figure 19A-D. *Results of multivariable linear model representing the association between the milk yield, on the Y axis, with the frequency of veterinary visits (A, top left), the number of veterinarians (B, top right), the number of employees (C, bottom left), and the amount of working hours per day (D, bottom right). Values on the axes were normalized between 0 and 1.*

Intramammary infection

Overall, 246 composite milk samples were collected from Holstein Friesian (39.4%), Brown Swiss (26.0%), Reggiana (17.5%), Italian red pied (14.2%) and Piedmontese (2.8%) cows (Table 14).

Table 14. *Characterization of sampled cows. The table presents the median sample size (min.-max.), the proportion of cow breeds, the median (min.-max.) parity and the median (min.-max.) number of days in milk (DIM) of the sampled cows, by area.*

Area	**Sample size**	**Brow Swiss (%)**	**Holstein Friesian (%)**	**Black/red spotted (%)**	**Piedmontese (%)**	**Reggiana (%)**	**Lactation no. (min-max)**	**DIM (min- max)**
AL	8.5 (6-18)	14.6	43.9	24.4	17.1	0.0	4 (1-8)	94 (15-432)
LU	13 (9-20)	45.8	24.1	30.1	0.0	0.0	3 (1-10)	180 (7-360)
RE	20(19-23)	16.4	48.4	0.0	0.0	35.3	3 (1-7)	163.5 (9-495)

The IMI prevalence, adjusted for DIM and number of lactations, was 71.3% (CI: 60.7-81.9). It did not differ between RE and AL (RR = 0.92, CI: 0.42-1.05) or LU (RR = 0.87, CI: 0.34-1.04). Also, no difference was observed for the total CFU between RE and AL (p = 0.271) or LU (p = 0.486). From 179 positive cows, 254 isolates were obtained. A single bacterial species was isolated from 117 cows, two species from 49, and three species from 13. The highest prevalence was observed for opportunistic (42.7%, CI: 30.5-54.9), followed by environmental (21.1%, CI: 12.5-29.7), contagious (19.4%, CI: 7.0-31.8), and other bacteria (8.5%, CI: 3.7-13.2) (Figure 21).

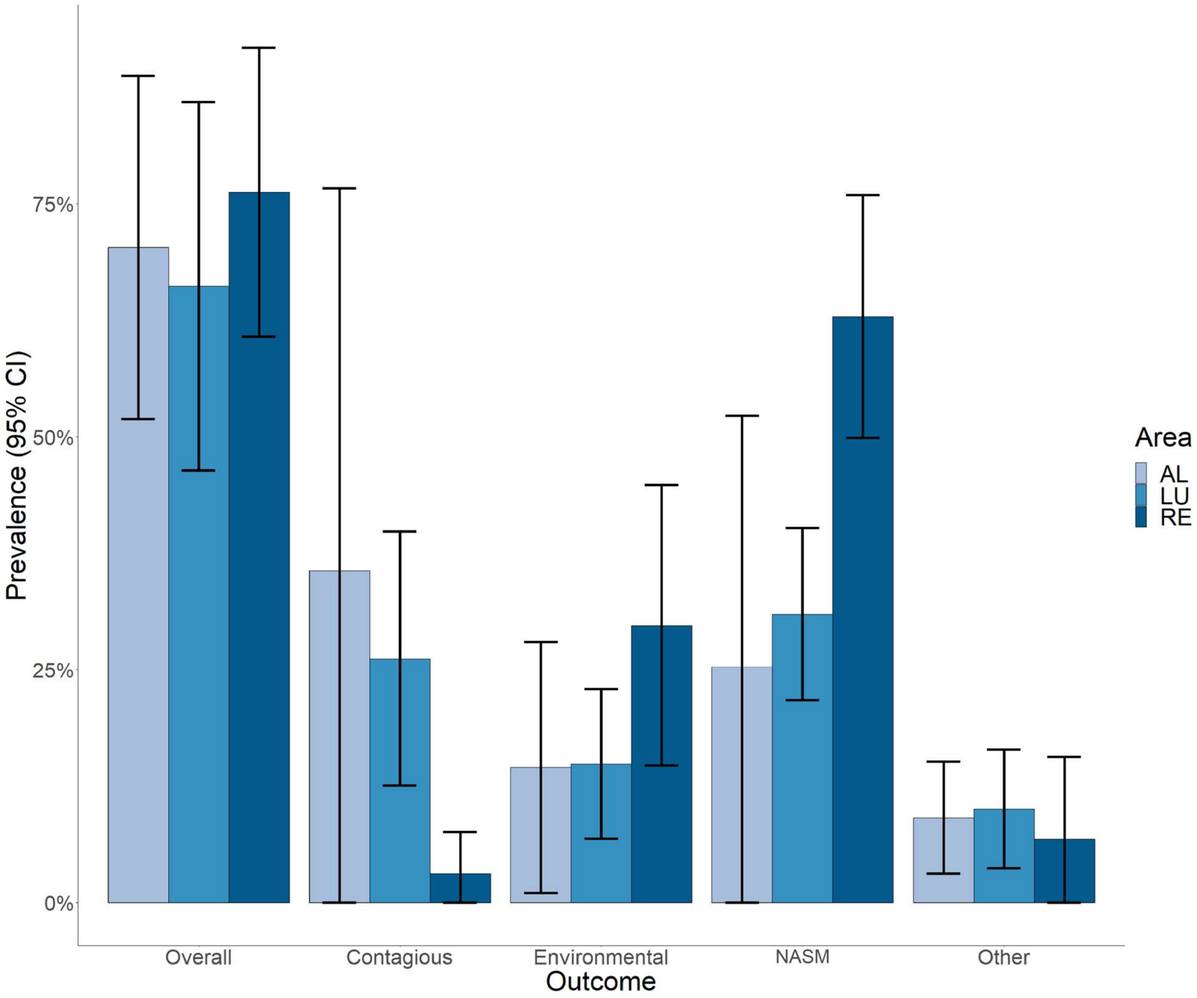


Figure 21. *Prevalence of intramammary infection by causative agents. The height of the bars reflects the prevalence of overall, contagious, environmental, NASM, and other agents, by area. Whiskers span from the lower to the upper limits of the 95% confidence interval. Prevalence and 95% confidence intervals were adjusted by DIM and number of lactations.*

The contagious IMI prevalence was significantly higher in both AL (RR = 11.41, CI: 1.75-12.32) and LU (RR = 8.38, CI: 2.27-10.77) than in RE (Supplementary Table 15). Out of 41 contagious IMI, *S. aureus* was the most frequently isolated species (73.2%), followed by *S. agalactiae* (22.0%), and *C. bovis* (9.8%).

Compared with RE, the prevalence of environmental IMI was not significantly lower in AL (RR = 0.486, CI:0.13-1.19) or LU (RR = 0.50, CI: 0.18-1.05). *A. viridans* was the most frequently isolated species (51.8%), followed by *S. uberis* (28.6%), *S. dysgalactiae* (5.4%), and *Lactococcus garviae* (3.6 %). Other isolated species were *Lactococcus lactis*, *Enterococcus faecalis*, *Enterococcus saccharolyticus*, *Streptococcus pluranimalium*, and *Streptococcus suis*.

Compared with RE, the prevalence of opportunistic IMI was lower in both AL (RR =0.40, CI: 0.08-0.98) and LU (RR = 0.49, CI = 0.23-0.83). The most frequent species were *S. xylosus* (21.7%), *M. sciuri* (20.0%), *S. chromogenes* (19.1%), and *S. haemolytitcus* (18.3%), followed *S. arlettae* (12.2%), *Staphylococcus simulans* (5.2%), *Staphylococcus microti* (3.5%), and *Macrococcus caseolyticus* (3.5%). Other 11 staphylococcal species were identified (*S. auricularis*, *S. capitis*, *S. epidermidis*, *S. equorum*, *S. fleurettii*, *S. hominis*, *S. hycus*, *S. schleiferi*, *S. simiae*, *S. succinus*, *S. vitulinus*), and multiple NASM species were simultaneously isolated from the same sample.

The IMI prevalence from other bacteria did not differ among areas (AL vs. RE RR = 1.34, CI: 0.28-3.52; LU vs. RE RR = 1.48, CI: 0.32-3.65). Other IMI were caused by genera *Corynebacterium*, *Bacillus*, and *Lactobacillus*.

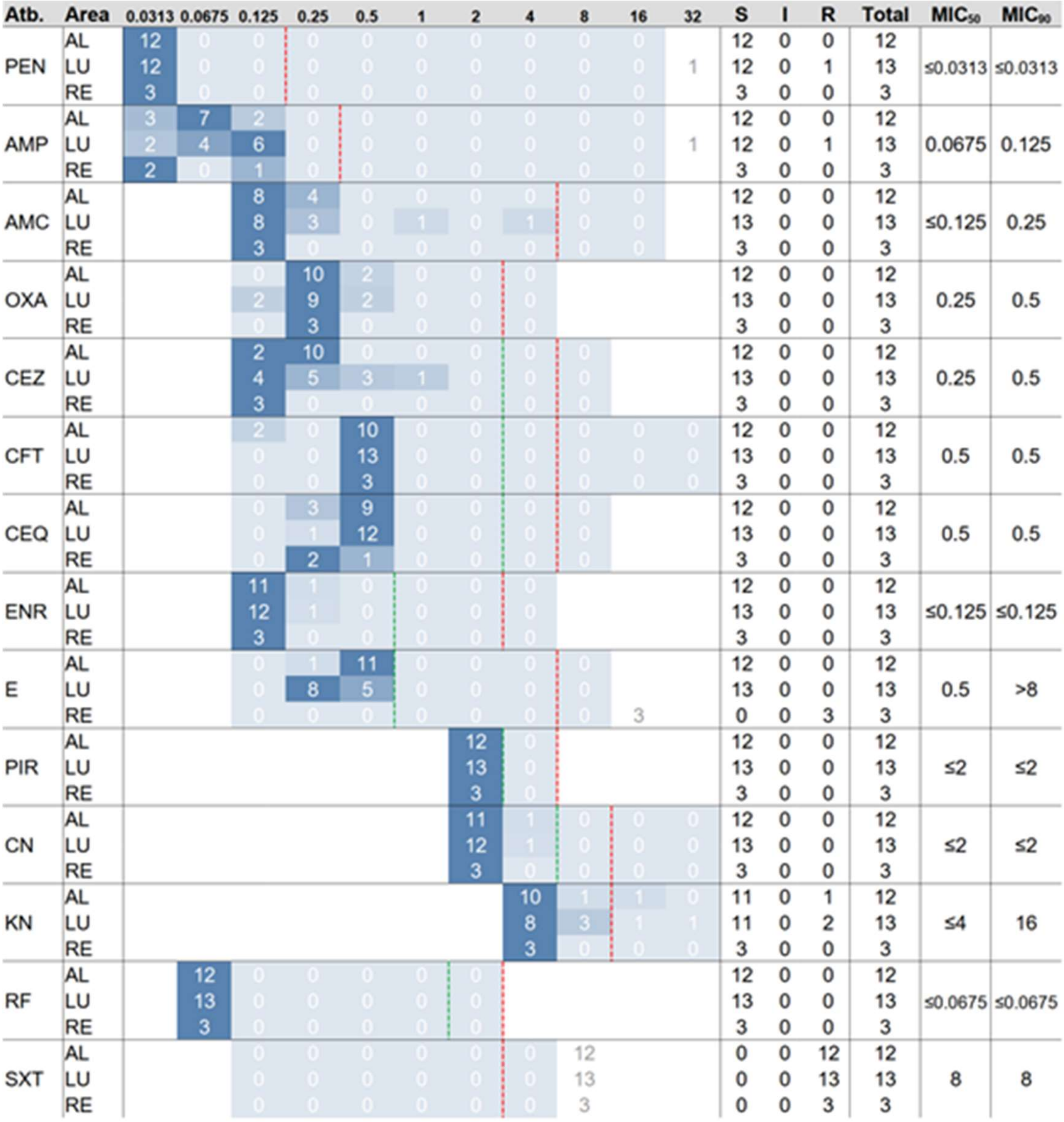
Three milk samples from the same LU herd were positive for *Prototheca zopfii*.

Antimicrobial resistance

Twenty-eight *S. aureus*, 14 *S. uberis*, eight *S. agalactiae*, three *S. dysgalactiae*, one *S. pluranimalium*, and one *S. suis* were tested for susceptibility to 14 antibacterials.

Of the *S. aureus*, all were resistant to SXT, whereas none was resistant to 1st, 3rd, or 4th generation cephalosporins (CEZ, CPZ, CEQ, respectively), to quinolones (ENR), lincosamides (PIR), and rifamycins (RF). One isolate was resistant to penicillines (PEN, AMP), but not in presence of beta-lactamase inhibitors (AMC). This strain was isolated from a LU herd where the most frequent treatments were 1st gen. cephalosporins and rifamycins, followed by sulphonamides in combination with diaminopyrimidines. Three isolates resistant to erythromycin came from a RE herd where the most frequent treatment was 1st gen. cephalosporins, followed by penicillines, macrolides, and lincosamides. One isolate, which was resistant to kanamycin, was collected from an AL herd were aminoglycosides were never recorded in the treatment registry. Only one MDR *S. aureus* (resistant to penicillines, cephalosporins, aminoglycosides, and sulphonamides + diaminopyrimidines) was isolated from a LU herd. MIC distributions for *S. aureus* are reported in Table 15.

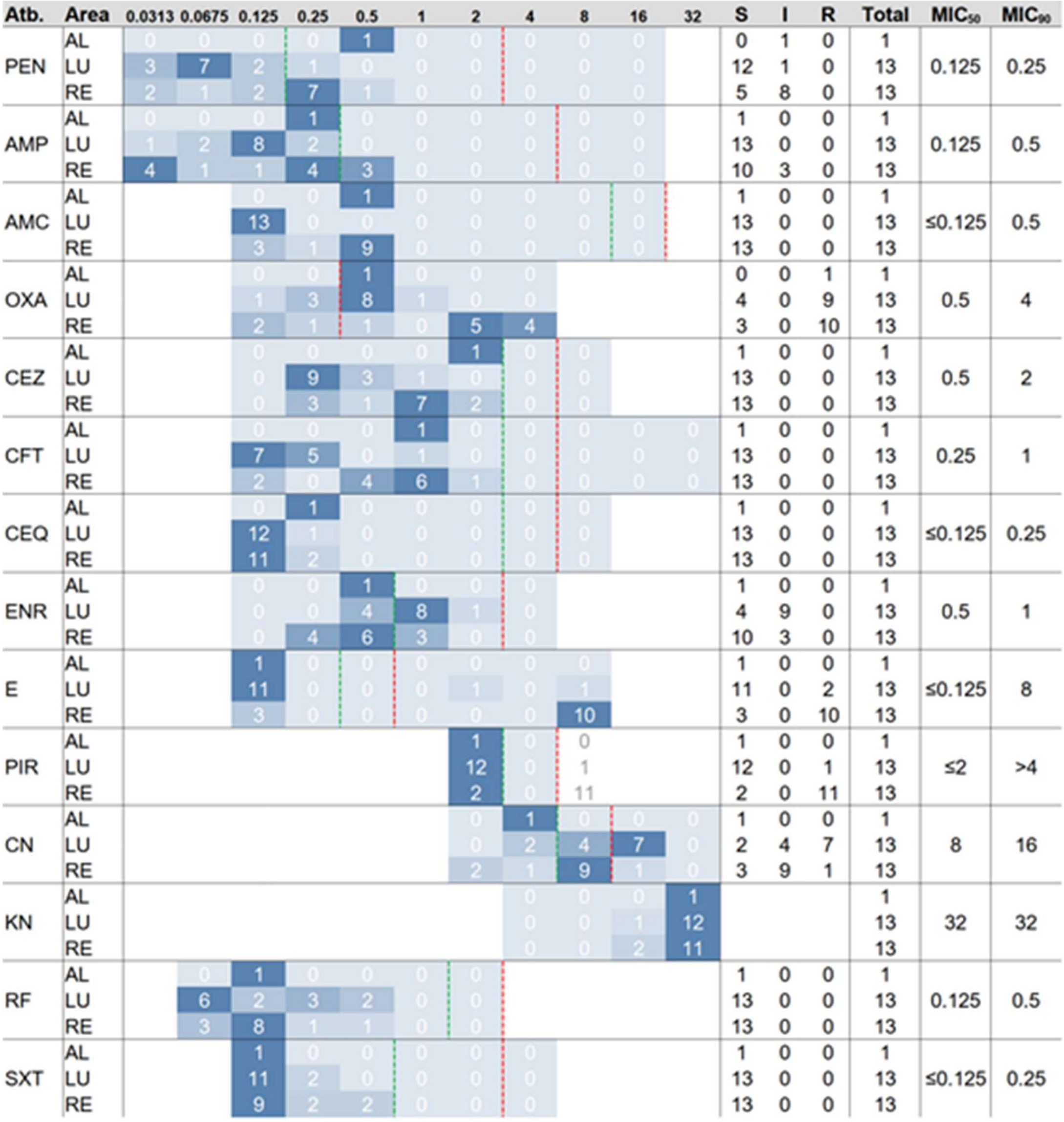
Table 15. *Antimicrobial susceptibility of Staphylococcus aureus isolates. Minimum inhibitory concentrations (MIC; 0.0313 to 32 ug/mL) of 14 tested antibacterials (Atb.) against Staphylococcus aureus isolates, by area. On the right, the counts of susceptible (S), susceptible to increased exposure (I), and resistant (R) isolates are reported, based on the available clinical breakpoints. The 50th and the 90th percentile MIC were reported (MIC*$_{50}$ *and MIC*$_{90}$*, respectively).*



Atb.	Area	0.0313	0.0675	0.125	0.25	0.5	1	2	4	8	16	32	S	I	R	Total	MIC$_{50}$	MIC$_{90}$
PEN	AL	12	0	0	0	0	0	0	0	0	0		12	0	0	12	≤0.0313	≤0.0313
	LU	12	0	0	0	0	0	0	0	0	0	1	12	0	1	13		
	RE	3	0	0	0	0	0	0	0	0	0		3	0	0	3		
AMP	AL	3	7	2	0	0	0	0	0	0	0		12	0	0	12	0.0675	0.125
	LU	2	4	6	0	0	0	0	0	0	0	1	12	0	1	13		
	RE	2	0	1	0	0	0	0	0	0	0		3	0	0	3		
AMC	AL			8	4	0	0	0	0	0	0		12	0	0	12	≤0.125	0.25
	LU			8	3	0	1	0	1	0	0		13	0	0	13		
	RE			3	0	0	0	0	0	0	0		3	0	0	3		
OXA	AL			0	10	2	0	0	0				12	0	0	12	0.25	0.5
	LU			2	9	2	0	0	0				13	0	0	13		
	RE			0	3	0	0	0	0				3	0	0	3		
CEZ	AL			2	10	0	0	0	0	0			12	0	0	12	0.25	0.5
	LU			4	5	3	1	0	0	0			13	0	0	13		
	RE			3	0	0	0	0	0	0			3	0	0	3		
CFT	AL			2	0	10	0	0	0	0	0	0	12	0	0	12	0.5	0.5
	LU			0	0	13	0	0	0	0	0	0	13	0	0	13		
	RE			0	0	3	0	0	0	0	0	0	3	0	0	3		
CEQ	AL			0	3	9	0	0	0	0			12	0	0	12	0.5	0.5
	LU			0	1	12	0	0	0	0			13	0	0	13		
	RE			0	2	1	0	0	0	0			3	0	0	3		
ENR	AL			11	1	0	0	0	0				12	0	0	12	≤0.125	≤0.125
	LU			12	1	0	0	0	0				13	0	0	13		
	RE			3	0	0	0	0	0				3	0	0	3		
E	AL			0	1	11	0	0	0	0			12	0	0	12	0.5	>8
	LU			0	8	5	0	0	0	0			13	0	0	13		
	RE			0	0	0	0	0	0	0	3		0	0	3	3		
PIR	AL							12	0				12	0	0	12	≤2	≤2
	LU							13	0				13	0	0	13		
	RE							3	0				3	0	0	3		
CN	AL							11	1	0	0	0	12	0	0	12	≤2	≤2
	LU							12	1	0	0	0	13	0	0	13		
	RE							3	0	0	0	0	3	0	0	3		
KN	AL								10	1	1	0	11	0	1	12	≤4	16
	LU								8	3	1	1	11	0	2	13		
	RE								3	0	0	0	3	0	0	3		
RF	AL		12	0	0	0	0	0					12	0	0	12	≤0.0675	≤0.0675
	LU		13	0	0	0	0	0					13	0	0	13		
	RE		3	0	0	0	0	0					3	0	0	3		
SXT	AL			0	0	0	0	0	0	12			0	0	12	12	8	8
	LU			0	0	0	0	0	0	13			0	0	13	13		
	RE			0	0	0	0	0	0	3			0	0	3	3		

All streptococci were susceptible to penicillines (PEN, AMP, AMC), cephalosporins (CEZ, CPZ, CEQ), quinolones (ENR), rifamycins (RF), and sulphonamides in association with diaminopyrimidines (SXT). Thirteen streptococci were susceptible to increased dosages (I) of penicillin and ampicillin, and twelve against enrofloxacin. Eight isolates were R and 13 were I to gentamycin, and twelve were resistant to pirlimycin. Twenty isolates were resistant to OXA (Table 16).

Table 16. *Antimicrobial susceptibility of Streptococcus spp. isolates. Minimum inhibitory concentrations (MIC; 0.0313 to 32 ug/ml) of 14 tested antibacterials (Atb.) against bacteria of the genus Streptococcus, by area. On the right, the counts of susceptible (S), susceptible to increased exposure (I), and resistant (R) isolates are reported, based on the available clinical breakpoints. No clinical breakpoints have been used for kanamycin. The 50th and the 90th percentile MIC were reported (MIC$_{50}$ and MIC$_{90}$, respectively).*



Atb.	Area	0.0313	0.0675	0.125	0.25	0.5	1	2	4	8	16	32	S	I	R	Total	MIC₅₀	MIC₉₀
PEN	AL	0	0	0	0	1	0	0	0	0	0		0	1	0	1	0.125	0.25
	LU	3	7	2	1	0	0	0	0	0	0		12	1	0	13		
	RE	2	1	2	7	1	0	0	0	0	0		5	8	0	13		
AMP	AL	0	0	0	1	0	0	0	0	0	0		1	0	0	1	0.125	0.5
	LU	1	2	8	2	0	0	0	0	0	0		13	0	0	13		
	RE	4	1	1	4	3	0	0	0	0	0		10	3	0	13		
AMC	AL			0	0	1	0	0	0	0	0		1	0	0	1	≤0.125	0.5
	LU			13	0	0	0	0	0	0	0		13	0	0	13		
	RE			3	1	9	0	0	0	0	0		13	0	0	13		
OXA	AL			0	0	1	0	0	0				0	0	1	1	0.5	4
	LU			1	3	8	1	0	0				4	0	9	13		
	RE			2	1	1	0	5	4				3	0	10	13		
CEZ	AL			0	0	0	0	1	0	0			1	0	0	1	0.5	2
	LU			0	9	3	1	0	0	0			13	0	0	13		
	RE			0	3	1	7	2	0	0			13	0	0	13		
CFT	AL			0	0	0	1	0	0	0	0	0	1	0	0	1	0.25	1
	LU			7	5	0	1	0	0	0	0	0	13	0	0	13		
	RE			2	0	4	6	1	0	0	0	0	13	0	0	13		
CEQ	AL			0	1	0	0	0	0	0			1	0	0	1	≤0.125	0.25
	LU			12	1	0	0	0	0	0			13	0	0	13		
	RE			11	2	0	0	0	0	0			13	0	0	13		
ENR	AL			0	0	1	0	0	0				1	0	0	1	0.5	1
	LU			0	0	4	8	1	0				4	9	0	13		
	RE			0	4	6	3	0	0				10	3	0	13		
E	AL			1	0	0	0	0	0	0			1	0	0	1	≤0.125	8
	LU			11	0	0	0	1	0	1			11	0	2	13		
	RE			3	0	0	0	0	0	10			3	0	10	13		
PIR	AL							1	0	0			1	0	0	1	≤2	>4
	LU							12	0	1			12	0	1	13		
	RE							2	0	11			2	0	11	13		
CN	AL							0	1	0	0	0	1	0	0	1	8	16
	LU							0	2	4	7	0	2	4	7	13		
	RE							2	1	9	1	0	3	9	1	13		
KN	AL								0	0	0	1				1	32	32
	LU								0	0	1	12				13		
	RE								0	0	2	11				13		
RF	AL		0	1	0	0	0	0					1	0	0	1	0.125	0.5
	LU		6	2	3	2	0	0					13	0	0	13		
	RE		3	8	1	1	0	0					13	0	0	13		
SXT	AL			1	0	0	0	0	0				1	0	0	1	≤0.125	0.25
	LU			11	2	0	0	0	0				13	0	0	13		
	RE			9	2	2	0	0	0				13	0	0	13		

S. agalactiae isolates were susceptible to all antibacterials, apart from two isolates from LU herds that were resistant to erythromycin, one of which was resistant also to pirlimycin. Nine MDR *S. uberis* (resistant to macrolides, lincosamides, and penicillines) were isolated from nine cows of a RE herd. One MDR *S. agalactiae* (resistant to penicillines, aminoglycosides, macrolides, and lincosamides) was isolated from a LU herd.

Antimicrobial use

Overall, IMI was the main reason for therapeutic treatment (50%), followed by urogenital diseases (10%). Other reasons for treatment include gastrointestinal infections (8%), musculoskeletal pathologies (7%), skin lesions and foot infections (6%), respiratory infections (5%), multi-organ or systemic diseases (5%), and metabolic disorders (4%) (Figure 20).

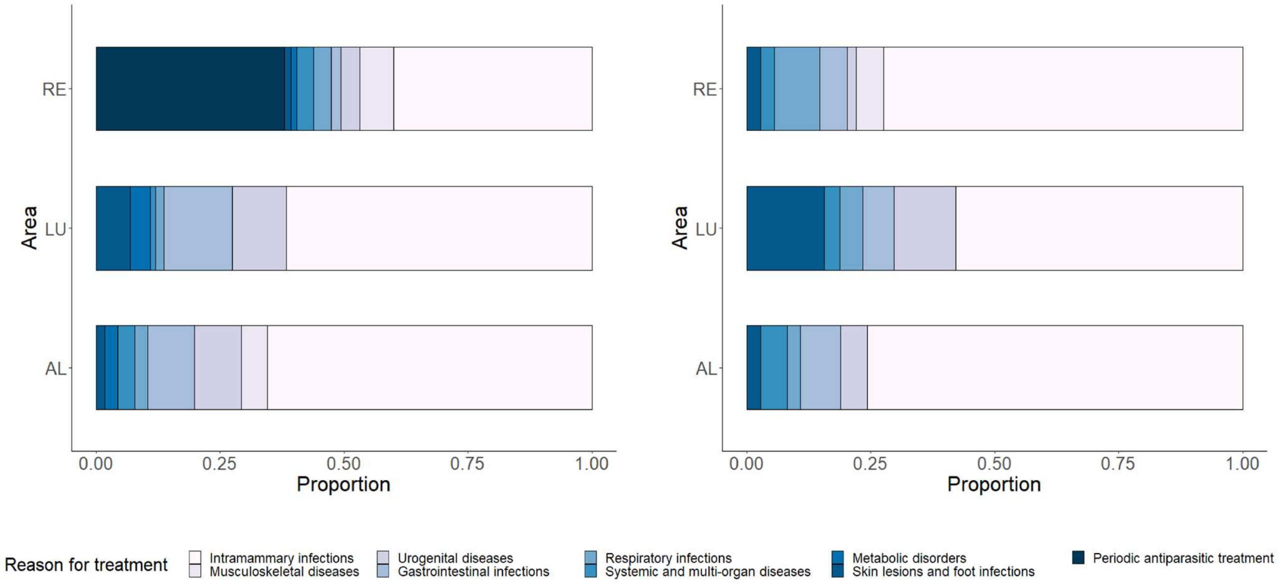


Figure 20. *Reason for treatment and antimicrobial treatment. The figure shows the proportion of each reason for treatment (left) and for antibacterial treatment (right), by study area.*

Periodic antiparasitic therapies accounted for 22% of the treatments but were implemented only by two RE farmers.

Up to 65% of the recorded treatments were antibacterial treatments. The proportion of antibacterial treatments over total treatments varied significantly among areas (p = 0.023) (AL = 73%, LU = 85%, RE = 56%). The overall ATI was 0.021 antimicrobial treatments per 100 cow-days (95%CI: 0.015-0.028) (Supplementary Table 2). The lowest antibacterial ATI was observed for AL herds, where it was significantly lower than in LU and RE herds (Table 17).

Table 17. *Treatment frequency. The table shows the median (min.-max.) number of treatments and antibacterial (atb) treatments per month, along with the adjusted antibacterial treatment rate (ATI) per 100 cowdays and treatment rate ratio (ATIR).*

Area	**Treatment per month (min-max)**	**Atb-treatment per month (min-max)**	**ATI / 100 cowdays (95% CI)**	**ATIR (95% CI)**
AL	0.68 (0.59-0.76)	0.45 (0.39-0.52)	0.009 (0.000-0.019)	0.165 (0.05-0.61)
LU	0.50 (0.36-1.41)	0.38 (0.30-0.47)	0.012 (0.005-0.020)	0.232 (0.08-0.64)
RE	2.38 (1.62-6.95)	1.75 (1.12-3.62)	0.027 (0.020-0.034)	1 (ref.)

The median number of cows treated with antibacterials per record varied by area ($p < 0.001$; AL = 2, LU = 3, RE = 1). The main reason for antibacterial treatment was IMI (78%), followed by gastrointestinal infections (8%), skin lesions and foot infections (4%), respiratory infections (4%), and urogenital diseases (3%). Overall, 28% of the treatments were a combination of multiple antibacterials (AL = 16%, LU = 22%, RE = 40%). The most administered antibacterial classes were 1st generation cephalosporins (36%), followed by rifamycins (20%) and penicillins (18%), macrolides (included lincosamides, 8%), sulfonamides (8%), aminoglycosides (4%), and tetracyclines (2%). Together, cephalosporins of 3rd and 4th generations accounted for 3% of the total.

Discussion

Herd characterization

As expected, herds with more employees, veterinarians and whose farmers spent more time on premises were more productive than the others. The questionnaire results showed that most aspects did not differ between herds in AL and LU compared to RE, and a fair degree of homogeneity within areas was observed. Cows were fed locally produced hay, were raised mostly free stall, and were artificially inseminated. In AL and LU, cows tended to live longer and grazed freely more frequently. External biosecurity measures were implemented by most farmers. However, scarce attention was paid to new introductions, quarantine, disposal of carcasses, vaccination plans, and contact with wildlife and domestic species, as demonstrated by a LU farmer who recently introduced *S. aureus* to the herd via an infected cow despite claiming strict biosecurity. As for internal biosecurity, no one reported an eradication strategy or routine testing for IMI pathogens.

Most RE farmers declared that they employ multiple specialized veterinarians, who frequently visit the farm. These findings were confirmed by the treatment registries of RE farms, where there were a high number of visit records, but fewer cows treated per record, suggesting a consistent presence of veterinarian on the premises. On the other hand, AL and LU farms usually employed only one veterinarian and had fewer records of veterinary visits per month, but with more cows treated per record. Also, LU farms had a higher proportion of antimicrobial treatments, possibly suggesting that the veterinarian was asked to address problems as they occurred, rather than consulted to prevent them.

In Italy, farmers are allowed to sell raw milk directly to consumers, but they are required to conduct fortnightly assessments of food safety. As long as the milk meets the safety standards, no consequence occurs (e.g., fines, milk destruction, farm inspection, etc.). On the other hand, farmers delivering milk to a dairy are often rewarded for high milk quality. Therefore, two approaches coexist: punishing and rewarding. In the rewarding system, farmers received report that let them know how they scored compared to other farmers, while in the punishing system, they only confront legal requirements. Except for one farmer, all RE farmers sold milk to the local dairy, of which they were co-owners. They were rewarded for high milk quality and provided with a monthly report that let them compare their SCC and CFU to other farmers, instead of meeting only the legal requirements. All but one RE farmers who participated in the milk quality reward system, could recall previous SCC results more often than AL and LU farmers who did not participate in such systems, even if they underwent more frequent controls for raw milk. This suggests that when the farmers have no way to regularly compare with colleagues, their awareness is reduced. The veterinarian can help recognize and address the problems, but for this to happen, dialogue and a collaborative relationship are needed (Roberson et al., 2003). However, no pattern of remembrance was observed for CFU. This could depend on the fact that SCC is a clinical indicator which directly describes the health of the udder, while CFU is a hygiene indicator, and its fluctuations are less interesting for farm management as long as it does not exceed the legal limits.

Information obtained through the questionnaire was compared with information collected from other sources. For instance, lameness and mastitis were the most concerning problems for RE farmers and the most frequent reasons for treatment regardless of the area, while other problems were not perceived as concerns. As examples, a RE farmer omitted reproductive problems but reported a cluster of abortions by *C. burnetii*; a LU farmer concealed a contagious mastitis problem, while having had multiple treatments for *S. agalactiae* in the last year; another LU farmer disregarded a *P. zopfii* infection as a threat to the herd; and an AL farmer denied having any health issues. In all these cases, the existence of a health problem was demonstrated by treatment records. This comparison of different information sources allowed us to shed light on farmers' awareness, and, although we did not measure it, the findings were suggestive of a need for further study.

It was not possible to distinguish AL and LU from RE farms using a single data source. However, gathering all the information, it was possible to identify farm types that partially overlap with the geographic areas.

We observed the two types among AL farms; two small family farms owned by elderly farmers, and two medium-large farms, with cows raised free-stall in recently built structures, managed as a family business. The first type were similar to most medium-sized LU farms. They also shared a similar geographic context. Indeed, AL farms were located within a natural reserve, surrounded by uncultivated woods, while LU farms, although neighboring medium-sized municipalities, were encircled by steep slopes which reduced the availability of arable land. Moreover, the lack of maintenance favored the encroachment of the forest into agricultural land. The second type of AL farm was more alike to the only RE farm selling dairy products to the customer instead of delivering milk to the local dairy. They share the large herd size, the presence of a milking parlor and the free-stall housing method. The remaining five RE farms were similar to each other: medium-sized, tie-stall, and delivering milk to the cooperative dairy. The gentler topology of the Emilian side of the Apennines favors the cultivation of most of the territory and the presence of many dairy cattle herds. Also, human settlements are not concentrated on the valley floor but are dispersed in a network of connected villages, instead.

Mastitis

Although the analysis of quarter milk performs better than composite milk, we chose composite milk for the analysis due to logistic reasons related to herds accessibility, specimens' storage, and transportation. However, there is evidence that composite milk performs similarly to quarter milk for certain pathogens (Maisano et al., 2019).

Similarities in the IMI pathogens were recognizable within geographic area. The prevalence of contagious pathogens was significantly higher in AL and LU compared to RE, with *S. aureus* and *S. agalactiae* being isolated from several cows within the same herd. Since the 1960s, the five-point plan has been effectively implemented to control contagious IMI (Bradley, 2002), but an accurate diagnosis and the systematic collaboration between farmer and veterinarian is essential for the control plan to be effective (Roberson, 2003). The lack of routine laboratory testing could be among the reasons for the higher prevalence of contagious IMI in AL and LU herds, where veterinary services were lacking and more difficult to access to. On the other hand, a higher prevalence of opportunistic and environmental IMI was observed in RE herds. Against environmental IMI, good hygiene practices and high animal welfare are among the most effective measures (Breen, 2019; NMC, 2023b; Smith et al., 1985). However, their clinical significance depends on clinical signs, so that it is not always advisable to treat (Bradley, 2002).

The lack of clinical breakpoint and epidemiological cut-offs render difficult the interpretation of many pathogen-drug combinations. Therefore, AST was performed only for *S. aureus* and *Streptococcus* spp. isolates. We did not observe any difference in antimicrobial resistance between RE and AL or LU. Indeed, most of the isolates were susceptible to almost all antibacterials. Eleven MDR isolates were identified from three herds; of them, nine *S. uberis* isolates with identical resistance patterns were isolated from a single herd. Although the identical resistance pattern provided insufficient evidence to support the spread of one single strain, it could support the hypothesis that *S. uberis* can, under certain circumstances, act as a contagious pathogen (Davies et al., 2020; Maciel-Guerra et al., 2021; Zadoks et al., 2003). We compared AST results with antimicrobial use even though the small sample size prevented us from statistically assessing the existence of a relationship. Indeed, apart from the resistance of *S. aureus* to erythromycin in one RE herd where macrolides were used, the other resistances were not explained by the treatment records.

The incidence of antimicrobial treatments was lower than recently reported in dairy cattle herds in other areas (Stevens et al., 2016; Tomazi and dos Santos, 2020), although a direct comparison was unavailable due to the different ATI calculation method. We observed a significantly lower ATI in AL and LU than in RE. However, the most used antimicrobial classes did not differ among areas, being first-line drugs. Third and fourth generation cephalosporins were the least frequently used. This differs from what is reported elsewhere (Stevens et al., 2016; Tomazi and dos Santos, 2020), but it may be due to the EU regulation that prevents the use of antimicrobial drugs reserved for human medicine.

The five RE herds similar to each other based on questionnaire characteristics, were alike for IMI prevalence as well. They had lower contagious IMI, but higher environmental and opportunistic IMI. than the AL and LU herds. The different types of IMI agents affecting AL and LU compared to RE could partially depend on aspects that are not of purely veterinary medical interest. Likely, the milk-quality-based rewarding system and the constant monitoring provided by the Parmigiano Reggiano production increased RE herd profitability and farmers' awareness. Also, RE farmers had a consistent and collaborative relationship with the vet, as demonstrated by the treatment registry, which is essential for the implementation of an effective mastitis control strategy (NMC, 2023a). On the other hand, in AL and LU, the low herd productivity and the rarefaction of farms and human settlements led to reduced veterinary services. This lack resulted in scarce monitoring and unmanaged problems, in a vicious cycle. Although it is difficult to say which is the cause and which is the consequence, cows' health likely represents the first issue to address in order to increase the profitability of these herds.

Conclusion

Dairy cattle herds are part of the unique Apennine landscape, where areas undergoing depopulation exist near thriving areas. This study examined a yet undescribed niche agricultural system and documented the characteristics of nearly all remaining farms in two of the most depressed areas for dairy production in the northern Apennines. We observed that the type and the epidemiology of mastitis pathogens differed between thriving areas and those undergoing rural abandonment. Measuring udder health through the prevalence of IMI is essential for understanding the health status of the dairy herd, yet it is only a starting point. The higher contagious IMI prevalence observed in depopulated areas suggests the need for the implementation of eradication plans and increased surveillance. A census of the remaining herds would help clarify herds' peculiarities, while continuous and detailed monitoring of the health status of the herds might counteract the rarefaction of dairy mountain herds.

Although of regional relevance only, the findings of this study can be useful to guide further studies in marginal areas, where the lack of agricultural and veterinary service jeopardize the survival of small and medium-sized dairy businesses. Moreover, for the specific area where it was conducted, the present study provided a baseline for the implementation of pathogen-specific eradication or control plans. However, an additional effort is necessary to foster the collaboration between the remaining farmers and the veterinarians, which seems essential for those herds to stay in business.

Afterword

Like other sectors, livestock production was questioned about its environmental impact. The growing demand for products of animal origin on a global scale, combined with the greater sensitivity that European consumers have towards ethical and environmental issues related to the food they consume are the crucial points for the future development of livestock production. Two of the main viable solutions indicated by the FAO and scientific research to improve the sustainability of dairy cattle farming are the improvement of herd efficiency and the smart use of local resources. This research project deal with both aspects through data analysis and on-field research.

The first case study analyzed a large cohort of dairy farms, representative of the Piedmontese and, partially, of the Italian situation. It defined the state of the art of dairy production in Piedmont, which is at the same time one of the most productive and most polluted regions in Europe. It described the relationship between individual- and herd-level risk factors for four health indicators. The result is a picture of a livestock sector which on the one hand, in the plains, produced at a high level and faced the typical problems of intensive farming (e.g., hyperketonemia). On the other hand, in marginal areas, medium-small sized, less productive farms face high mastitis incidence and long reproductive intervals.

The second case study focused precisely on these marginal areas, comparing the cattle farms present in three different areas, although very close geographically. The geography of the territory partly reflected the distribution of farms, but the main factor that seems to determine the fortune or misfortune of the dairy sector in an area is the profitability of the farms and consequently the presence of veterinary services that offer monitoring of the herds. In fact, the frequency of veterinary visits on premises was the factor that seems to impact production more than any other. The reduced productivity, also caused by health problems, might lead to a rarefaction of the herds, in turn linked to the disappearance of veterinary services, which closes the vicious circle leading to reduced monitoring of the herds and a consequent difficulty for the farmers to cope with problems that, in some cases, they don’t even know they have.

Agriculture and livestock production in marginal areas are of uttermost importance not only from an economic perspective, but also -and possibly mostly- for environment protection, wildlife management, and for the social cohesion of communities. Therefore, national and local administration should support those activities as they are of public concern. However, more awareness is needed by breeders about animal health and welfare in order for the use of local resources to be effectively a response to climate change and become one of the strategies of the livestock sector to improve its sustainability.

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Supplementary materials

Supplementary Table 1. *Cow breeds distribution. The distribution of the cattle breeds in the original database, and how they were coded for the analysis.*

Breed	**Coded**	**Obs. Freq. in the working database (%)**
Friesian Holstein	As it is	3,760,324 (83.88)
Cross-bred	As it is	257,237 (5.74)
Italian red roan	As it is	237,495 (5.30)
Brow Swiss	As it is	77,075 (1.72)
Oropa red roan	As it is	48,539 (1.08)
Valdostana (red)	Valdostana	44,010
Valdostana (black)	Valdostana	1,552
Sub tot.		45,562 (1.01)
Jersey	As it is	14,908 (0.33)
Piedmontese	As it is	11,580 (0.26)
Grauvieh (Grey Alpine)	As it is	10,645 (0.24)
Pustertaler	As it is	10,155 (0.23)
Brown	As it is	2,579 (0.06)
Abondance	As it is	2,162 (0.05)
Angler	Other	2,700
Tarina	Other	1,013
Montbèliarde	Other	391
Pinzgau	Other	345
Normande	Other	171
Red Swedish	Other	70
Black spotted Polish	Other	33
Podolica	Other	27
Red spotted Yugoslavian	Other	24
Belgian Blue	Other	8
Saler	Other	8
Charolaise	Other	3
Red Norwegian	Other	3
Sub tot.		4,796 (0.1)
Total		4,483,057

Supplementary Table 2. *Variables for cluster analysis and multiple factor analysis. Variables were grouped into 13 categories.*

Variable	**Variable category**
Pasture	Structures
Housing method	Structures
Infirmary	Structures
Calving room	Structures
Milking system	Structures
Employee	External biosecurity
Veterinarians	External biosecurity
New introductions	External biosecurity
Contact with wildlife	External biosecurity
Contact with domestic	External biosecurity
Biosecurity measures	External biosecurity
Frequency of bedding material changes	Internal biosecurity
Antiparasitic control plan	Internal biosecurity
Vaccination plan	Internal biosecurity
Carcasses management	Internal biosecurity
Necroscopy on abortions	Internal biosecurity
Work hours	Awareness
Frequency of veterinary visits	Awareness
Frequency of milk SCC checks	Awareness
Frequency of milk CFU checks	Awareness
Remembrance of previous SCC results	Awareness
Remembrance of previous CFU results	Awareness
Years of experience	Farmer’s education
Specific high school diploma	Farmer’s education
Courses attendance	Farmer’s education
Lactating cows	Animals
Calves (< 1 y. o.)	Animals
Heifers	Animals
Milk yield	Milk production
Duration of lactation	Milk production
Grass	Diet
Concentrate	Diet
Natural mating	Reproduction
Age at first insemination	Reproduction
Synchronizing oestrus	Reproduction
Colostrum evaluation	Nursery
Colostrum bank	Nursery
Respiratory	Perceived health problems
Digestive	Perceived health problems
Orthopaedic	Perceived health problems
Reproductive	Perceived health problems
Metabolic	Perceived health problems
Mastitis	Perceived health problems
Overall mortality	Perceived health problems
Proportion of abortions	Perceived health problems
Proportion of dead calves	Perceived health problems

Supplementary Table 3. *Housing method, bedding material, and litter changes. The table presents proportion of housing method, median (min. - max.) of bedding material quantity, and median (min. - max.) frequency of litter changes by area.*

Area	**Free range** (row %)	**Tie Stall** (row %)	**Bedding kg/cow** (Min.-Max.)	**Litter changes/day** (Min.-Max.)
AL	2 (50.0)	2 (50.0)	1.7 (0.2-3.2)*	1.5 (0.2-2))
LU	1 (16.7)	5 (83.3)	1.7 (1.7-1.7)	2.5 (2-3)
RE	1 (16.7)	5 (83.3)	2.8 (1.0-8.1)	2 (1-2)

*: very low quantity of bedding material were reported by a farmer that left the whole herd on pasture every day.

Supplementary Table 4. *Pasture use and duration. The table presents the proportion of herds grazing on pasture and the median (min. – max.) duration of pasture, by area.*

Area	**No pasture** (row %)	**Part of herd** (row %)	**Whole herd** (row %)	**Pasture length** (Min.-Max.)
AL	1 (25.0)	1 (25.0)	2 (50.0)	180 (180-180)
LU	4 (66.7)	0 (0.0)	2 (33.3)	240 (240-240)
RE	4 (66.7)	2 (33.3)	0 (0.0)	257.5 (150-365)

Supplementary Table 5. *Farmers' age, experience, workhours, and education. The table represents the median (min. – max.) farmers' age, their median (min. - max.) experience in dairy cattle farming in years, and median (min. - max.) workhours a day by area. Also, it displays the proportion of farmers who received a specific education in cattle farming in high school or through courses.*

Area	**Age**	**Experience**	**Workhours**	**High school (row %)**	**Courses (row %)**
AL	50 (32-68)	34 (17-53)	9 (2-10)	1 (25.0)	4 (100)
LU	49 (32-57)	34 (6-42)	5.8 (2-7.5)	0 (0.0)	2 (33.3)
RE	49.5 (37-60)	28.5 (6-45)	8.3 (6-10)	2 (33.3)	4 (66.7)

Supplementary Table 6. *Infirmary presence in the farms. The table represents the proportion of farms having an infirmary, by area.*

Area	No infirmary (%)	Outside (%)	Inside, when necessary (%)	Permanent, not exclusive (%)	Permanent (%)
AL	2 (50.0)	0 (0.0)	1 (25.0)	1 (25.0)	0 (0.0)
LU	0 (0.0)	1 (16.7)	2 (33.3)	0 (0.0)	3 (50.0)
RE	3 (50.0)	0 (0.0)	2 (33.3)	1 (16.7)	0 (0.0)

Supplementary Table 7. *Waste storage and cultivable land by area. The table diplays the proportion of farm having structures for manure and sewage storage, the median (min. – max.) duration of storage, and the median (min. – max.) surface of cultivable land, by area.*

Area	**Manure storage (%)**	**Manure storage length (min-max)**	**Sewage Storage (%)**	**Sewage storage length (min-max)**	**Cultivable land ha (min-max)**
AL	3 (75.0)	30 (30-30)	2 (50.0)	90 (15-180)	112.5 (15-210)
LU	6 (100)	112.5 (10-240)	1 (16.7)	90 (90-90)	30 (0-40)
RE	6 (100)	122 (30-255)	2 (33.3)	91 (60-122)	25 (13.5-100)

Supplementary Table 8. *Diet formulation and feeding frequency. The table represents the proportion of herds having their diet prepared by different professionals and the median (min. – max.) frequency of feedings a day, by area.*

Area	**Farmer**	**Nutritionist**	**Veterinarian**	**Feed industry**	**Feeding freq.**
AL	2 (50.0)	1 (25.0)	1 (25.0)	0 (0.0)	2 (1-3)
LU	2 (33.3)	0 (0.0)	0 (0.0)	4 (66.7)	3 (2-5)
RE	2 (33.3)	0 (0.0)	1 (16.7)	3 (50.0)	2 (2-7)

Supplementary Table 9. *Reproduction and calving management, and proportion of abortions. The table presents the median (min. – max.) age at first insemination, the median (min. – max.) duration of stay in the calving room before and after parturition, the proportion of cows giving birth per year, and abortion per calving, by area.*

Area	**Age at first insemination**	**Stay in calving room before calving**	**Stay in calving room after calving**	**Calving proportion**	**Abortion proportion**
AL	20 (18-24)	40 (20-60)	31 (2-60)	85.0 (80.0-100)	0.0 (0.0-3.0)
LU	17.25 (16-20)	30 (2-90)	10 (10-10)	90. (60.0-100)	5.5 (0.0-7.4)
RE	15.25 (14-24)	62.5 (60-65)	10 (10-10)	91.8 (76.5-100)	3.5 (2.5-6.0)

Supplementary Table 10. *Colostrum evaluation, storage, and administration. The table shows the median (min. – max.) proportion of calves' mortality, the proportion of farmers performing colostrum evaluation, storage, and ways of administration, by area.*

Area	**Calves mortality**	**Colostrum evaluation (%)**	**Colostrum bank (%)**	**Bucket feeding (%)**	**Feeding bottle (%)**	**Under the cow (%)**
AL	0.0 (0.0-8.0)	4 (100)	3 (75.0)	1 (25.0)	2 (50.0)	1 (25.0)
LU	0.0 (0.0-0.0)	2 (33.3)	3 (50.0)	0 (0.0)	6 (100)	0 (0.0)
RE	1.1 (0.0-19.2)	4 (66.7)	2 (33.3)	0 (0.0)	6 (100)	0 (0.0)

Supplementary Table 11. *New introductions. The table shows the median (min. - max.) proportion of newly introduced animals, and frequency of introductions per year, by area. Also, the proportion of origin of introduced animals are presented, by area.*

Area	**Prop. of new introductions**	**Frequency of introductions**	**From exhibition or multiple herds (%)**	**Single >50km far farm (%)**	**Single <50km far farm (%)**
AL	0.0 (0.0-12.5)	1 (1-1)	0 (0.0)	1 (25.0)	0 (0.0)
LU	5.3 (0.0-28.6)	1.5 (1-2)	2 (33.3)	0 (0.0)	1 (16.7)
RE	7.5 (0.0-23.1)	5 (1-6)	1 (16.7)	0 (0.0)	2 (33.3)

Supplementary Table 12. *Contacts with other domestic and wild animals. The table shows the proportion of herds that had contact with other domestic and wild species, by area.*

Area	**Other domestic species, partially separated**	**Other domestic species, non separated**	**Deer**	**Roedeer**	**Muflon**	**Wild boar**	**Small mammals**	**Wolf**
AL	1 (25.0)	0 (0.0)	1 (25.0)	3 (75.0)	1 (25.0)	3 (75.0)	3 (75.0)	4 (100)
LU	0 (0.0)	1 (16.7)	2 (33.3)	3 (50.0)	1 (16.7)	3 (50.0)	2 (33.3)	2 (33.3)
RE	1 (16.7)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	1 (16.7)	2 (33.3)	0 (0.0)

Supplementary Table 13. *Biosecurity measures and carcass disposal. The table presents the proportion of farmers adopting different biosecurity measures and the way carcasses were disposed, by area.*

	Biosecurity measures			**Carcasses disposal**		
Area	**Shoe covers**	**Full clothing**	**Any biosecurity measure**	**Outside**	**Inside, away from other animals**	**Inside, near other animals**
AL	0 (0.0)	2 (50.0)	2 (50.0)	1 (25.0)	2 (50.0)	1 (25.0)
LU	1 (16.7)	3 (50.0)	4 (66.7)	2 (33.3)	4 (66.7)	0 (0.0)
RE	4 (66.7)	0 (0.0)	4 (66.7)	1 (16.7)	3 (50.0)	2 (33.3)

Supplementary Table 14. *Dairy herd improvement program (DHI) participation and other performance and health monitoring, and farmer's awareness. The table displays the proportion of herds participating in DHI, controlled by public veterinary services, and the dairy, by area. Also, it shows the median (min. – max.) frequency of controls on bulk-tank milk for SCC and CFU and the number of controls the farmer remembered, by area.*

Area	**DHI**	**Public vet.**	**Dairy**	**Control frequency**	**SCC recall**	**CFU recall**
AL	3 (75.0)	1 (25.0)	0 (0.0)	25 (24-26)	0 (0-1)	0.5 (0-1)
LU	4 (66.7)	2 (66.6)	0 (0.0)	17.5 (11-24)	1 (0.2)	1 (0-2)
RE	4 (66.7)	0 (0.0)	2 (33.3)	18 (11-24)	1.5 (0.3)	1 (0-1)

Supplementary Table 15. *Prevalence of intramammary infection by causative agents and bacterial load. The table shows the prevalence proportion with 90% confidence interevals (CI) of individual-cow milk samples positive to contagious, environmental, non-aureus staphylococci and mammalicocci (NASM), and other bacteria, by area. Mean ± standard deviation of colony forming unit (CFU/10ul) are also reported, by area. Prevalence and 95% CI were adjusted for DIM and number of lactations.*

Area	Overall	Contagious	Environmental	NASM	Other	CFU/10ul
AL	73.2 (54.9-85.8)	31.7 (1.3-70.1)	14.6 (3.2-25.8)	31.7 (2.7-47.9)	9.8 (4.1-14.2)	41.7 ± 44.6
LU	68.7 (49.6-82.7)	27.7 (14.8-37.7)	15.7 (8.2-21.6)	31.3 (23.2-38.7)	9.6 (4.7-15.4)	37.1 ± 45.0
RE	75.4 (63.2-89.2)	4.1 (0.0-6.9)	30.3 (17.2-42.4)	62.3 (52.0-73.8)	6.6 (0.0-14.3)	32.2 ± 36.6

Supplementary Table 16. *Antimicrobial treatment incidence rate. The table shows the antimicrobial treatment incidence rate (ATI) of each antimicrobial class or combination of classes, by area.*

Antimicrobial class	AL	LU	RE
Aminoglycosides	0.128	0.738 ± 0.743	-
Betalactams	0.131 ± 0.048	0.145 ± 0.174	1.088 ± 0.757
Betalactams + 1st gen. cephalosporins + rifamycins	-	-	0.139 ± 0.172
Betalactams + macrolides	0.024	0.059 ± 0.008	0.165 ± 0.105
1st gen. cephalosporins	0.378 ± 0.223	0.539 ± 0.339	1.071 ± 1.119
1st gen. cephalosporins + rifamycins	0.092	0.200 ± 0.011	0.560 ± 0.674
3rd gen. cephalosporins	0.073	0.209	-
4th gen. cephalosporins	-	-	0.373 ± 0.515
Lincosamides + aminoglycosides	0.043 ± 0.009	0.122 ± 0.019	0.360 ± 0.460
Macrolides	-	-	0.165
Rifamycins	0.146 ± 0.000	0.411 ± 0.153	0.214 ± 0.182
Sulphonamides	-	-	0.066 ± 0.023
Sulphonamides + diaminopyrimidines	0.024	0.184 ± 0.076	0.117 ± 0.069
Tetracyclines	0.034 ± 0.021	0.094 ± 0.077	0.042 ± 0.036