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Technological and biotechnological innovations in agrifood commodities for sustainable rural development

Coordinator of Ph. D. Course: *Prof. Giuseppe Maiorano*

Supervisor: *Prof. Patrizio Tremonte*

Co-Supervisor: *Prof. Elisabetta Salimei*

Ph. D. Candidate

Dr. Costantino Caturano

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Abstract

The present dissertation highlights the results obtained from the activities conducted within the framework of the PhD focused on the project "Technological and biotechnological innovations in agrifood commodities for sustainable rural development". The European agriculture, and the food systems in the last decades were characterised by profound structural changes resulting in a food production depletion of from natural processes and an increased dependency on industrially produced inputs and fossil fuels. The negative social and environmental outcomes of these developments have been widely analysed and documented. The concentration of agricultural production and the increasing polarisation of agricultural structures has led to significant problems in both intensive farming areas and less favoured areas. There is a large risk that the very substantial public and private sector investments currently going into building a "knowledge-based bioeconomy" will further aggravate these problems, as this will reinforce the agricultural sector's focus on producing cheap raw materials. To promote a more balanced development of rural areas, according to several research found in literature, it seems of utmost importance to steer structural changes in directions that foster a more sustainable development, and that contribute to addressing the social, environmental, and economic imbalances and challenges. Hence, the transformation and the adaptive capacity of the agricultural sector and of rural economies have become key questions. The notion of sustainable development (SD) is increasingly characterizing the political horizon of many governments.

Based on this consideration, a common organisation of the markets in agricultural products and a new governance structure are the main innovations of the 2021-2027 Common Agricultural Policy (CAP), approved by the European Parliament. In particular, the CAP 2021-2027 is characterized by an alignment with the climate and sustainable development objectives. In fact, the European Parliament wants to bind farmers to practices that are more respectful of the climate and the environment, in exchange for direct funding. At least 30% of direct aid (the first pillar of the CAP) is destined for voluntary ecological schemes that could end up increasing the income received by farmers as well. Another 35%, that of the second pillar of the CAP (the Rural Development Plans), will be dedicated to any type of measure linked to the climate or the environment, while the remaining 30% of the funding should support farmers in the fight against change climate, also considering the sustainable management of natural resources and the protection of biodiversity. Moreover, the marginal and internal areas will have a strategic role to enable sustainable development at global level. In order to really get the so-called Green

Deal off the ground, a new strategy is necessary establishing a strong alliance between the reasons of the agriculture and those related to ecosystems and climate protection.
The activities conducted in Chapter 1 highlighted that the development in a rural context can begin by using a "district" approach to manage the area. For this purpose, the entire province of Benevento was chosen as the area that better represent the internal and marginal agri-food system. So, Benevento was used as a sample area for validating and verifying the "district approach". The agri-food system of the inland areas, even where it identifies district forms rich in potential, needs development strategies in the management of agricultural systems. The geographical area of Sannio can be identified by two distinct and lively districts, one dedicated to zootechnics and the other to vine and olive products. However, it lives in a threatened condition, clinging to the fragile ecosystems of the Mediterranean, while facing serious ecological, financial, and social challenges. A lack of successful farmers, climate change and COVID-19 restrictions have made this system vulnerable, despite its long tradition. Yet, agri-food and agropastoral systems in inland areas plays a crucial role in a new era, a new post-Covid pandemic time, by increasing resilience to climate change, preserving, and restoring ecosystems and biodiversity, but above all through the development and promotion of a healthy and safe food system with a sustainable and rational use of natural sources. Through the activities outlined in Chapter 1, the characteristics of the selected geographical area were investigated, and pivot chains were identified. Specifically, the bovine chain for milk production appears to be the most interesting and, at the same time, full of fragilities and impending innovations.
Chapter 2 provides reflections and discussions on a new financial model capable of ensuring One Health (OH) and ecological transition in cattle breeding. Economic health is here considered as the starting point of the new One Health financial model, ethical and scientific, proposed as a model in embryo, in which specific rules should govern public funds, private investments and trade. In this way, economic losses due to the negative side effects of human activities can be progressively reduced and the entire planet will benefit from this process.
Recalling Dr. Bernard Url's conclusive speech in the ONE2022 conference: "We have to change the financial model in which we are locked". Since investors bet on innovation only considering its impact/success and the extent of economic return, we need to build a way to direct investments toward ethical and science-based choices. National and international rules concerning public/private investments and trading could regulate the flow of money, directing it exclusively toward companies that meet a broad range of OH parameters, ranging from environmental impact up to food safety, human health, work conditions, duration of

employment, number of layoffs per decade, wage conditions, gender, and social equality. A OH conformity certificate for enterprises - listed in the stock market or not - should be awarded considering various aspects of the OH, each of which should be analysed using a proper checklist. Each analysed checklist could then be merged in a final document declaring (or not) the compliance of a specific enterprise with a minimum number of the considered parameters useful to evaluate its OH status. This in-embryo One Health Financial Model is based on money exclusively invested in certified enterprises. In this model, money can be effectively used in the effort to improve human society and global conditions, avoiding economic losses due to negative side effects on the holistically considered health, in the effort to effectively purpose the OH goals. Dedicated agencies and databanks preserving all data concerning certified enterprises are needed. Data, when shared in open access, could efficaciously improve public engagement, addressing consumers' choices toward certified producers who comply with the OH objectives.

The introduction of sustainable and one heath solutions to improve food quality and competitiveness remains an interesting new element to pursue as it would give a clear identity to the Sannio dairy sector. A strategic element, the latter, that would also allow the valorisation of competitive segments downstream, such as dairy production from healthy, genuine, sustainable, and clearly identifiable milk.

Furthermore, it should be taken into strong consideration that the intensification of livestock breeding in the last century has contributed to the numerous negative impacts of agriculture on the environment and on animals and human health. Among them, just to name a few, zoonotic epidemics, antibiotic resistance, consumption of animal products associated with increased risk of cancer. The evolution of farming towards sustainable systems that respect humans, animals and the ecological state of the planet is a key aspect of the transitions of the agri-food system. In this context, existing solutions are no longer sufficient, and it is necessary to develop new solutions, research and innovation approaches. While in recent years research and innovation in the agricultural sector has been organized top-down and has been outsourced by farms, many studies have highlighted the need to develop research approaches and innovation open to the participation of a variety of actors (researchers, practitioners, government, and civil society) in a context of global transition.

In Chapter 3, the most appropriate innovation strategies were identified to improving competitiveness compatible with sustainability and agroecology. To produce non-obvious, widely shared and more efficient innovative ideas, a method based on sharing knowledge and experience within the specific district area was proposed. This approach represents an evolution of the citizen science model and would facilitate the definition of innovations and the promotion of agroecological transitions by building a common horizon with farmers, technicians, and researchers. Several studies point out that a group with a heterogeneity of actors can lead to the design of original and varied ideas, if methods are mobilised to overcome fixation effects. Among these, the Knowledge-Concept-Proposal (KCP) method, based on the organisation of design workshops, aims to control these fixation effects. Therefore, the research actions were conducted with the intention of involving citizens and the various actors of the food district itself in the exploration of innovative solutions for the milk supply chain. To this end, a co-designing process (based on workshops and comparisons) was implemented involving researchers, livestock professionals and citizens with the aim of producing knowledge and developing innovative solutions for the dairy systems of the Alto Sannio area. Based on the data that emerged from the analysis of the quantitative research literature and derived from the sharing of knowledge and experience through the KCP method, the central part of the doctoral thesis was addressed to the development of an innovation based on hay management and the valorisation of the beekeeping heritage of Alto Sannio.

Bees and hay in cattle feeding appear to be the two themes that best identify the concept of health in its broadest sense as well as the link to territoriality. Hay alone or as a vehicle for microorganisms can significantly affect animal welfare and milk quality. It is widely known that pollinating insects provide important ecosystem services that supports global biodiversity and environmental health. However, it should be emphasised that the microbial supply from the use of silage significantly influences rumen activity and thus milk quality. Therefore, combining the benefits of hay with those of the microbial population is an interesting auspice. The identification of a microbial biotype compatible with the forage essences of the district area as well as indicator of the environmental well-being of the area would fully meet the need for non-obvious innovations. Results related to the validation of biotechnological innovation are reported in Chapter 4. In particular, the innovation based on the combination of hay and microbial biodiversity of the bee herd has produced positive effects on sustainable rural development and One Health concepts. The innovation has removed the unhealthy veil from the cattle sector and improved both animal welfare and milk quality. For example, the introduction of a specific lactic acid bacteria from beekeeping material into hay management has improved the milk fatty acid profile. An innovation in line with the times of biodiversity and satisfying both milk quality and consumer healthiness.

Chapter 1

Internal area with district characteristics and pivot supply chain

The agri-food and rural development world has experienced significant changes in recent years. The evolution towards globalized and highly complex food supply systems has been accompanied by growing competition, reduced state subsidies as well as concerns about quality, output, and the environment. At the beginning of the 21st century, the agri-food industry is urgently searching for new solutions. In this context, there are two key questions:

1. It is possible, in EU, to produce typical foods eco-friendly and respectful of stringent European safety food standards?
2. Typical products and technological innovation can live together?

The production of typical food in accordance with eco-friendly and safety standards is possible but it's not that simple. Traditional technologies were born in times when foodborne diseases and pathogens were not well known, so these technologies are not very attentive to Good Hygienic Practices (GHP), and to HACCP system. Therefore, in order to produce safe typical foods, in some cases, it is necessary to partially modify the production technology by inserting, for instance, a process of sanitization of the raw material, such as pasteurization. However, so the peculiar characteristics of the products can be lost. In the recent past, environmental issues were not as felt, as now, by consumers and producers so, to obtain typical eco-friendly products in some cases it was necessary to modify farming practices and waste management.

Regarding the relation between typical products and technological innovation, the question is not if typical products and technological innovation can coexist, because it is impossible for typical products to remain unchanged in an evolving ecological, technical, and social environment, but how to govern this coexistence.

Since the end of 1900, new production criteria have been needed to satisfy the increasing volumes of foods processed with high hygienic standards. In many cases, this trend involved drastic modifications to food processing that have deeply affected the relationship between the production environment and product characteristics. The effects were different and variable depending on the product considered, but in majority of the cases, they have impaired the territorial links and sensory features of products. On the other hand, technological evolution has always accompanied the production of typical products, even those certified by the EU. Furthermore, if process innovations are well conducted, respectful of tradition they can contribute to improve the characteristics (especially safety and shelf life) of typical foods.

The Cork Conference (1996) played an important role in the debate in the 1990s on the future of EC agricultural and rural policies. It gave birth to the "decalogue" which established the principles behind European rural development. In the following years rural development became the "second pillar" of the Common Agricultural Policy (CAP), and the new CAP for 2014-2020 has confirmed and reinforced this priority. In addition, the programmers of the EC Structural Funds have resulted in an acceleration of the new regional policies. These are increasingly aimed at integrated development of the whole region, both urban and rural, and at sustaining the process of economic and social cohesion in the EU. They also highlight the growing importance in EU policies of economic activity other than agriculture in rural areas (Papadopoulou et al, 2011).

Thus, at present EU polices are aimed at diversification of agricultural and rural activities, with the strategic direction being: improving the environment and the landscape, improving the quality of life, promoting diversification and creating local work opportunities. In rural areas - defined as settlements of historic, social and cultural value or generators of a "cultural atmosphere", capable of linking the economy, the social sphere and the local authorities together in a dense network of relationships - diversification of agriculture activities and their multi-functionality may help them to improve the quality of the landscape (Bryant et al, 2011; Carullo et al, 2013a; Carullo et al, 2013b; Russo et al, 2014a; Russo et al, 2014b; Siciliano, 2012). Traditional agricultural production method plays a recognised role in maintaining the rural matrix, and are an integral part of the landscape, society and economy (Herzfeld and Jongeneel, 2012; Strano et al, 2012).

Some studies have highlighted how typical products, thanks to pilot initiatives co-financed by EU, have helped to give added value to the area and to protect the local community. The development of diversified activities in rural areas has made it possible to protect and safeguard these areas. These activities offer innovative products which help to create a suitable environment for tourism and cultural activities (Carmona-Torres et al, 2011; Febles-González et al, 2011).

The "identity of typical products" factor fits in perfectly with the "identity of the landscape" factor. Together they resemble a cultural marker and encourage improvements in the social and economic well-being of the communities to which they belong. They thus provide concrete opportunities for possible growth in new sectors, such as tourism and recreational activities in rural environments (Erickson et al, 2011).

However, at present these positive possibilities are often unattractive in areas which are greatly suited for such activities, as Southern Italy. This is because they lack the services and support structure which are necessary for both agriculture and other multifunctional activities, as well as any plans aimed at developing local resources.

Exploring the recent developments, doctoral activities highlight the latest research on understanding and promoting sustainable food systems. Featuring a range of different case studies, the activities investigate different models of rural development for food production, examine the implications for a sustainable future, analyzes future challenges, and suggests new strategies for future agri-food development in a world fast exceeding its resources.

The research is aimed to identifying areas which have the necessary characteristics to allow them to be defined as Typical Agricultural Districts.

The concept and development model based on the agri-food district has a rather long history and has its roots in the economic changes' characteristic of the 1970s and 1980s. However, because of its characteristics, it is still the subject of interest at both the economic-organisational and the judicial level. In the Italian economy of the 1970s, a new model of real development emerged consisting of a set of companies, mainly small and medium-sized, characterised by a tendency towards horizontal and vertical integration and production specialisation, generally concentrated in a specific territory and linked by a common historical, social, economic, and cultural experience (Becattini, 1987). In particular, the local systems of small enterprises are exalted, i.e., the enterprises established within the industrial districts, immersed in an institutional fabric with which they interact, and which have codes of conduct to the point advantages in terms of increases in competitiveness and efficiency to be determined.

For these reasons, the national legislation tries to regulate this new phenomenon with law 317/1991. It, therefore, gives for the first time a regulatory definition of the district: *"a territorial area characterised by a high concentration of small businesses characterised by a particular production specialisation, where there is a specific relationship between the presence of companies and the resident population"* (Law n. 317/1991).

In the agri-food sector, with Legislative Decree 228/2001 were introduced two types of the district:

Rural districts: *"local production systems, as per art. 36, paragraph 1, of law no. 317/91, characterised by a homogeneous historical and territorial identity resulting from the integration of agricultural activities (...) and other local activities, as well as the production of goods or services of particular specificity, consistent with natural and territorial traditions and vocations"*.

Quality agri-food districts: *"local production systems, also of an inter- regional nature, characterised by interrelation and productive interdependence of agricultural and agri-food*

companies, as well as by one or more certified and protected products by the current community or national legislation, or by typical traditional products" (Legislative Decree n. 228/2001).
In light of this legislation, many regions have recognised the quality of industrial and agri-food districts present in their territories, with different purposes, functions, juridical subjectivity, and different forms of support (Zecca et al, 2014). In this situation, several districts were formed on the national part and remained so until 2017.
These districts have had a strong relaunch with the Law n. 205 of 27 December 2017, which provide the definitions of food districts model with two main objectives:

1. Gives new impetus to the experiences of the agri-food and rural districts already recognised by Italian regions;
2. Promote the birth of new realities through the possibility of accessing dedicated loans (Law n. 205/2017).

The recognition of the Food Districts is granted by the Regions and the autonomous Provinces to which they belong. At the same time, the Ministry of Agricultural, Food and Forestry Policies (MAFFP) holds the National Register of the Food Districts.
In 2019, the MAFFP also defined, in agreement with the Ministry of Development Economics, the criteria and methods for implementing specific interventions in the Food Districts, which District Food Contracts (Ministerial Decree n. 7775/2019). Its purpose is to favoring processes of reorganisation of the relations between the different subjects of the supply chains operating in the territory of the food district through the financing of investments and innovations capable:

- to promote collaboration and integration between the issues of the supply chains;
- to stimulate the creation of better market relationships;
- to ensure, as a matter of priority, positive effects on agricultural production.

It is done by asking the regions to send the list of recognised districts to establish the national register of food districts (it becomes the continuously updated population of possible beneficiaries of the call). In the National Register of food districts, there are 139 food districts recognised by Italian regions based on regional legislation (last updated 19.10.2022). In the first call (2019-2022) 20 district contracts were presented and were under evaluation.
To find the most suitable areas for creating typical agricultural districts, the chosen criteria are the following:

- typical food product with a quality or typical product label;
- historical/archaeological context with particular aspects which are linked to the food products;
- interesting landscape linked to the food products;

- traditional food process linked to food waste as source of valuable compounds.

Development in a rural context can begin by using a “district” approach to manage the area. Let us being by defining “district” as a geographical area in which there are significant interactions between the economic, social and cultural elements of the area and natural elements of the landscape, and that these are capable of generating endogenous development processes and of taking advantage of the opportunities offered by the world economic network (Becattini, 2000; Iacoponi et al, 1995). Thus the “district” concept implies that there are firms or, more generally, products (handicrafts, cultural, agricultural, etc.) which, acting together in synergy, create its social and economic context (Arfini and Zanetti, 1997).

Definitions of the district environment often overlook the importance of the quality of the landscape in defining such an area, despite the fact that is widely recognised that this is of fundamental importance if one is to encourage personal development and generate new and more varied activities (De Montis, 2014).

Regarding the case study of the present doctoral project, the entire province of Benevento was chosen as the area that better represent the internal and marginal agri-food system. So, Benevento was used as a sample area for validating and verifying the “district approach”. The province of Benevento has a wide variety of landscapes linked to agricultural and food production. Agriculture is practised in the rural areas with intensive olive and vineyard cultivation on the hills, and extensive agriculture on the arable land. Most of the agriculture in the province is specialised and there are particular areas which are characterized by their production of typical certificated products. Most of these are concentrated on the hills of Taburno and consist of the vineyards and the network of old and new vintners which give character to the landscape, while in Colline Beneventane area there are olive groves plants. Finally, the meat chain characterizes the Fortore area.

1.1 Alto Sannio Area: firms, environmental and economics features

Sannio Area represents the area under analysis and covers a territory in a predominantly hilly and mountainous lands located in a portion of the Campanian Apennines. There are 68 municipalities with a predominantly rural character (rural manufacturing and rural cultural) and relatively homogeneous morphological characteristics in the territory (Figure 1.1). In this area, most of the municipalities are also recognized as “internal areas” but rich in important environmental and cultural resources and highly diversified in their nature. These features intuitively suggest that the municipalities within the geographical area under analysis are characterized by similar environmental opportunities for the growth of farms and other economic activities.

Figure 1.1 - Sannio Area municipalities of Campania region, located in Benevento province.

The doctoral activities conducted in the preliminary phase and reported in this chapter, aim to provide two innovative contributions to a specific Food District. Firstly to discuss notion of Food District by stressing its relationship with other territorial features (i.e., environmental, social, and economic) that complement the agricultural dimensions. Secondly, to propose a methodological approach for the identification of food district considering biological and agricultural features of territories as well as crucial aspects related to their environmental, social and economic contexts. Moreover, an indicator is developed to assess the vocation of territories to enter a food district. In line with the bottom-up approach inspiring the function of food district, the indicator aims to provide a useful informative base for supporting policymakers and other relevant stakeholders in the discussions and participatory processes concerning the creation of food district.

The purpose of this section is to provide a detailed description of the structural and managerial characteristics of representative Alto Sannio dairy farms, contextualizing the livestock activities carried out in the rural territory of Benevento province, paying particular attention to fragile contexts threatened by depopulation and abandonment.

1.1.1 Materials and methods

1.1.1.1 Food production characterization

Data were collected by firms' interviews active in organic production, processing and retailing in the 68 municipalities of area under analysis. The analysis is based on an ad hoc questionnaire with closed questions (Likert scale or dummy variables) and open answers (Table 1.1). The presence of open answers helped to better contextualise the interpretation of some responses. The questionnaire was organized in different sections on the basis of the purpose of the paper: (i) production features and quality management; (ii) raw materials supply and vertical coordination with agriculture; (iii) distribution channels. Moreover, an opening section aims to collecting general information about the firms in order to describe the sample.

Table 1.1 – Information collected by questionnaire.

Main data and information requested	*Brief description*
1. *Farming entrepreneur and farm: general data*	Name, address, contact data, farm localisation (i.e., altitude about sea level), soil characteristics, irrigable area, crop rotation, breeding system, type of management (i.e., single, family, cooperative).
2. *Total agricultural area (TAA) and utilised agricultural area (UAA)*	Total surface in hectares (ha), agricultural area of property or for rent, UAA used for feed production.
3. *Productive use of UAA*	Total surface (ha), average annual production (tons), reuse of agricultural products within the farm (%), percentage of pasture, grassland, grains, legume, hay, silage, arboreal crops. Grazing period and stocking rate.
4. *Other information*	Third-party activities (ha*year), multifunctional activities (i.e., agritourism, educational farm).
5. *Structural farm characteristics*	Type of stall (free or tie), year of construction, presence of barn, facilities (e.g., storeroom), silos, manure storage, reuse of manure on farm soils, milk room.
6. *Description of fam characteristics*	Free or permanent animal housing, farm cheese making, farm butcher shop.

7. Farm automation and technology innovation level	Presence of tractors, agricultural machinery for fodder and grain production, mixer wagon (for feeding unifeed), type of milking (manual or mechanized), photovoltaic system, systems for the recovery of heat or wastewater, ventilation systems.
8. Hygienic and sanitary standards	Major zoonosis and cattle disease (i.e., brucellosis, tuberculosis, bovine herpes virus).
9. Herd characteristic and composition	Breed, number of heads divided according to sex, age, and physiological stage.
10. Other management data	Fertility rate, number of calve per year, duration of lactation, daily milk production, milk fat and protein contents (%), birth weight, insemination (natural of artificial), weaning period, mortality rate.
11. Feedstuffs	Number of diets formulated according to physiological stage, feeding pasture or concentrates based, soybean or other legume utilised, mineral or vitamins supplements, dietary forage to concentrate ratio, use of silage, use of by-products.
12. Description of diets administered	Calves until weaning, heifers, dairy cattle, dry cattle.
13. Possible nutritional disorders	Acidosis, ketosis, lameness, collapse.
14. Milk quality	Average microbial load, somatic cells.
15. Milk uses	Sale of fresh milk or of milk products to regional or extra-regional market.
16. Other species raised	Sheep, goat, swine, poultry.

The sample was composed by 900 firms. The sample size was selected choosing a level of relative error (around 6%), taking into account the population size. For the selection of the firms to be interviewed, we start from the list of the firms certified in each municipality. The selection was based on two criteria, as the geographical distribution of the firms within the area, and the representativeness of different sectors. Then, a request to participate in the survey was sent to each firm, obtaining a final sample of around 50 firms per region including processors and retailers. Specifically, in order to study the processing firms, and at the same time the vertical coordination with the retailing sector, the sample selected was composed of 85% of farms, 10% of processing firms, and the remaining 5% was composed of retailers. Concerning the retailers, 92% are shops specialized in traditional certified food products.

For a better definition and analysis of the characteristics of agricultural activities made by farmers, information regarding geographical location, legal form, and management traits were collected by the means of a questionnaire. A detailed description of agricultural land use, as well as all the collateral activities, structural farm characteristics, level of automation and computerization, with particular attention to animal milking and feeding, was requested. Moreover, information about characteristics of the herd and animal management, feeding system, diet administration, and dietary components were also considered. Finally, data about milk characteristics were requested.

All the information collected and reported in Table 1.1 were analysed, and a comparison between three selected farms (Figure 1.2) was made also considering the peculiarities of Sannio area. The three typical dairy farms of Alto Sannio area were identified and named as small, medium, and large, according to the herd consistency, i.e. 39, 50, 70 heads respectively.

Figure 1.2 – Localisation of the selected farms in Alto Sannio area (Source: Google Earth, 2022).

1.1.1.2 Environmental impact assessment and diet composition of selected farms

Considering the interest in agriculture sustainability and food quality and safety issues, in recent decades the agricultural sector is faced with the challenge of producing and using food and feed with low environmental impact, respecting human and animals' wellbeing and health (EEA, 2022). On this regard, according to Leroy and colleagues (2022), is important to consider that livestock farming well-managed could improve soil and ecosystems' health also through the cultivation of marginal lands and the use in animal nutrition of no-food product, as forage or by-product.

According to data analysis provided by Istat (2021), during 2015 – 2020 period the legumes were characterized by an increasing demand, although the Italian cultivation of legumes suffered a decline which affects both food and industrial plants. However Italian soybean cultivation accounts annually around 265 thousand hectares, with a production over 950.000 tons (Istat, 2021). Soybean meal, co-product after extraction, is one of the main protein sources commonly used in animal diets. Large part of soybean is derived from genetically modified organisms (GMOs) and is cultivated in non-European countries (Dotas et al, 2014). Soybean represents the most used protein-rich feed because of its good balance in amino acids and its low content in antinutrients (Manceron et al, 2014; Cherif et al, 2018), and during 2021 – 2022 period, soybean price was characterized by record price of 615 euro/tons (ISMEA, 2022). Taken together, these aspects motivate the possible inclusion of alternative vegetal protein sources from local origin in the diet of dairy cattle, also emphasizing the link with the territory. For this purpose, the attention was focalised mainly on faba bean (*Vicia faba* L.), which could represent an interesting alternative protein source for dairy cows (Cherif et al, 2018). Faba bean is an herbaceous crop widely grown in winter season, largely cultivated in inner areas of Centre-South Italy, characterized by 22-45% of starch and about 36% of protein, used both in humans and animals' nutrition (Punia et al, 2019).

According to FAO (2014), carbon footprint of a product quantified the total amount of greenhouse gas (GHG) emitted during its lifecycle, expressed in kilograms of carbon dioxide equivalents (kg CO_2-eq). Carbon footprint include emissions calculated considering all agricultural inputs, machinery, livestock, soils, processing, transportation, preparation of food, and waste disposal. For this reason, one kg of wheat, or one kg of milk, have different carbon footprints, since their life cycles are different and are responsible for different amount of greenhouse gases (FAO, 2014). More in detail, for the conversion of non- CO_2 gases, the global warming potential over a 100-year period is applied according to Forster and colleagues (2021), which considers the warming potential of methane (CH_4) from fossil origin and of nitrogen oxide (N_2O) to be respectively 29.8 and 273 times that of CO_2.

In this thesis work, the study of environmental impact of livestock farms even after a possible innovation action was assessed on a year basis considering geographical localisation of farms and their production category, herd characteristics, ingredients used for animal diets, and manure and waste management. More in detail, for each farm the potential environmental impact of the introduction of alternative protein source (faba bean) in the diets of dairy cattle was estimated as compared to the dietary ingredients traditionally used.

Based on the information collected from the questionnaires (above paragraph *Food production and characterization*), diets were studied for each farm according to animal physiological state. Diets were formulated used Sistema Plurimix® ver. 2.45.63 released by Fabermatica srl (http://www.fabermatica.com/index.php/it/software/soluzioni-per-la-zootecnia/sistema-plurimix), as widespread and consolidated management software tool of reference in the Italian livestock scene.

To calculate the carbon footprint emitted on a year basis from the milk production process of each farm, two different software were utilised: i) Latte Sostenibile mobile application (https://www.crpa.it/nqcontent.cfm?a_id=22113&tt=crpa_www&sp=crpa&print_in=1) was used to estimate the carbon footprint associated with the milk production and expressed in terms of tons of CO2-eq produced per tons of milk, and in terms of enteric methane produced annually (Bertolini et al, 2022); ii) Global Livestock Environmental Assessment Model - Interactive (GLEAM-i 3.0, https://gleami.apps.fao.org/) was used to estimate the carbon footprint related to feeds utilised in selected dairy farms, considering the kg of CO_2 annually produced before and after the introduction of faba bean as diet ingredient, calculated as the ratio between the difference (after – before) and before value.

Both models are based on Life Cycle Assessment (LCA) and Geographic Information System (GIS) methodologies to assess interactions between livestock and the environment, and to support stakeholders in their efforts toward adopting more sustainable practices to mitigate the environmental impact linked to livestock productions (FAO, 2022).

The main common steps behind the use of GLEAM-i, and partially of Latte Sostenibile software, includes the selection of geographical region, production systems for different animal species (bovine, buffalo, sheep, goat, poultry, swine), identification of case studies, input of the main characteristics of herd (animal numbers, live weights, mortality rate, fertility, and production), feed (dietary ingredients), and manure management. The results show details about the emissions intensity.

Starting from the data previously described, small, medium, and large farms were studied simulating the effects of the introduction of alternative protein source (faba bean) in cow diets.

Diet composition

According to dairy cows' physiological state, animal nutritional requirements, and farm management systems, diets were simulated respectively for small (Table 1.2), medium (Table 1.3), and large (Table 1.4) farm.

Table 1.2 – Diet simulated for dairy cattle (n=5) raised in the small farm.

	Diet
Ingredients, %	
Hay (*Lolium perenne, Hedysarum coronarium, Trifolium* spp.)	61.51
Commercial mixed feed*	10.25
Corn meal	9.23
Barley meal	8.20
Soybean meal (44%, CP)	4.66
Whole oat	2.82
Durum wheat bran	2.82
Buffer[§]	0.51
Chemical composition (g/kg DM) **and energy content** (UFL/kg DM)	
CP	155.1
EE	26.8
Ash	77.2
Starch	134.6
NDF	399.0
ADF	240.9
ADL	36.4
UFL	0.81

Legend: DM= dry matter; CP= crude protein; EE= ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; ADL = acid detergent lignin; UFL = Forage unit for lactation (1 UFL = 7.12 MJ NEl/ kg, Sauvant et al., 2004).

* Labelled ingredients: corn meal, dehulled soybean meal mixed feed, grains wheat bran, dehulled sunflower meal, glutinate corn meal, corn distiller, barley meal, sugar cane molasses, calcium carbonate, calcium salts from fatty acids, sodium chloride, sodium bicarbonate, monocalcium phosphate. Composition of compound feed (percentage, as fed): Protein: 22.0, Fat: 5.20, Crude Fibre: 7.00, Ash: 8.70, Sodium: 0.50.

[§] Labelled ingredients: sodium bicarbonate, wheat bran, calcium carbonate, magnesium, common wheat. Composition of compound feed (percentage, as fed): Magnesium: 9.5, Sodium: 9.0, Calcium: 8.7.

Table 1.3 - Diet simulated for dairy cattle (n=8) raised in the medium farm.

	Diet
Ingredients, %	
Hay (*Lolium perenne*, *Trifolium* spp.)	58.78
Corn meal	11.76
Barley meal	11.76
Commercial mixed feed*	5.88
Durum wheat bran	5.88
Commercial mixed feed**	5.10
Mineral feed§§	0.47
Buffer§	0.37
Chemical composition (g/kg DM) **and energy content** (UFL/kg DM)	
CP	146.0
EE	26.6
Ash	69.6
Starch	166.5
NDF	373.2
ADF	228.6
ADL	30.3
UFL	0.83

Legend: DM= dry matter; CP= crude protein; EE= ether extract; NDF = neutral3 detergent fiber; ADF = acid detergent fiber; ADL = acid detergent lignin; UFL = Forage unit for lactation (1 UFL = 7.12 MJ NEl/ kg, Sauvant et al., 2004).

* Labelled ingredients: corn meal, wheat bran, wheat middling, sunflower meal, barley meal, dry corn grains, calcium carbonate, sugar cane molasses, sodium bicarbonate, sodium chloride, hydrogenated fats, soybean oil, dicalcium phosphate. Composition of compound feed (percentage, as fed): Protein: 15.0, Fat: 5.50, Crude Fibre: 7.50, Ash: 7.50, Sodium: 0.50.

** Labelled ingredients: dehulled soybean meal, dehulled sunflower meal, glutinate corn meal, wheat bran, wheat middling, molasses, sodium bicarbonate, hydrogenate palm fatty acids, calcium carbonate, barley, sugar cane molasses, sodium chloride, dicalcium phosphate, magnesium oxide. Protein: 34.0, Fat: 4.00, Crude Fibre: 8.69, Ash: 10.59, Sodium: 1.00, Magnesium: 0.55.

§ Labelled ingredients: calcium and magnesium carbonate, mono-dicalcium phosphate, magnesium phosphate, sodium bicarbonate, barley meal, alfalfa protein concentrate, dried *Saccharomyces cerevisiae*. Composition of compound feed (percentage, as fed): Calcium: 17.0, Phosphorus: 3.2, Sodium: 7.5, Magnesium: 7.5.

§§ Labelled ingredients: sodium bicarbonate, wheat bran, calcium carbonate, magnesium, common wheat. Composition of compound feed (percentage, as fed): Magnesium: 9.5, Sodium: 9.0, Calcium: 8.7.

Table 1.4 - Diet simulated for dairy cattle (n=10) raised in the large farm.

	Diet
Ingredients, %	
Hay (*Lolium perenne*, *Trifolium* spp., *Avena* spp.)	55.13
Commercial mixed feed*	22.05
Barley meal	12.86
Faba bean	9.19
Mineral feed§§	0.40
Buffer§	0.37
Chemical composition (g/kg DM) **and energy content** (UFL/kg DM)	
CP	175.4
EE	35.0
Ash	76.5
Starch	109.6
NDF	340.5
ADF	211.6
ADL	25.6
UFL	0.84

Legend: DM= dry matter; CP= crude protein; EE= ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; ADL = acid detergent lignin; UFL = Forage unit for lactation (1 UFL = 7.12 MJ NEl/ kg, Sauvant et al., 2004).

* Labelled ingredients: dehulled soybean meal, corn, linseed extruded, wheat middling, soybean, pea bean, corn distillers, calcium carbonate, palm hydrogenated fatty acid, sodium chloride, dicalcium phosphate. Composition of compound feed (percentage, as fed): Protein: 23.8, Fat: 8.40, Crude Fibre: 4.20, Ash: 8.20, Sodium: 0.37.

§ Labelled ingredients: calcium and magnesium carbonate, mono-dicalcium phosphate, magnesium phosphate, sodium bicarbonate, barley meal, alfalfa protein concentrate, dried *Saccharomyces cerevisiae*. Composition of compound feed (percentage, as fed): Calcium: 17.0, Phosphorus: 3.2, Sodium: 7.5, Magnesium: 7.5.

§§ Labelled ingredients: sodium bicarbonate, calcium carbonate, sodium chloride, magnesium oxide, monocalcium phosphate, barley meal, sulphurous. Composition of compound feed (percentage, as fed): Calcium: 13.5, Phosphorus: 1.00, Sodium: 13.5, Magnesium: 5.2.

As possible innovation, the partial substitution, isoenergetic and isonitrogenous, of corn grain (0.3 kg/capo * d) and soybean meal (0.3 kg/capo * d) with faba bean (0.6 kg/capo * d) in the diets has been considered from an environmental point of view.

Environmental impact assessment

Following the main guidelines of Latte Sostenibile and GLEAM-i software, to estimate the potential annual environmental impacts of livestock farms, details on data entered for herd, feed, and manure modules, are summarised in Table 1.5.

Table 1.5 - Gleam-i module parameters described for small, medium, and large farm.

Parameters	Units	Small farm	Medium farm	Large farm
Herd module				
Number of adult females	#	10	14	23
Number of adult males	#	1	2	5
Milk yield	kg	4648.5	4132.0	8057.4
Milk protein content	%	4.0	3.3	3.3
Milk fat content	%	4.3	4.0	4.1
Age at first parturition	months	27	26	24
Weight at birth	kg	80	80	60
Live weight adult females	kg	600	700	650
Live weight adult males	kg	600	700	650
Replacement rate of adult females	%	31	31	40
Fertility rate	%	70	70	70
Death rate young female	%	1	0	0
Death rate young males	%	3	0	0.1
Death rate adult animals	%	1	0	0.1
Feed module *	%			
Manure module				
Solid storage	%	100	100	100

* Please, see data reported in Tables 1.2, 1.3, 1.4, respectively for small, medium, and large farm.

Furthermore, kg CO_2 related to feed production and utilization was calculated assuming that corn grain and soybean meal were partially replaced by faba bean as above mentioned.

1.1.1.3 Economic and Social features

From an economic standpoint, the geographical area under consideration has been characterized using data available in public data bank.

Social features represent important factors that define the initial geographical area under analysis. As already emphasized, the notion of a food district assumed in this paper basically adopts an institutional perspective, which concerns the actions and processes oriented to formally recognize areas that prove to meet specific requisites, related to organic farming and agroecological capacities. This perspective assumes a development model that emphasizes the participation of the local community in the processes constituting district. In this context, the existence of local communities predisposed to cooperation and innovation in order to create an innovative food district plays a crucial role in the preliminary evaluation for territorial planning. Indeed, while the geographical area under consideration in the analysis could be potentially undefined from a technical standpoint, its initial extension (i.e., the number of municipalities included in the analysis) depends crucially on the expression of interest from the relevant stakeholders to constitute the food district since this preliminary requisite that basically has to be met before a technical evaluation of the characteristics of the territories involved can be performed.

1.1.2 Results and discussion

1.1.2.1 Agri-food in the Alto Sannio area

The area under analysis (Sannio) is characterized by small-sized firms with high level of mechanization and automation but poor investment capacity in innovation and especially in biotechnology in favor of eco-compatible characters (Figure 1.3).

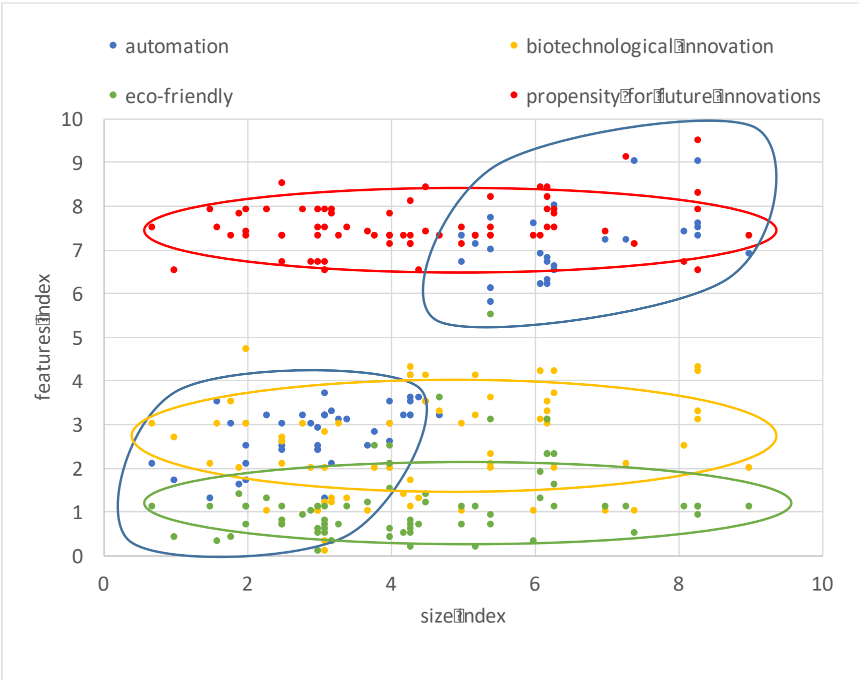

Figure 1.3 - Principal features (automation of process, biotechnology innovation, eco-friendly approaches, and future innovation propensity) of agricultural firms respect to their size. A relationship can be found between the size and the automation. While the firms highlight low (in innovation and in eco-friendly index) or high (in propensity for future innovation) values regardless of their size.

Despite these limitations, farms involved in questionnaire showed a medium-high level of specialization (Figure 1.4). The territorial area is highly specialized in the zootechnical production of the White Vitellone of the Central Apennines (PGI), as showed in Figure 1.5. There are, 2,321 pivot farms and more than 46,200 cattle. The results reveal a local production system characterized by the presence of farms organized in one or more certified and recognized agri-food chains in accordance with current legislation. In particular, there is the livestock sector for meat, in which the White Vitellone of the Central Apennines PGI stands out (Reg. CE n.510/06) and the complementary chains of dairy farming, cereal farming and the new experimental chains.

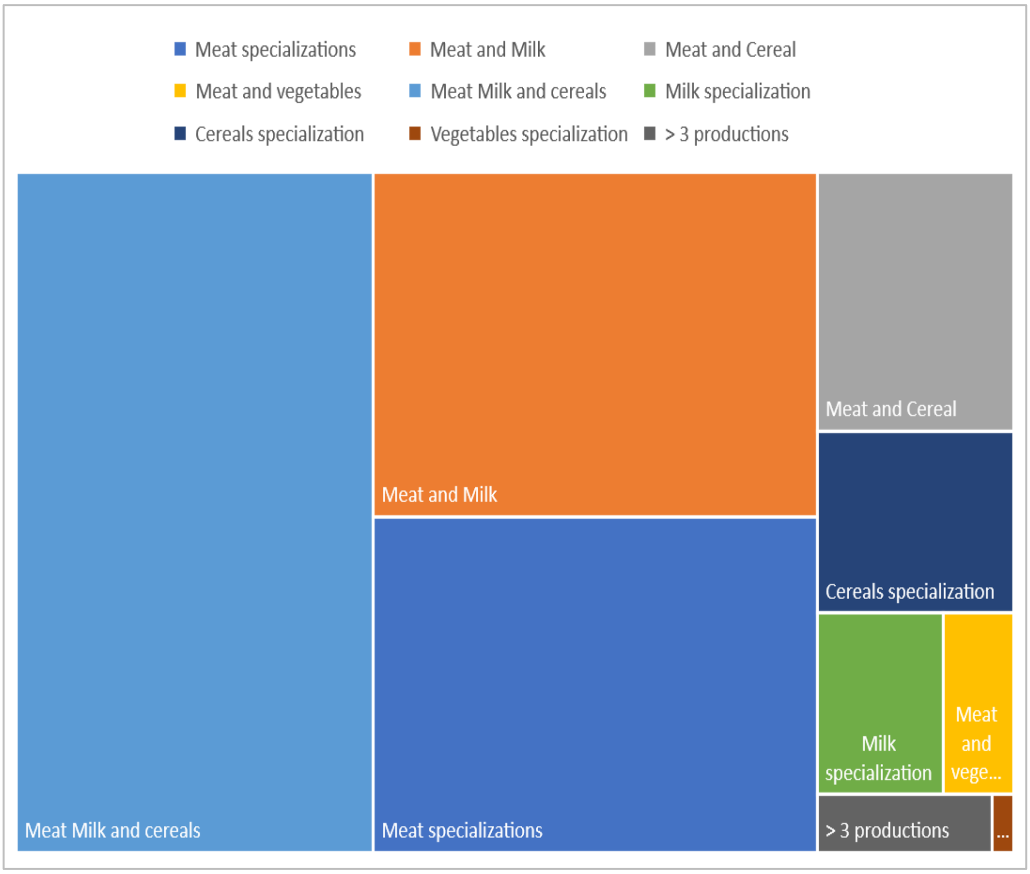

Figure 1.4 - Specialization level of the total interviewed farmers.

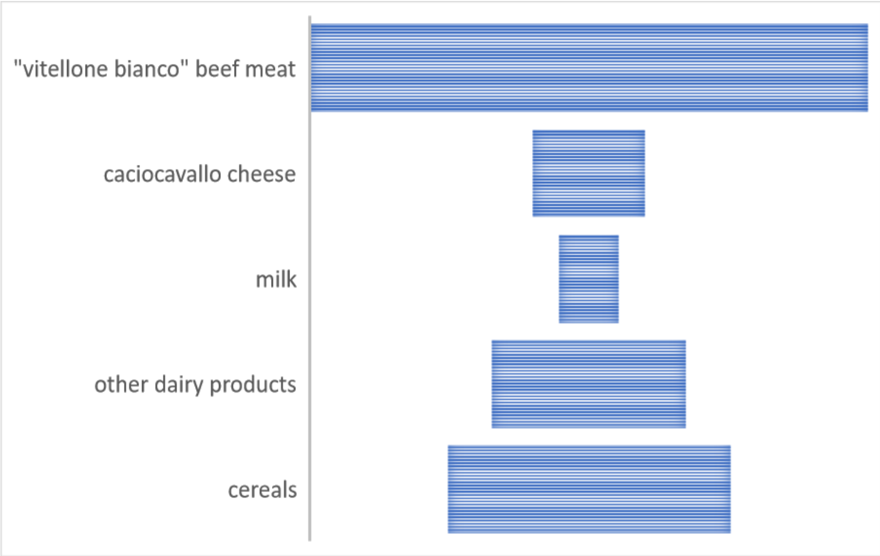

Figure 1.5 - Principal food production obtained from zootechnical supply chain in Sannio area.

Despite the specialization for the meat production, an important quota of cattle of the Marchigiana is destined for the comeback to obtain the mixed Marchigiana-spotted red breed, widely used to obtain high quality milk, 60% of which is placed in the dairy market and used for the well-known "caciocavallo" cheese. Just the “Castelfranco in Miscano Caciocavallo cheese” represents one of the dairy excellences of Sannio area. The zootechnical supply chain of Sannio, correlated to the cereal one, has the constant objective of increasing the added value of the cattle breeds in the territory, thanks also to the enhancement and promotion work carried out by the Consortium for the Protection of the PGI "Vitellone Bianco dell'Appennino Centrale". In this field, since the feeding system is fundamental for achieving the required quality standards, a high level of attention is reserved for cereals, partly intended for animal feed, for the management of fodder and pastures of the area; activities that find a clear link with the protection of the environment, the rural landscape and the protection of the territory. Moreover, in a complementarity view with animal husbandry (Figure 1.6), unexpected supply chains are developing especially in the upper Fortore area, such as that of the "dry" cherry tomatoes of San Bartolomeo in Galdo or that of legumes.

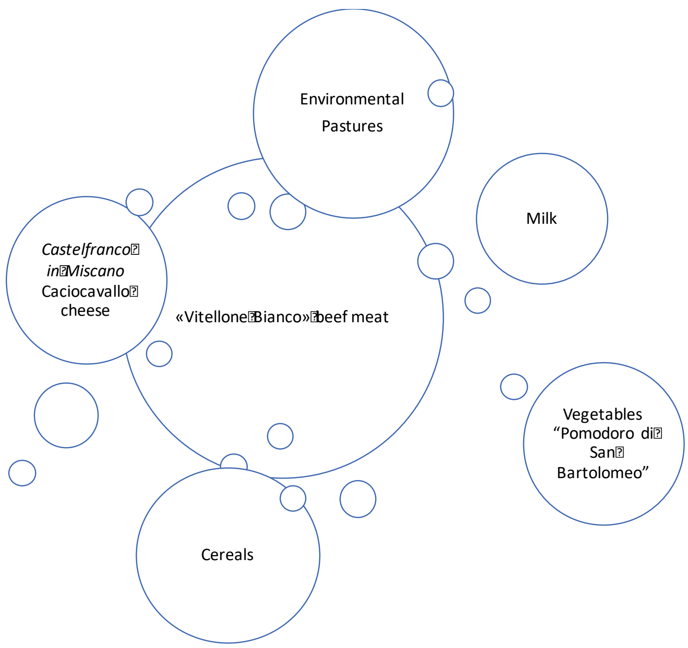

Figure 1.6 - Interactions between Vitellone dell'Appennino Centrale and complementary supply chains.

Therefore, the Sannio area has a strong concentration in certified agri-food productions consistent with the traditions and characteristics of the territory such as the Vitellone dell'Appennino Centrale PGI (Reg. CE n.510/06), with a specialization index above the regional average. This supply chain, which can be considered a driving force, has also developed together with other complementary production chains, with relations of integration and interdependence between agricultural companies and companies in the agri-food processing and distribution sector of certified products. The main complementary supply chains are dairy farming with the production of Caciocavallo (Registered under the EC Reg. No. 1263/96 and the subsequent EC Reg. Cappelli), and cereal cultivation of Saragolla and Marzellina, varieties of durum wheat recognized as "Traditional vegetable products" of the Campania Region.

1.1.2.2 Alto Sannio dairy chain

Following the information collected from the questionnaires, the three selected farms showed similar aspects as the localisation in hilly area, the intensive farming system family managed, and the exclusively vocation to milk production and to grains and legume cultivation. However, some differences were found about total agricultural surface, total number of animals raised, herd management, average milk production and hygienic aspects (Table 1.6).

Table 1.6 – Summary of information collected from the three selected farms: small, medium, and large.

Main data and information requested	***Small farm***	***Medium farm***	***Large farm***
1. *Farm data*	* - Hilly area at about 500 m a.s.l. - 23 ha irrigable area, crop rotation with grains and legume in organic production - Intensive livestock system managed by family.	* - Hilly area at about 680 m a.s.l. - No irrigable area, crop rotation with grains and legume - Intensive livestock system managed by family.	* - Hilly area at about 850 m a.s.l. - No irrigable area, crop rotation with grains and legume - Intensive livestock system managed by family.
2. *Total agricultural area (TAA) and utilised agricultural area (UAA)*	- 23 ha of TAA, entirely used for feed production.	- 21 ha of TAA for rent, entirely used for feed production.	- 45 ha of TAA, entirely used for feed production - 20 ha of property.
3. *Productive use of UAA*	- Annually total grains (3.9 t * ha), forage (3 t * ha) were re-used as feed - Pasture and silage were not present - Arboreal crop (olive).	- Annually total grains (4.5 t * ha), forage (13 t * ha) were re-used as feed - Pasture and silage were not present - 0.5 ha of arboreal crop (grapevine).	- Annually total grains (4.2 t * ha), total legume (6.0 t * ha) and 75% of forage (8.8 t * ha) were re-used as feed - Pasture, silage, and arboreal crops were not present.
4. *Other information*	- None.	- None.	None.
5. *Structural farm characteristics*	- Tie stall, built in 2000 - Barn, storeroom, silos, manure heap and milk room.	- Tie stall, built in 1998 - 2 Barns, 2 storeroom, manure heap and milk room.	- Tie stall, built in 1980 - Barn, storeroom, 3 silos, manure heap and milk room.
6. *Description of fam characteristics*	- Partial outdoor access - No cheese making or farm butcher shop.	- No outdoor access - No cheese making or farm butcher shop.	- No outdoor access - No cheese making or farm butcher shop.

7. *Farm automation and technology innovation level*	- Tractors and machinery for foraging - Mechanical milking in stall - No photovoltaic, recovery of heat or wastewater, or ventilation systems.	- Tractors, and machinery for forage production - Mechanical milking in stall - No photovoltaic, recovery of heat, or ventilation systems. - Wastewater recovery system.	- Tractors, and machinery for forage and grain production - Mechanical milking in stall - No photovoltaic, recovery of heat or wastewater, or ventilation systems.
8. *Hygienic and sanitary standards*	- None.	- None.	- None.
9. *Herd characteristic and composition*	- Total of 39 Pezzata rossa breed bovines - 6 males from 0 to 24 months - 19 females from 0 to 24 months - 10 dairy cows - 4 dry cows.	- Total of 50 Pezzata rossa crossbred bovines - 10 males from 0 to 24 months - 21 females from 0 to 24 months - 14 dairy cows - 5 dry cows.	- Total of 70 Italian Friesian breed - 20 males from 0 to 24 months - 20 females from 0 to 24 months - 23 dairy cows - 7 dry cows.
10. *Other herd management data*	- 70 % or fertility rate - 1 calve per year at 27 months - lactation 180 d - 25 L of milk/cow * d - 4.0 % protein - 4.3 % fat - 70 - 80 kg of birth weight - Artificial insemination - 70 d weaning period - 1% mortality rate.	- 70 % or fertility rate - 1 calve per year at 26 months - lactation 200 d - 15-20 L of milk/cow * d - 3.3% protein - 4.0 % fat - 80 kg of birth weight - Artificial insemination - 90 d weaning period - 0% mortality rate.	- 60 % or fertility rate - 1 calve per year at 24 months - lactation between 240 and 260 d - 30 L of milk/cow * d - 3.3% protein - 4.1 % fat - 60 kg of birth weight - Artificial insemination - 100 d weaning period

			- 0% mortality rate.
11. Feed used	- Four different diets formulated by nutritionist for different physiological stages - forage *ad libitum* - use of soybean meal - use of mineral supplements.	- Four different diets formulated by nutritionist for different physiological stages - ratio forage: concentrate = 75:25 - use of soybean meal - use of mineral and vitamins supplements.	- Four different diets formulated by nutritionist for different physiological stages - ratio forage: concentrate = 50:50 - use of soybean meal and faba bean - use of mineral and vitamins supplements - pasta co-products.
12. Description of diets administered	**	**	**
13. Possible pathology	- Lameness problems.	- None.	- Fertility problems.
14. Milk quality	- 78 000 CFU of microbial load.	- 30 000 CFU of microbial load.	- High microbial load and somatic cells.
15. Milk uses	- Milk for regional cheese industry.	- Milk for extra-regional cheese industry.	- Milk for regional cheese industry.
16. Other farm species	- None.	- Sheep, swine, and poultry.	- None.

Legend: * Personal data about name, address and contact details were not reported due to privacy reasons; ** diets description will be detailed below.

Campania is particularly suited to animal husbandry and livestock farms are present on large part of the regional territory (Inform, 2020). However, Campania livestock sector has several peculiar characteristics within the regional territory in terms of agricultural vocations within the different municipalities and, consequently, in terms of land use.

According to Carillo et al (2005), Campania livestock farms are characterized by similar traits as low degree of productive specialization with a prevalence of mixed production orientation, general farm and agricultural areas' fragmentation, more small farms than large ones when number of reared animals and utilized agricultural area are considered. In other words, farms are differentiated in terms of size and production organization, even within the same productive area, i.e., there are more specialized production techniques in milk than in meat sector, partially justified by the weight of milk supply chain in regional economy, also considering the use of raw milk for several dairy products, many of which recognised Protected Designation of Origin or Protected Geographical Indication products.

1.1.2.3 Context analysis of Italian livestock sector

Italian livestock sector accounted over 16 billion of euro, representing more than a third of total agricultural production. Among the primary products, milk represents more than 30% of livestock production, while meat accounts for more than 60% (Macrì, 2017), and eggs and honey account only for 8%.

Large part of agricultural and livestock production comes from intensive systems localized in flat areas of northern Italy, i.e., Piemonte, Lombardia, Veneto, and Emilia Romagna regions, characterized by more favourable contexts in terms of logistic and economy (Macrì, 2017). However, equally valuable are the extensive livestock farms more widespread mainly in mountainous and marginal areas of Alps and Apennines localised in central and southern part of Italy, which contribute to the valorisation of local products as well as performing soil, water, and environment protection and preservation functions.

In recent decades, a gradual decrease has been observed in numbers of both farms (-13% from 2010 to 2013) and raised animals (- 6% from 2000 to 2010) (Macrì, 2017; CREA, 2021). Nevertheless, there has been a gradual expansion of active livestock farms, which have increased both the average number of animals and the available cultivated area. In other words, small farms are gradually disappearing on behalf of few but large farms. Consequently, also data from the 7th Italian Agricultural Census in 2020 confirm the decline in the number of farms, from 1.6 million in 2010 to 1.13 million in 2020, and the progressive increase of farm size in terms of utilised agricultural area (UAA, defined according to Eurostat (2023) as total area taken up by arable land, permanent grassland, permanent crops and kitchen gardens used by the holding, regardless of the type of tenure or of whether it is used as a part of common land), which is varied from 5 hectares per farm in 1982 to 11 hectares per farm in 2020. Anyway, in more than 60% of farms UAA is less than 5 hectares and, among them, in more than 20% UAA is less than one hectare (CREA, 2022).

In Italy there are more than 246,000 livestock farms, which represent at about 28% of total agricultural activities investigated by the Census (CREA, 2022). When the adult bovine unit

(ABU, i.e., one ABU is equivalent to one cattle, one horse/donkey, or 6 sheep/goats) is considered, there are more than 9 million of ABU in our country, and once again the northern regions confirm their livestock vocation, in fact Lombardia, Veneto, Emilia Romagna and Piemonte regions detain more than 60% of national livestock population (Figure 1.7).
After the slowdowns in livestock sector growth, occurred during 2019 – 2020 period because of the COVID-19 pandemic, a positive change affecting both meat (+6.4% cattle, +10% pig, +6.3% goat and sheep, +9.7% poultry) and dairy (+2.5%) production has been reported (CREA, 2022).
According to CREA report (2022), focusing on the Italian agri-food sector, our country holds the European record for the number of food products labelled as Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) for a total of 316 products, plus 4 products designed as Traditional Specialty Guaranteed (TSG). On this regard, it must be considered that traditional agri-food products are expression of Italian cultural heritage, whose method of processing, preservation and seasoning are preserved over time.

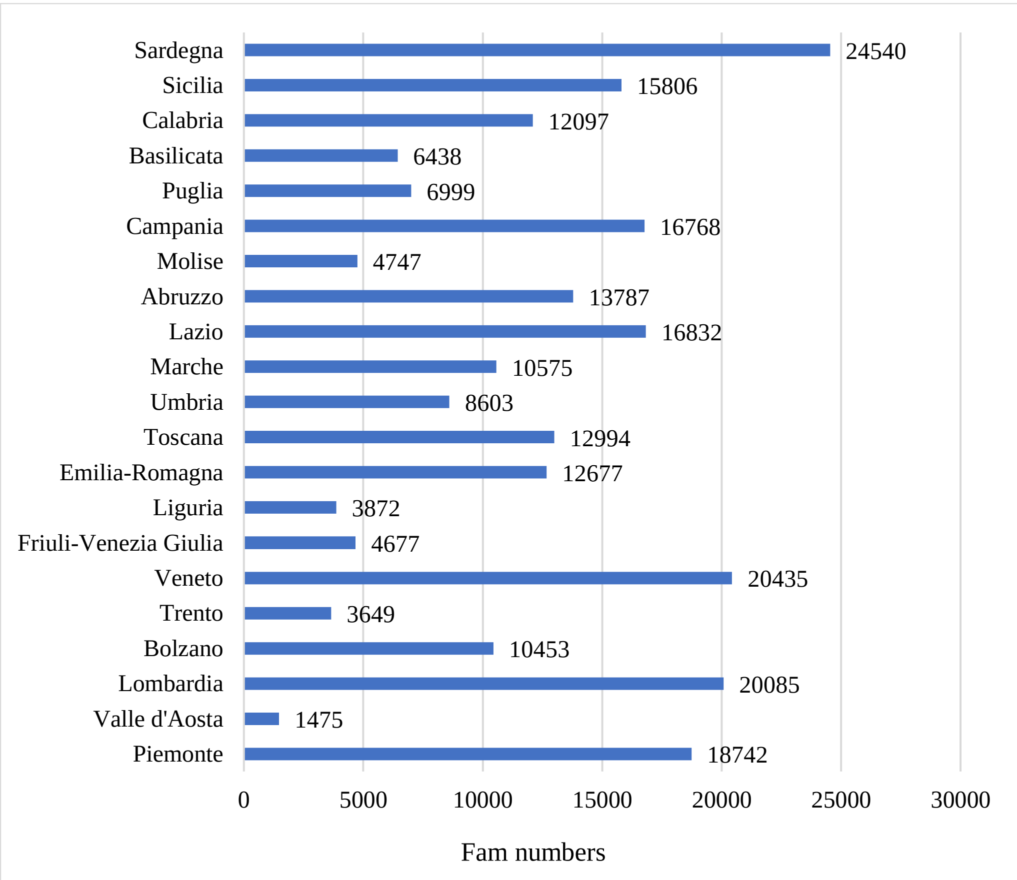

Figure 1.7 – Regional livestock farms during 2019-2020 period. Data from CREA, 2022.

1.1.2.4 Livestock supply chain and agri-food district in Campania region

Campania region, localised in southern part of Italy, is characterized by heterogeneous physical traits. About half of the total areas consists of hill, while about 35% consists of mountain and only about 15% is occupied by flat areas, where the highest density of population is registered. Moreover, Campania is the third most populated region in Italy, following Lombardia and Lazio (CREA, 2021).

When the territory of Campania region is analysed from an agricultural point of view, the UAA accounts for 40.3% of total regional area, which is slightly lower than the national average, but significantly lower than of Southern-Italy values (Table 1.7).

Table 1.7 - Consistency of Italian agricultural territory. Data from CREA, 2021.

	UAA, 000 ha	**TAA**, 000 ha	**UAA/TAA, %**
Campania region	551.1	1367.1	40.3
Southern regions	6329.3	12373.0	51.2
Italy	12777.0	30206.8	42.3

Legend: UAA= utilised agricultural area; TAA= total agricultural area.

Agricultural economy in Campania region is consistent with the national and southern trend (Table 1.8): the main activity deals with cultivation of perennial and permanent crops and animal breeding (CREA, 2021). Although livestock farming is widespread throughout the entire regional area, some specializations exist as related to territorial specificities (altitude, pedological aspects, temperature, humidity, precipitation). For instance, cattle farming is characterized by a uniform distribution in all the provinces, while buffalo farming is primarily concentrated in Caserta and Salerno provinces. Instead, extensive or semi-extensive farming system are adopted in sheep and goat farms, mainly located in marginal areas where pastures represent their main food source for large part of the year (Regione Campania, 2022 a).

Consequently, more than 90% of farms in Campania are in the hill or mountain areas (Inform, 2020) and they are characterized by peculiar territorial potentialities which could contribute to improve the quality standards of the animal source foods. Besides preserving rural culture and traditions, from an environmental point of view it has to be considered that livestock farming often represents the economic activity more remunerative able to ensure the land protection and preservation, avoiding the marginalisation of the land, i.e., degradation and abandonment phenomena (Leroy et al, 2022).

Table 1.8 - Comparison between Campania, southern regions, and Italian raised animals in 2020. Data from CREA, 2021.

	Campania	**Southern regions**	**Italy**	**Campania:southern regions**, %	**Campania:Italy**, %
Bovine	164 191	1 279 840	5 632 978	12.8	2.91
Buffalo	29 623	316 998	412 889	9.34	7.17
Ovine	17 796	4 770 227	6 346 310	0.37	0.28
Caprine	51 744	700 784	1 061 373	7.38	4.88
Swine	90 505	612 474	8 846 231	14.8	1.02
Poultry	2 518 599	20 995 466	137 492 323	12.0	1.83

Moreover, observing in detail the characteristics of Campania farms reported in Inform report (2020), is possible to highlight some points of strengths, weakness, opportunities, and threats as reported in Table 1.9.

Table 1.9 - Points of strengths, weakness, opportunities, and threats of livestock sector in Campania.

Strength points	**Weakness points**
- Presence of typical production as PDO, PGI and TSG. - Presence of farms which use farm products in animal feed. - Increased structural investments and efficient marketing strategies. - Traceability system of products and loyalization of customers.	- Reduced average size of farms as an obstacle to the development of production and to the improvement of farmers' incomes. - Prevalence of small businesses. - High average age of holders of farms and difficult vertical integration capacity.
Opportunities	**Threats**
- Large demand for products with high standards of quality, hygiene, and safety.	- Support livestock income especially in marginal areas, promoting competitiveness, sustainability, and animal welfare. - Enhance the role of the certification system to protect the quality and specificity of products in the national and international markets

Legend: PDO= protected designation of origin; PGI= protected geographical indication; TSG= traditional speciality guaranteed.

1.1.2.5 Critical issues and strengths of the livestock supply chain in Campania region and Alto Sannio area

Dairy supply chain

Dairy supply chain plays an important role in relation to the correlation between economic and social sectors of the same territory. Nevertheless, in Campania, as in general in central and southern part of Italy, the limited size of farms has a significant impact on the real potentialities of dairy sector, partly because of the limited dynamism of economic operators. In fact, the fragmentation of the farm production system is reflected in the fragmentation of companies that process and sale milk and derivatives.

As far as the dairy sector is concerned, in Campania there are many family-run dairies which are increasingly linked to farms, creating an alternative commercial context to the traditional one (represented by the large-scale distribution), thanks to the increase of on-farm shops or the expansion of agritourism and accommodation facilities. However, is important to mention the presence of typical and recognized productions including the PDOs Mozzarella di Bufala Campana, Caciocavallo Silano, and Provolone del Monaco (Regione Campania, 2022 b), that play an important role in strengthening these niche productions in a competitive context that turns to globalization, while boosting the linkage with the territory in social and cultural terms. Generally, due to environmental and structural limits, Campania farms have higher production costs compared to farms in northern Italy or Europe, particularly when the feeding costs are considered. As far as the competitiveness of farms is concerned, also the consumption trends should be taken into account. For instance, during last decade, consumers expressed their interest in healthy and safe diet also from an environmental point of view, besides the common motivations of their choice, such as taste, tradition, and culture. It is therefore necessary to understand consumer desires and at the same time use the innovation to create new markets (Regione Campania, 2022 b). Moreover, also a close synergy between primary producers, stakeholders, policy makers, and research institutions is necessary to protect and improve the modern dairy sector.

Meat supply chain

Meat supply chain has a strong impact in Campania economy, especially related to the cattle sector, both in terms of production and of employment capacity due to the close interdependence among feed and dairy industry, slaughtering and meat processing sectors (Regione Campania, 2022c). Despite this, in last decades meat consumption varied, reflecting changes in habits of both food consumption and lifestyles. In fact, an increasing attention to quality and safety of products is observed, so that for instance sensitivity to health and cultural aspects prevails over the satisfaction of nutritional needs of consumers, resulting in a generalised decline of meat consumption. Consequently, current trends seem to drive the demand of meat toward high quality fresh products, including organic and certified products.

Among the excellences of meat supply chain in Campania region, the case of "Vitellone bianco dell'Appennino Centrale", as Protected Geographical Indication, should be reported. This label refers to meat from Chianina, Marchigiana and Romagnola breeds cattle (male and female), aged between 12 and 24 months, and with significant common morphological characteristics as black pigmentation of skin, tongue and palate, somatic structure, and particular coat named "fromentino" (light blonde) for the first three months after birth. In addition, these animals are characterized by the same growth characteristics, carcass yield (about 64%), high meat quality with low cholesterol content. Moreover, "Vitellone bianco dell'Appennino centrale" PGI is the only denomination given to raw meat in Italy, and cattle intended for PGI meat production, identified and registered in herd books, are raised and fed according to the standard protocols reported in the disciplinary regulation (Regione Campania, 2022 c).

Diet composition

Observing the diets detailed respectively in Tables 1.2, 1.3 and 1.4, it should be noted that dietary crude protein (CP), ether extract (EE), and energy (UFL) contents varied from 15.5% CP, 26.8% EE, 0.81 UFL, to 14.6% CP, 26.6% EE, 0.83 UFL, and 17.5% CP, 35.0%, 0.84 UFL, respectively for small, medium, and large farm. So that, although crude protein and energy contents were similar in all diets, diet of large farm is characterized by a higher fats content. All farms use self-produced forages, characterized by local herbaceous essences. Small farm is the only one that use soybean meal as raw material, while in both medium and large farm soybean meal is included as ingredient of commercial feeds used.

Generally, protein in feedstuffs is expressed as crude protein (CP), which represents the percentage of nitrogen detectable in feed, multiplied by 6.25 (Erikson and Kalscheur, 2020). Moreover, crude protein includes true proteins together with other nitrogenous compounds, i.e., amino acids, peptides, nucleic acid, ammoniacal compounds and nonprotein compounds. According to Erikson and Kalscheur (2020), cattle can use amino acids for enzymes, milk proteins, immunoglobulins, and tissues production. However, an excess of dietary nitrogen could negatively affect the amount of nitrogen introduced in the environment, so that a balanced nitrogen administration is required in the diet to maximize the nitrogen utilized by the animals while reducing the amount of nitrogen lost in the environment.

On the other hands, it is important to focalize the attention about the fat supplementation level in ruminant diets. Although the improve of dietary energy density is the main reason to increase fats in diets, this could also improve milk fat percentage, without loss of milk yield (Palmquist and Jenkins, 2017; Plata-Peréz et al, 2022). Moreover, milk fatty acid profile could be affected by the fat diets but also depends on interaction of several factors, i.e., diet composition, feed intake, ruminal fermentation, lipid metabolisms (Plata-Peréz et al, 2022). However, studies on fat digestibility showed that added more than 5% of oils in ruminant diet could decrease the fibre digestion and the rumen activities (Lucas and Loosli, 1944; Palmquist and Jenkins, 2017).

Environmental impact assessment

Natural resources, represents the dietary basis of ruminant livestock. Diet composition varies due to localization, season and animal feeding preferences, and production system (Archimède et al, 2011; Manceron et al, 2014). However, a diet properly formulated for dairy cow should consider physical characteristics of feeds and interactions of nutrients including water, carbohydrates, proteins, fats, mineral, and vitamins (Erikson and Kalscheur, 2020).

Data about GHG emissions simulated with the above-mentioned application on a year basis for the selected farms were reported in Table 1.10.

Table 1.10 - GHG emissions reported for the selected farms.

Environmental parameters	**Small farm**	**Medium farm**	**Large farm**
tons CO_2-eq/tons of milk	0.32	0.31	0.15
tons enteric CH_4/year	2.70	3.10	5.20
tons CO_2-eq from CH_4/year	69.0	79.0	130
tons CO_2-eq from CH_4/tons of milk	0.16	0.13	0.07

Observing the obtained results, the large farm has been found less impactful in terms of tons CO_2-eq/tons of milk, compared to small and medium farms, due to the more productive efficiency. On this regard, it should be noted that the large farm is characterized by the higher annual average milk production. However, if the absolute values are considered, the total CH_4 produced by small and medium farms is halved compared to the large farm (Table 1.10).

Given these environmental impacts of the selected farms, the partial replacement of soybean meal and corn grain with faba bean can contribute to the reduction of the GHG emissions from feed production, accounting for -40%. However, the simulated impacts of the dietary substitution with faba bean represents 10% of CO_2 emission, while it increases the CH_4 and N_2O emissions, +32.5% and + 80.6% respectively. It has to be considered that the presence of antinutritional factors in faba bean, as protease inhibitors, lectins, phenolic compounds and saponins (Diaz et al, 2006), could affect feed intake, ruminal fermentation, nutrient digestion, and performances of dairy cows (Cherif et al, 2018). However, the inclusion of faba bean in dairy cow diets can increase the starch supply, shifting the ruminal fermentation toward more propionate production and less methane production, due to the inverse relationship between propionate formation and methane synthesis in the rumen (Cherif et al, 2018).

Daily several products are made by dairy sector from the same raw materials, through different manufactured production lines, having a great environmental impact (Palmieri et al, 2017).

Total Italian GHG emissions expressed in CO_2 equivalent, from 1990 to 2018, have decreased about 17%, excluding the emission related to the land use, and land-use change and forestry (LULUCF) (ISPRA, 2020). Based on data reported by ISPRA (2020), in the same period, a decrease trend of the most important GHG gases was observed, i.e., -20.5%, -10.8% and -32.0%, respectively for CO_2, CH_4 and N_2O. The total emissions form the different productive sectors in Italy remains unvaried for 1990 – 2018 period, although the greatest part of total GHG emissions is related to the energy sector (80%), followed by industrial process (8%), agriculture sector (7%) and waste management (about 5%) (ISPRA, 2020). More in detail, for the agricultural sector, from 1990 to 2018, GHGs trend showed a reduction due to the decrease of farm activity, according to data presented by Macrì (2017) and CREA (2021) which underline the contraction of farm and animal raised numbers. However, in 2018, the national agriculture sector is responsible respectively of 44.7% of CH_4, 59.4% of N_2O and only 0.1% of CO_2 productions (ISPRA, 2020).

According to the obtained results and the data collected, it should be noted that even in the smallest livestock farm several alternative management strategies that can be introduced, i.e., selection of the most appropriate ingredients with high quality level to limit for instance the abnormal ruminal fermentation processes, corrected manure management, could boost the development of livestock sector especially in marginal areas of Centre-South Italy, from economic and environmental protection points of view.

1.1.3 Conclusion: social and environmental features

The system of relations between agricultural enterprises and local enterprises active in other sectors is present but further margins of development still unexplored.

In particular, strategies in order to the develop of a system that pushes to the aggregation and to the overcoming entrepreneurial dwarfism encoming economies of scale and scope are needed. Moreover, the communities show a high predisposition to cooperation and to create a district. This aspect plays a crucial role in the preliminary evaluation for territorial planning, as well as in the definition of the scope of the food district. Indeed, while the geographical area under consideration in the analysis could be potentially undefined from a technical standpoint, its initial extension (i.e., the number of municipalities included in the analysis) depends crucially on the expression of interest from the relevant stakeholders to constitute the district since this preliminary requisite that basically has to be met before a technical evaluation of the characteristics of the territories involved can be performed. In short, the initial definition of the extension of the geographical area under analysis depends on the preliminary willingness of the local communities to cooperate with each other to constitute an ecoregion, which is also a fundamental requisite for the effective functioning of the ecoregion over time. This requisite seems to be by and large met in the case of the Sannio Apennines area. As regard the evaluation of environmental features, the results show the presence of 68 municipalities with a predominantly rural character and homogeneous morphological characteristics in the territory. In this area, most of the municipalities are also recognized as "internal areas", significantly distant from centres offering essential services (high education, health, and mobility) but rich in important environmental and cultural resources and highly diversified in their nature and secular enthronization processes. These features intuitively suggest that the municipalities within the geographical area under analysis are characterized by similar environmental opportunities for the growth of quality certified farming and other economic activities that could be well developed through the establishment of a district with the aim of realizing agreements for the sustainable management of local resources.

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Chapter 2

Consideration for a New One Health Financial Model

The dissertation of the PhD thesis before entering the heart of the experimental activity aimed to reflect and discuss a new financial model capable of ensuring One Health and ecological transition in cattle breeding. Economic health is here considered as the starting point of the new One Health financial model, ethical and scientific, proposed as a model in embryo, in which specific rules should govern public funds, private investments and trade. In this way, economic losses due to the negative side effects of human activities can be progressively reduced and the entire planet will benefit from this process. Despite the considerable efforts made in the context of the OH approach, war causes tragic and devastating effects on the physical and mental health of human beings, their lives, pandemic and zoonotic threats, animals, plants and, last but not least, the environment. War is incompatible with OH. Huge efforts for peace are therefore urgently needed.

The present activity was conducted in collaboration with my tutor and other researchers from University of Molise. The acquired results found editorial placement in an International Scientific Journal in the form of an original research article entitled "***From the Intersection of Food-Borne Zoonoses and EU Green Policies to an In-Embryo One Health Financial Model***" (Figure 2.1).


foods MDPI

Review

From the Intersection of Food-Borne Zoonoses and EU Green Policies to an In-Embryo One Health Financial Model

Alessandra Mazzeo, Patrizio Tremonte *, Silvia Jane Lombardi, Costantino Caturano, Arianna Correra and Elena Sorrentino

Figure 2.1 - Mazzeo A, Tremonte P, Lombardi SJ, **CATURANO C**, Correra A, Sorrentino E (2022) - From the Intersection of Food-Borne Zoonoses and EU Green Policies to an In-Embryo One Health Financial Model. Foods, 11(18), 2736.

2.1 From the Intersection of Food-Borne Zoonoses and EU Green Policies to an In-Embryo One Health Financial Model

Zoonotic agents are pathogens with an unrestricted host spectrum. In nature, their survival occurs in reservoir animal species, which generally do not present clinical symptoms and, therefore, are difficult to identify. Promiscuity between farmed animals and wildlife increases the risk of transmission of pathogens and their consequent adaptation to new host species, including human beings. Therefore, promiscuity increases the risk of emergence of new zoonoses. According to the World Organisation for Animal Health (OIE), zoonoses represent 60% of human infectious diseases and 75% of the emerging ones; 80% of pathogens of animal origin have strong potential as bioterrorism agents (OIE, 2022a). Deforestation and destruction of natural areas produce promiscuity, pushing wild species to invade new areas and to arrive in anthropic environments. In high-income countries, domesticated animals are as much a potential reservoir of high-risk zoonoses as the wildlife animals in equatorial rainforests or wet markets.

Companion and zoo animals - with limited syndromic monitoring in place - remain an underestimated but potentially high-risk disease reservoir for emerging zoonoses (Bowsher et al, 2021). Through the global commercialisation of food, food-borne zoonoses (FZs) can also reach individuals who have never been in contact with infected animals or their environment.

FZs are transmitted to human beings indirectly, both through food obtained from infected animals - which are contaminated at their origin - and through food previously contaminated in the various steps of production, sale, and domestic use. Once infected, consumers generally become a source of infection for animals and humans, as well as a source of contamination for food and the environment.

Human beings, animals, and the environment constitute a cohesive and inextricable system, in which human and animal health are interdependent and linked to the health of the ecosystem in which they live. Therefore, they must be considered under the framework of One Health (OH) (OIE, 2022a).

Since organic farming contributes to environmental and climate protection, long- term soil fertility, high levels of biodiversity, a safe environment, and high animal welfare standards (European Commission, 2022a), the European Commission (EC) has set a target of at least 25% of the European Union’s (EU’s) agricultural land being under organic farming by 2030 (European Commission, 2022b). To achieve this goal and help organic agriculture reach its maximum potential, the EC proposes an action plan for organic production in the EU (European Commission, 2022b). Then, improvement of human health can be achieved through better environmental conditions and healthier food. The EU's organic logo gives a coherent visual identity to organic products produced in the EU. This makes it easier for consumers to identify EU organic products, and helps farmers to market them (European Commission, 2022c). Thus, OH overlaps the European Green Deal plan and its relaunched Farm to Fork Strategy.

Nevertheless, zoonoses and animal infectious diseases cause decreased breeding yields and reduced income. Consequently, the cost and market price of farm products become uncompetitive with respect to the price of industrial food. In other words, zoonoses cause lower revenues, hindering the growth of organic farming expected in the framework of the EU Green Deal. In such scenarios, zoonosis control becomes a key element to align EU policies aimed at achieving the goal of "ZERO environmental impact" by 2050.

2.1.1 One Health

The OH approach is adopted in world policies by:

- The World Health Organization (WHO, 2022a)
- The Food and Agriculture Organization of the United Nations (FAO) (FAO, 2022a)
- The World Organisation for Animal Health (OIE) (OIE, 2022b)
- The European Union (EU) (OHEJP, 2022)
- The USA, where the Centers for Disease Control and Prevention (CDC-Atlanta, GA) host the National Center for Emerging and Zoonotic Infectious Diseases, which works "to protect people at home and around the world from emerging and zoonotic infections ranging from A to Z - anthrax to Zika - since we are living in an interconnected world where an outbreak of infectious disease is just a plane ride away" (NCEZID, 2022).

Recently, the Global Health Summit, held in Rome in May 2021, stated the need to adopt the OH approach in the *Rome Declaration*, issued at the conclusion of the summit (European Union, 2022). On 12/12/2021, the FAO, OIE, WHO, and the United Nations Environment Programme (UNEP) adopted the following definition: "*One Health is an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals and ecosystems. It recognizes the health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and interdependent. The approach mobilizes multiple sectors, disciplines, and communities at varying levels of society to work together to foster well-being and tackle threats to health and ecosystems, while addressing the collective need for clean water, energy and air, safe and nutritious food, taking action on climate change, and contributing to sustainable development*".

Although health, food, water, the environment, and energy are very broad topics with specific and sectoral concerns, interdisciplinary and cross-sector collaborations could provide more effective tools both to protect us from emerging infectious diseases and antimicrobial resistance, and to promote the biodiversity and integrity of ecosystems.

Furthermore, OH can help to address the full spectrum of disease control - from prevention to detection (a lesson learned during the ongoing COVID-19 pandemic), through preparedness, response, and management - and to improve and promote health.

The OH approach can be applied at the national, community, regional, and global levels, and relies on shared and effective governance, communication, collaboration, and coordination. Using this approach will make it easier for people to better understand the co- benefits, risks, trade-offs, and opportunities to promote equitable and holistic solutions (OIE, 2022b).

Firstly, the One Health High-Level Expert Panel (OHHLEP) advisory board focuses on preparing a policy-relevant scientific assessment of emerging health crises arising from the human–animal–ecosystem interface, on developing a long-term strategic approach to reduce the risk of zoonotic pandemics—with an associated monitoring and early warning framework—and on the synergies necessary to improve and institutionalise the OH approach, as well as in the areas that drive the risk of pandemics (OIE, 2022c).

The WHO, OIE, and FAO work together to control and prevent health risks arising from the human–animal–ecosystem interface. They are developing global strategies and tools to ensure a coherent and harmonised approach worldwide, and to better coordinate national and international human, animal, and environmental health policies. In October 2017, the second Tripartite Strategy document was published, with a particular focus on the following aspects: strengthening national services for human and animal health and food safety; strengthening and modernisation of early warning and surveillance/monitoring systems; prediction, preparedness, and response to emerging, re-emerging, and neglected infectious diseases; encouraging and promoting coordinated research to achieve a common understanding of the highest-priority zoonotic diseases; and challenges posed by food safety that require a multisectoral approach in the context of improving food security (OIE, 2022d).

The One Health Surveillance Codex (OHS Codex) was established to provide a frame- work for the OH community to constantly share practical solutions applicable for stakeholders from different One Health Surveillance sectors. The OHS Codex framework includes four high-level action principles, which support, respectively, collaboration, knowledge exchange, data interoperability, and dissemination, which are summarized as follows. (i) The *collaboration* principle represents the need for positive interaction and communication between actors, and it is considered to be the foundation of OH surveillance; without the willingness and ability to collaborate and communicate, surveillance systems will remain sectorial and fragmented. This principle collects tools and resources that facilitate the understanding of surveillance across sectors and disciplines, whilst also showing that these solutions can be found in environments where collaboration works. (ii) The *knowledge* principle represents the need for mutual scientific understanding and expertise in the evolution of knowledge of other sectors; the aim of this principle is to provide surveillance professionals and stakeholders with guidance on sources of knowledge. (iii) The *data* principle represents the ability to understand, reuse, and interpret surveillance data across sectors and disciplines. (iv) The *dissemination* principle represents the distribution of surveillance results to stakeholders and surveillance actors, including industries and policymakers (Filter et al, 2021).

The EC's Green Deal is an integral part of the OH strategy to implement the United Nations' Agenda 2030 and the Sustainable Development Goals (SDGs), which are an urgent and universal call for action by all high- and low-income countries. The EU will work with all partner countries to increase climate and environmental resilience to prevent these challenges from becoming sources of conflict, food insecurity, and forced migration. Some parts of the EU Green Deal, such as the Farm to Fork Strategy and the Biodiversity Strategy, explicitly refer to OH. In recent decades, the OH concept has expanded from the medical and veterinary sciences to include a rapidly growing range of synergistic disciplines, including food safety, food security, public health, health economics, environmental ecosystem health, social sciences, and animal health and welfare. It is now recognised that environmental factors—including chemical contaminants in animals and animal products, veterinary drug residues, and pesticides—play significant roles that call for holistic transdisciplinary approaches to move towards safe and sustainable food systems (Bronzwaer et al, 2021).

2.1.2 Food-Borne Zoonoses and EU Animal Health Laws

Zoonosis control in the EU is regulated by Directive 2003/99/EC, which lists in LIST A the zoonoses to be subjected to mandatory control, including the main FZs, such as brucellosis and tuberculosis in cattle and buffaloes, salmonellosis in poultry and turkeys, and trichinellosis. The individual Member States (MSs) activate National Control Plans (NCPs) in primary production. NCPs are mandatory and possibly co-financed by the European Commission (European Commission, 2022d). They are harmonised in order to make the results comparable, thanks to methods of analysis developed, validated, and disseminated by the European Union Reference Laboratories (EURLs) (European Commission, 2022e), which transfer them to individual

National Reference Laboratories (NRLs) of each Member State (MS) which, in turn, disseminate them extensively to the laboratories of their own national territory. Other optional plans can be activated, based on the epidemiological situation of specific territories as regards the zoonoses included in LIST B of Directive 2003/99/EC. The NCPs are based on the following aspects: diagnosis (serological diagnosis is adopted if there is no possibility of taking samples useful for direct diagnosis); identification and elimination of infected animals or of the entire herd hosting them; attribution of the sanitary qualification “Officially Free” (OF) to breeding and, progressively, to the entire province or region and to the MS in which the specific zoonosis has been eradicated; the prohibition of vaccination (generally mandatory); biosecurity measures, which must be adopted in a strict way, because the presence of the NCP causes the absence of natural or artificial immunological responses against the specific zoonotic agent, due to the pathogen- free territory and to the prohibition of vaccination, respectively. While vaccination can be authorised in the event of a serious emergency with attribution of the sanitary qualification “Free” (F), antibiotic therapy is not allowed. In case of vaccine authorisation, the OF qualification can be newly acquired when only non-vaccinated animals are present in livestock. NCPs are divided into “eradication plans”, if the zoonosis is present in the animal population, and “surveillance plans”, which are limited to checking whether the eradicated zoonosis is re-emerging. The surveillance, in turn, is divided into active surveillance, which involves official controls on farms aimed at identifying infected animals, and passive surveillance, which induces the activation of official controls only when suspected cases are signaled. Data relating to the results obtained in primary production under the application of NCPs, reported human cases, test results in food control activities, test results in feed control activities, and antimicrobial resistance converge in *The European Union One Health Zoonoses Report*, published jointly by the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), in open access and on an annual basis. In the report published in 2021 relating to data for the year 2020 (EFSA, 2021), *Salmonella* (Le Minor and Popoff, 1987) remains the most frequently reported etiological agent in episodes of FZs in the EU, and the pathogens considered in relation to the foods of greatest risk were found to be *Salmonella* in eggs and their derivatives, norovirus in crustaceans and molluscs (including bivalves), and *Listeria monocytogenes* (Murray et al, 2022) in fish and fish products (Parte et al, 2020). Correlation between human brucellosis and non-OF territories persists (EFSA, 2021). Regarding *Salmonella* infections, on 17 February 2022, the United Kingdom (UK) reported a cluster of cases with monophasic *Salmonella* Typhimurium sequence type 34 infection. As of 18 May 2022, 324 cases had been reported in 12 EU/EEA countries and the UK, including two distinct strains. As of 3 June 2022, 392 cases of monophasic *S*. Typhimurium have been identified in the EU/EEA and the UK (*n* = 370 confirmed cases and *n* = 22 probable cases). In addition, cases have been identified in Canada (*n* = 4), Switzerland (*n* = 48), and the United States (*n* = 1), bringing the total number of cases to 445 globally (ECDC, 2022a; Joint ECDC-EFSA, 2022). Most cases were detected in persons below 10 years of age, and 41% of all cases were hospitalised. The two strains are multidrug-resistant, and some tested isolates also exhibit resistance to disinfectants based on quaternary ammonium compounds and hydrogen peroxide, but are susceptible to azithromycin, ciprofloxacin, meropenem, and third generation cephalosporins. Epidemiological investigations have suggested some specific chocolate products, produced in a plant in Belgium, as likely vehicles of infection. Two monophasic *Salmonella* Typhimurium strains matching the outbreak strains were identified in the buttermilk line at the Belgian plant between December 2021 and January 2022. The buttermilk was provided by an Italian supplier, where *S. Typhimurium* was not detected. On 8 April 2022, on the basis of official controls, the Belgian food safety authority decided to revoke the production authorisation of the indicated plant due to lack of transparency and insufficient guarantees for safe production. All at-risk products produced at the closed plant have been recalled. National competent authorities in several countries issued public warnings. This outbreak has evolved rapidly, with children most at risk of severe infection. The plant closure and the global recall of all potentially hazardous products have reduced the risk of exposure. However, eight cases cannot be explained by consumption of chocolate products, suggesting that there may also be other sources of infection. The ECDC has published, in open access, the data on antimicrobial resistance (ECDC, 2022b) and, jointly with EFSA, *The European Union Summary Report on Antimicrobial Resistance in Zoonotic and Indicator Bacteria from Humans, Animals and Food in 2018/2019* (ECDC, 2022c).

2.1.3 EU Control Programmes

EU co-funded veterinary programmes have proven to be a catalyst for achieving improvements in public and animal health, reductions in disease prevalence/incidence, safeguarding of public health (in the case of zoonoses), disease prevention/management in the context of the EU Animal Health Strategy, and economic benefits for the EU as whole by protecting the value of the sector, contributing to market stability, guaranteeing safe trade, increasing extra-EU trade, and reducing human health costs. Significant differences in MSs’ veterinary systems and livestock facilities lead to variability in the implementation of programmes, risking jeopardising the results achieved at the EU level - particularly when dealing with transboundary diseases

(European Commission, 2022f). Regulation (EU) No 652/2014 laying down provisions for the management of expenditure relating to the food chain, animal health, and animal welfare concerns diseases with impacts on human health, diseases with impacts on animal health (taking into consideration their potential spread and the morbidity and mortality rates in animal populations), diseases and zoonoses that risk being introduced and/or re-introduced into the EU territory from third-party countries, diseases with the potential to generate a crisis situation with serious economic consequences, and diseases with impacts on trade with third-party countries and on intra-EU trade. It also concerns the main FZs, including bovine tuberculosis, bovine brucellosis, echinococcosis, campylobacteriosis, listeriosis, salmonellosis, trichinellosis, and verotoxigenic *Escherichia coli* (Castellani and Chalmers, 1919) infections (Parte et al. 2020; EUR-Lex. Regulation (EU) No 652/2014). Regulation (EU) 2016/429 on transmissible animal diseases and amending and re- pealing certain acts in the area of animal health "Animal Health Law" (EUR-Lex. Regulation (EU) 2016/429) concerns the main FZs, including infection with *Brucella* (Meyer and Shaw, 1920) - specifically, *B. abortus*, *B. melitensis*, and *B. suis*; infection with *Mycobacterium bovis* (Karlson and Lessel, 1970), *M. caprae*, and *M. tuberculosis*, included in the *Mycobacterium tuberculosis* complex; and infestation with *Echinococcus multilocularis* (Leuckart, 1863; Parte et al, 2022; Vuitton et al 2011). It was amended and corrected in 2017, 2018, and 2020 (EUR-Lex. Commission Implementing Regulation (EU) 2020/2002). In 2020, in Northern Europe, 20 MSs were officially brucellosis-free in cattle, and 17 MSs were officially tuberculosis-free in cattle, while these zoonoses persisted in the Mediterranean area. Italy, Portugal, and Spain activated co-funded eradication programmes for bovine brucellosis as well as for bovine tuberculosis (also activated in Ireland and Malta) (EUR-Lex. Regulation (EU) 2016/429), while in Greece only the eradication programme concerning ovine and caprine brucellosis (*B. melitensis*) was co-funded (European Commission (EC), 2022g). Greece reported the highest prevalence of *Brucella*-positive ruminant herds, and Spain reported the highest prevalence of tuberculosis in cattle.

2.1.4 Control of Non-Regulated Diseases in Cattle and Buffalo (Cattle Diseases Listed under Category C, D, or E in the EU Animal Health Law)

NCPs are in force both for the most important zoonoses and for the most important animal infectious diseases that lack zoonotic potential. For diseases not included in the European Union Animal Health Law Categories A or B under Commission Implementing Regulation (EU) 2020/2002, approximately one- third of control plans (CPs) are voluntary and can be limited to a well-defined territory of the MS; their funding structure is divided between government and private resources. Countries that have already eradicated diseases such as enzootic bovine leukosis, bluetongue, infectious bovine rhinotracheitis, and bovine viral diarrhoea have also implemented CPs for other diseases in order to further improve the health status of cattle in their country, increasing the commercial value of animals and animal products. Consequently, the gap in the health status of farmed animals could progressively increase among the EU MSs.

2.1.5 The EU Green Policies concerning the Food System

In the European Union, OH overlaps the European Green Deal plan launched by the European Commission (EC Green Deal plan) to achieve the goal of "ZERO environmental impact" by 2050. In this scenario, the devastating impact of war must be considered (Figure 2.2).

Figure 2.2 - One Health improvements deriving from EU green policies in comparison with peacetime.

The Green Deal relaunches the Farm to Fork Strategy for a healthy and environmentally sustainable food system, providing specific measures to make the economy circular, while concomitantly reducing the use of pesticides, fertilisers, and antibiotics, so as to limit the alarming antimicrobial resistance that is spreading worldwide (EUR-Lex, 2020; European Commission 2022a and b).

2.1.6 Public Engagement

Public engagement can be stimulated through the improvement of food labelling, which should include information concerning the production environment, aimed at facilitating consumers' choices in the direction of healthy and, at the same time, sustainable diets. A reward mechanism is triggered for farms that adopt the circular economy and that produce in compliance with the objectives of the EU Green Deal. On the other hand, consumers receive beneficial effects on their health, in terms of both food safety and the improvement of environmental conditions (European Parliament, 2022a). Labelling is a key factor for food safety in agrifood chains. It is often characterised by asymmetric information. Producers and marketers tend to be better informed than consumers about the potential risks of food. The use of innovative strategies to communicate information on food risks can help reduce the divergence between assessed and perceived risks. In this respect, innovative labels - such as traffic-light labels or the use of nanotechnologies - could be valid alternatives. Furthermore, technologies such as Agri- Food 4.0, Blockchain, and the Internet of Things can be useful tools to inform consumers in real time, while also supporting the supply chain decision-making process and improving the coordination process involving farmers, industries, and consumers risks (Santeramo et al, 2021). In this respect, innovative labels - such as traffic-light labels or the use of nanotechnologies - could be valid alternatives. Furthermore, technologies such as Agri-Food 4.0, Blockchain, and the Internet of Things can be useful tools to inform consumers in real time, while also supporting the supply chain decision-making process and improving the coordination process involving farmers, industries, and consumers (Santeramo et al, 2021).

2.1.7 Fighting the Antimicrobial Resistance

The Green Deal includes - among the objectives of primary importance - the reduction in the use of antibiotics in livestock production, in order to combat antimicrobial resistance (AMR), which is a global emergency that has increased during the COVID-19 pandemic. This is referred to as the silent, second pandemic (John Hopkins Bloomberg School of Public Health, 2022). In addition to the wide use of antimicrobials in animals (Chang et al, 2022), plant agriculture frequently uses antibiotics to enhance crop yields. This means that fruits and vegetables have also become potential sources of AMR (Humboldt-Dachroeden et al, 2021). Multidrug resistance (MDR) is continuously expanding worldwide, and poses a challenge in treating infections, necessitating the use of reserve antibiotics, which can have higher cost-to-benefit ratios and a lower safety profile. Among the MDR germs, "the ESKAPE pathogens" have had the greatest impact on healthcare-associated infections—a group of six pathogens with the capacity to elude the bactericidal activity of antibiotics: *Enterococcus faecium* (Orla-Jensen, 1919), *Staphylococcus aureus* (Rosenbach, 1884), *Kleb- siella pneumoniae* (Trevisan, 1887), *Acinetobacter baumannii* (Bouvet and Grimont, 1986), *Pseudomonas aeruginosa* (Migula, 1900), and *Escherichia coli* (Parte et al, 2020). The ESKAPE group is characterised by pathogenic and transmission, resistance traits - which are represented by enzymatic inactivation, target changes, and alteration of cell permeability through loss of porins or increased expression of efflux pumps - and mechanical protection through biofilm formation (Arbune et al, 2021). In 2019, an estimated 1.27 million deaths were attributable to bacterial AMR. At the regional level, the all-age death rate attributable to resistance was highest in western sub-Saharan Africa, and lowest in Australasia. Lower respiratory tract infections accounted for over 1.5 million resistance-associated deaths in 2019, making this the most burdensome infectious syndrome. The six main pathogens for resistance - associated deaths *E. coli*, followed by *S. aureus*, *K. pneumoniae*, *Streptococcus pneumoniae* (Chester, 1901), *A. baumannii*, and *Pseudomonas aeruginosa* - were responsible for more than 900,000 deaths attributable to AMR in 2019 (Parte et al, 2020). One pathogen–drug combination - methicillin-resistant *S. aureus* - caused more than 100,000 deaths attributable to AMR in 2019, while six others each caused between 50,000 and 100,000 deaths: multidrug-resistant (excluding extensively drug-resistant) tuberculosis, *E. coli* resistant to third generation cephalosporins and to fluoroquinolones, *A. baumannii* and *K. pneumoniae* resistant to carbapenems, and *K. pneumoniae* resistant to third generation cephalosporins (Murray et al, 2022). In the EU food system, the AMR monitoring and reporting cover the following food- producing animal populations and foods: broilers; laying hens; fattening turkeys; cattle less than one year old; fattening pigs; fresh meat from broilers; and fresh meat from turkeys, pigs, and cattle. AMR surveillance concerns the monitoring and reporting of antimicrobial resistance of the following bacteria: *Salmonella* spp., *Campylobacter coli*, *C. jejuni* (Doyle, 1948; Véron and Chatelain, 1973), indicator commensal *E. coli*, *Salmonella* spp., and *E. coli* producing extended-spectrum β-lactamases, AmpC β-lactamases, and carbapenemases. In addition, it may cover indicator commensal *E. faecalis* and *E. faecium* (European Commission, 2022h). In the United States of

America, Congress issued the *Disarm Act of 2021* (U.S. Congress.Gov. H.R.4127—DISARM Act of 2021), in order to develop an innovative strategy to fight the increase in antimicrobial resistance (U.S. Congress.Gov. H.R.3932—PASTEUR Act of 2021).

2.1.8 Discussion

NCPs and CPs for animal diseases provide benefits for animals, farmers, producers, and consumers, because they improve animal health and welfare, reduce the use of antibiotics, and - in the case of zoonotic diseases - improve the safety of animal products. NCPs and CPs reduce direct and indirect losses due to diseases. Their implementation involves associated costs for testing and administrative work; however, the cost is usually considered to be outweighed by the benefits (Hodnik et al, 2021). Nevertheless, in non-OF territories, zoonoses and animal infectious diseases cause lower productivity and often lower reproductive capacity of infected farm animals. The de- creased breeding yield produces increased cost of farm products that, in turn, induce higher and uncompetitive market prices, e.g., in the framework of Regulation (EC) 853/2004, raw milk must come from cows or buffaloes belonging to a herd that is OF or F from brucellosis. In non-OF nor F herds, raw milk may still be used when coming from cows or buffaloes that do not show a positive reaction to tests for brucellosis, nor have any symptoms of the disease. In this situation it is mandatory to have the authorisation of the competent authority in order to make the milk undergo a compulsory heat treatment useful to reveal a negative reaction to the phosphatase test. The cost of additional analyses is generally charged to farmers and, consequently, most of them prefer to discard this milk. Furthermore, infected livestock or infected heads are culled in the frame of zoonosis and animal infectious disease NCPs, and the cost of restocking is not fully covered by indemnities. Thus, zoonoses and animal infectious diseases cause decreased revenues, hindering the growth of organic farms, and hampering the fulfilment of the EU Green Deal objectives (Mazzeo et al, 2021). In 2020, EU data on zoonoses and related zoonotic agents reported a drastic decrease in the numbers of human cases (EFSA, 2022). This was evidently influenced not only by the UK's exit from the EU, but also by the COVID-19 pandemic and the resulting restriction measures imposed in EU MSs. This worldwide event, which today sees the rise of variants of concern (VOCs) of SARS-CoV-2 in selected countries with unconsolidated economies and in which the administration of vaccines is slow, highlights the importance of the OH approach, which is useful in designing a new health system to be applied homogeneously at a global scale not only as an ethical necessity, but also as an indispensable safeguard to prevent that those who have been left behind from becoming victims and, at the same time, sources of new emergencies (Galien Forum

Africa, 2021). The Economic Community of West African States (ECOWAS) has borne a significant burden of zoonotic disease impacts. To address zoonotic disease threats in ECOWAS, a One Health Zoonotic Disease Prioritization (OHZDP) was conducted in December 2018 to prioritise the zoonotic diseases of greatest regional concern and develop the next steps to address these priority zoonoses through a regional, multisectoral, OH approach. ECOWAS was the first region to use the OHZDP process to prioritise zoonotic diseases of greatest concern. With the identification of priority zoonotic diseases for the region, ECOWAS member states can collaborate more effectively to address zoonotic disease threats across the region using a OH approach. Strengthening national- and regional-level multisectoral OH coordination mechanisms allow ECOWAS member states to advance OH and has a significant impact on improving health outcomes for both people and animals living in a shared environment (Goryoka et al, 2021). Since it is not possible to stem the spread of pathogens by constructing disjointed barriers, it becomes imperative to act simultaneously at all levels and on a global scale, financing actions that guarantee uniform conditions of protection. This is necessary to counteract the enormous biological plasticity of microorganisms which, together with their rapid reproduction cycles, makes them easily globalised. In the EU, to limit the spread of zoonoses reported as official cases, actions based on the essential protection of the environment are necessary through the concrete implementation of the Farm to Fork Strategy and the pursuit of the Green Deal; in fact, important zoonoses included in Directive 2003/99 EC, although originally linked to animal reservoirs, can be found today in vegetable foods to be consumed raw, as preferential and hazardous vehicles of transmission to humans (Boqvist et al, 2018). Furthermore, animal diseases threaten global food security. African swine fever (ASF)—a viral haemorrhagic disease characterised by high morbidity and high mortality in domestic and wild swine (but lacking zoonotic potential)—is progressively expand- ing in Asia and Europe (FAO, 2022a). In particular, in 2017, ASF cases increased in wild boar in Ukraine, (Smith and Roberts, 2022); in 2021, ASF continued to be reported in wild boar across Europe, and fre- quent outbreaks in domestic pigs continued to be reported in Romania, with small numbers of outbreaks also reported in Ukraine, which is currently under military attack (OIE, 2022e). The probable escape of infected wild boars from territories exposed to military attacks or human factors due to the consequences of war (e.g., population displacement) could cause the further spread of outbreaks in neighboring EU MSs. This is only a minor consequence of the tragedy of the ongoing war, which sees human lives sacrificed and the mass exodus of the exhausted Ukrainian human population, in whom 32,000 new tuberculosis (TB) cases have been estimated in 2020 (with almost 11,000 cases estimated to be drug-resistant TB), and patients have had to stop their required lengthy treatment (WHO, 2022c). ASF has been present on the island of Sardinia since 1978, without consequences for the Italian mainland, where ASF was later on confirmed on 6 January 2022 in wild boars; the ongoing outbreaks first spread in the regions of Piedmont and Liguria (north-west Italy), and then reached the region of Lazio (Central Italy). Control measures at the event level, including domestic control measures in one domestic outbreak that occurred in a farm hosting nine swine (i.e., disinfection, *ante* and *post mortem* inspections; official disposal of carcasses, byproducts, and waste; zoning; traceability; surveillance within the restricted zone; surveillance outside the restricted zone; stamping out; screening; official destruction of animal products; movement control) and wild control measures (i.e., screening; official disposal of carcasses, byproducts, and waste; movement control; surveillance outside the restricted zone; ante and *post mortem* inspections; official destruction of animal products; surveillance within the restricted zone; zoning) were put in place to avoid the major zooeconomic problems caused by ASF—in particular in the export of fine Italian delicatessen products (Coxon et al, 2022). The *African swine fever virus* (ASFV), in the genus *Asfivirus* (International Committee on Taxonomy of Viruses, 2022), does not induce neutralising antibodies, making difficult the efforts for the production of vaccines. A new candidate vaccine for ASF has been developed recently. It uses an attenuated whole virus which, by inducing antibodies towards many viral antigens, is able to protect swine against infection, but may present the risk of retromutation to the virulent virus. Therefore, the "reversion to virulence" test is an important milestone as part of a series of safety studies. Recently, the U.S. Department of Agriculture's Agricultural Research Service (ARS) announced that a vaccine candidate for ASF had passed an important safety test required for regulatory approval, moving the vaccine one step closer to commercial availability (U.S. Department of Agriculture, 2022). Furthermore, Ukraine remains the only country in Europe where rabies is widespread, with about 1600 rabies cases in animals and sporadic cases in humans (Makovska et al, 2021; FAO, 2022b). Rabies is the deadliest of all known zoonoses and it is lethal to mammals. It negatively impacts on food security and livelihood (FAO, 2022c). Veterinary bodies in countries bordering Ukraine and in other MSs have made exceptions to peacetime restrictions for bringing pets across borders in a bid to aid refugees. Consequently, the risk of introducing rabies in free countries is feared (Ukraine's EU, 2022). In Asian countries (e.g., Cambodia, Indonesia, Vietnam), where dogs and cats are used for human consumption, rabies can be considered a food-related zoonosis in workers at dog and cat slaughterhouses, and it can be considered a food-borne zoonosis for the consumers of infected dogs and cats. In Vietnam, workers at dog slaughterhouses are vaccinated in the framework of a national program for rabies control and prevention (Vu et al, 2021). There is a strong policy impetus for the OH cross-sectoral approach to address the complex challenge of zoonotic diseases—particularly in low/lower- and middle-income countries (LMICs), where there is limited policy visibility on zoonotic diseases—especially high-burden endemic diseases that disproportionately affect marginalised rural populations (Asaaga et al, 2021). Since 1 January and as of 22 June 2022, 3413 laboratory-confirmed cases and 1 death from monkeypox have been reported to the WHO from 50 countries/territories in 5 WHO regions. In a week, 1310 new cases were reported, and eight new countries reported cases. As of 21 July 2022, 15,848 confirmed cases had been reported. The unexpected appearance of monkeypox and the wide geographic spread of cases indicate that the monkeypox virus might have been circulating below levels detectable by the surveillance systems, and that sustained human-to-human transmission might have been undetected for a period of time (CDC, 2022; WHO, 2022b). In 2020, a total of 4594 suspected cases of monkeypox, including 171 deaths (case fatality rate = 3.7%), have been reported in 127 health zones from 17 out of 26 provinces in the Democratic Republic of the Congo. Communicating monkeypox- related risks and engaging at-risk and affected communities, community leaders, civil society organisations, and healthcare providers - including those at sexual health clinics - in prevention, detection, and care, is essential for preventing further secondary cases and ensuring effective management of the current outbreak (WHO, 2022c). The WHO has been considering declaring monkeypox a Public Health Emergency of International Concern (PHEIC). In 2019, the U.S. Food and Drug Administration (FDA) approved a smallpox and monkeypox vaccine. This is a good example of preparedness (U.S. Food & Drug Administration, 2022). The recent “One Health of Peripheries” proposal highlights violence as a cause of morbidity and mortality, and among the approaches to address its complexity is the prevention of violence against animals. Geographical peripheries are heterogeneous, encompassing entire countries, areas circumscribed within countries, cross-border regions, rural areas, indigenous territories, and favelas. In particular, the contextual effects of favelas on health are mediated by imposed risks and the lack of resources (e.g., money, time, infrastructure, knowledge), creating a vicious circle of vulnerability due to the increased burden of diseases that compromise individuals’ opportunities for economic and social inclusion. The contextual effects of favelas affect multispecies collectives, and this aspect is even more neglected. Animals are exposed and vulnerable to pollution, humidity, darkness, inadequate ventilation, malnutrition, and high population density. It is necessary to pro- mote the health of animals for their own sake, but also for the sake of the humans living with them (Baquero et al, 2021). Collaboration must be tailored to the surveillance objective and context, characterised by a wide range of factors (i.e., epidemiological, ecological, economic, social, and environmental); successful cross-sectoral collaboration is largely rooted in mutual trust and respect between the different actors. The need for a transition from traditional public health/biosurveillance to a health security intelligence approach to epi/pandemics requires delineating emerging threats from companion, livestock, and wildlife animal communities, which requires funds, the integration of early warning tools, open-source platforms, multisource and multispecies surveillance, proactive diagnostics, field testing technologies, and increased focus on necropsy in captive and wild animals. Diagnostic tools - able to separate immunoglobulin isotypes in order to specifically detect and quantify them simultaneously - could be useful in serological diagnosis aimed at individuating emerging threats, their epidemiological features, and their evolution during vaccination campaigns (Mazzeo, 2020). Furthermore, OH education must be incorporated into scientific, engineering, and humanities curricula, in order to build capacity in OH skills, with the goal of creating networks that will work to improve public health, food safety, food security, and sustainable agriculture. This can be achieved by establishing new perspectives on the interactions between plants, animals, and humans, recognising the threat of disasters and transboundary diseases to food security. The implementation of OH calls for identifying priority areas for added value of joint activities, and for the effective knowledge elicitation of experts from different and relevant disciplines. Consequently, OH may call for updated models for establishing and maintaining effective and timely collaboration and communication across and within disciplines. The establishment of OH approaches and networks can be of high value for countries aiming to establish or improve their OH activities, supporting science-based regulations in the areas of health, food, and the environment (Garcia et al, 2020).

2.1.9 Conclusions

Specific evaluation attributes need to be developed to allow the measurement of the impacts and benefits of collaborative surveillance versus a juxtaposition of isolated sectoral surveillance components (Bordier et al, 2020). In the EU, surveillance systems integrate elements of public and animal health systems and food chains to detect, assess, and control multistate food-borne infections. These systems are based on an investigation and notification approach (Early Warning and Response System-EWRS), the European surveillance portal for infectious diseases EpiPulse, and the Rapid Alert System for Food and Feed (RASFF). The exchange of information is facilitated and coordinated by the EFSA and ECDC and is followed up by the EC (Sarno et al, 2021). General requirements for the laboratory and bioinformatic components of whole- genome sequencing (WGS) and associated metadata for food-borne bacteria have been recently published by the International Organization for Standardization. These guidelines provide the basis for harmonisation of bioinformatic analysis and validation of the end-to- end WGS workflows (ISO/DIS 23418) (Sarno et al, 2021; ISO, 2022). Despite food-borne zoonosis control needs large investments, it can positively affect economies, so the parameter economic health should be included in the OH approach in order to fund eradication programmes in EU MSs facing financial difficulties (heightened by the ongoing COVID-19 pandemic) and, globally, in extra-EU low-income countries, as well as to monitor the effectiveness of interventions and their benefit/cost ratio. EU actions should include, in the case of economic needs, the financing of NCPs in MSs such as Greece which—despite the persistence of the highest notification rate of human cases for brucellosis in the EU, resulting in the highest number of confirmed domestically acquired cases in an MS, and the highest prevalence of *Brucella*-positive ruminant herds - does not benefit from the EC co-funded programme for brucellosis in cattle.

2.2 Future Directions

In 2012, for the first time, the World Bank highlighted the cumulative societal cost of infectious disease outbreaks. The moment at which a system is able to detect signals of possible threats was correlated with the possible cost of the system. Thus, the generation of high-quality zoonotic situational awareness at the earliest possible stages of an outbreak must be a priority for future health security systems (G20, 2021). Promoting the OH approach for food safety (including control of related zoonotic agents) and food security (including control of related animal and plant infectious diseases with no zoonotic potential) is a way to involve the younger generation in entering employment and training in agriculture and the food system. To this end, the obstacle represented by language barriers must be removed. Establishing a broader concept of OH that incorporates the food system as well as cultural and societal awareness is essential. Developing a holistic view of biomedical, biotechnological, and agricultural sciences can help in transforming traditional academics and researchers into OH practitioners who work in transdisciplinary teams to solve complex problems at the interface of human, animal, plant, and environmental health (Garcia et al, 2020). Given the unequal health status of EU farms with respect to both FZs and infectious animal diseases, it would be appropriate to introduce a new OH assessment parameter: economic health. This could be used to allocate European funds that would allow MSs facing economic difficulties to achieve eradication by reaching the same levels as the MSs with the most advanced animal husbandry. Recalling Dr. Bernard Url's conclusive speech in the ONE2022 conference, “We have to change the financial model in which we are locked” (EFSA, 2022). Since investors bet on innovation only considering its impact/success (In Focus News, 2022) and the extent of economic return, we need to build a way to direct investments toward ethical and science-based choices. National and international rules concerning public/private investments and trading could regulate the flow of money, directing it exclusively toward companies that meet a broad range of OH parameters, ranging from environmental impact up to food safety, human health, work conditions, duration of employment, number of layoffs per decade, wage conditions, gender and social equality, etc. A OH conformity certificate for enterprises - listed in the stock market or not - should be awarded considering various aspects of the OH, each of which should be analysed using a proper checklist. Each analysed checklist could then be merged in a final document declaring (or not) the compliance of a specific enterprise with a minimum number of the considered parameters useful to evaluate its OH status. This in-embryo One Health Financial Model is based on money exclusively invested in certified enterprises. In this model, money can be effectively used in the effort to improve human society and global conditions, avoiding economic losses due to negative side effects on the holistically considered health, in the effort to effectively purpose the OH goal. Dedicated agencies and databanks preserving all data concerning certified enterprises are needed. Data, when shared in open access, could efficaciously improve public engagement, addressing consumers’ choices toward certified producers who comply with the OH objectives. Despite the considerable efforts being carried out in the context of the One Health approach, war causes tragic and devastating effects both on physical and mental health of human beings, on their lives, on pandemic threats, on animals, on plants and last, but not least, on the environment. Huge efforts are urgently needed for peace.

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Chapter 3

Biotechnologies innovations in Alto Sannio milk chain, ONE Health and Territorial Identity

The evidence and analyses reported in the previous chapters have highlighted an interesting food district that can be identified in the "*Alto Sannio Beneventano*" area, which sees within it an interesting potential represented by the companies referable to the dairy chain. In fact, in the Benevento area the dairy chain shows a growing trend with its highest expression precisely in the areas that fall within the Alto Sannio. The farms, which are generally family-run and small, are not able to express their real potential. The limited dynamism makes it difficult to adopt innovations, a condition that condemns the farms and the entire chain to a low level of competitiveness. The cow's milk produced in Alto Sannio, as in much of the region, is processed into products that are technologically consolidated but stagnant in terms of markets. A behavior that encourages ruthless competition for the lowest price and a steady decline in consumption. A picture, therefore, far removed from the consumption trends of high quality, healthy and sustainable food. The direct involvement of many livestock farms and the transfer of knowledge and innovations to the very segment of the supply chain that is most reluctant to innovate seems desirable. The definition and identification of a food district is the answer to the call for the involvement of similar stakeholders.

The introduction of sustainable solutions to improve animal welfare and quality remains an interesting new element to pursue as it would give a clear identity to the Sannio food milk. A strategic element, the latter, that would also allow the valorisation of competitive segments downstream, such as dairy production from healthy, genuine, sustainable, and clearly identifiable milk. Furthermore, it should be taken into strong consideration that the intensification of livestock breeding in the last century has contributed to the numerous negative impacts of agriculture on the environment and on animals and human health, among several negative impacts, just to name a few, zoonotic epidemics, antibiotic resistance, consumption of animal products associated with increased risk of cancer (Diallo et al, 2018; Morand, 2020). The evolution of farming towards sustainable systems that respect humans, animals and the ecological state of the planet is a key aspect of the transitions of the agri-food system (Silbergeld, 2019; Garcia et al, 2020). In this context, existing solutions are no longer sufficient, and it is necessary to develop new solutions and research and innovation approaches. While in recent years research and innovation in the agricultural sector has been organized top-down and has been outsourced by farms, many studies have highlighted the need to develop research approaches and innovation open to the participation of a variety of actors (researchers, practitioners, government, and civil society) in a context of global transition (Coeugnet et al, 2023).

A method based on the involvement of a district would be a shared science approach with citizens, an evolution of the citizen science model, which would facilitate the definition of innovations and the promotion of agroecological transitions with the construction of a common horizon with farmers, technicians, and researchers. Several studies point out that a group with a heterogeneity of actors can lead to the design of original and varied ideas provided that methods are mobilized to overcome the fixation effects (Vourc'h et al, 2018). Among them, the KCP (Knowledge-Concept-Proposal) method, based on the organization of design workshops, aims to control these fixation effects (Le Masson et al, 2009). This method is used in organizations of all sizes and sectors to explore solutions that break with the existing method for its dual ability to support the exploration of disruptive solutions (Elmquist and Segrestin, 2009).

Co-design is an approach that involves the end user in a product or service development and design process. It is therefore a user-centered design method where the emphasis is on the active role of users. Therefore, the research actions were carried out with the intention of involving citizens and different actors of the same food district for the exploration of innovative solutions for the milk supply chain. To do this, a co-planning process was implemented (based on workshops and comparisons) involving researchers, breeding professionals and citizens with the aim of producing knowledge and developing innovative solutions for the dairy systems of the upper Sannio. The KCP method was used because, as mentioned above, it is known to support agroecological transitions, build a common horizon and stimulate innovations.

3.1 Knowledge, concept and proposal

The KCP method was used as tool of directed creativity in order to organize the innovative design with a collective of designers (Graceraj et al, 2019). It consists of three steps: a phase K of "Knowledge", a phase C of "Concept", and a phase P of "Proposal". The aim of phase K (Knowledge) is to share knowledge, identify missing knowledge, and to identify links between existing knowledge. This phase is essential in order to create a common knowledge base, especially in the case where the designers are diverse, and each has very heterogeneous knowledge about the purpose of the design. During phase K, a plenary presentation was given by the PhD student and by tutor on the links between human health, animal health, and the overall ecological state. Then, the participants were divided into three groups. Each group was led by a pair of facilitators (a thematic referent facilitator and a method referent facilitator); two facilitators were responsible for the global organization. Each group was associated with one of the three identified themes. Each participant had an individual knowledge sheet. Then, discussions organized by the facilitators allowed everyone to react to the knowledge of the sheet, express their expectations, or provide additional knowledge. The second knowledge sheet on an innovative example from the laying hen sector was distributed. This sheet was intended to shift the reflections of participants and open up the phase of exploring innovative solutions of the afternoon. During phase K, the facilitators transcribed the key ideas of the exchanges on a poster. At the end of phase K, each group presented to the others the exchanges they had on their subject. Phase K provides common knowledge based on the main challenges to the diversity of participants.

Phase C (Concept), a guided exploration of designers using the C tree of the C-K diagram, was used to generate new concepts and develop solutions from these ideas. During phase C of the workshop, the participants were in the same group as phase K. The key instruction was to explore concepts without taking current constraints into account. The participants had trend boards and Post-its® to explain their ideas. The facilitators were also there to support the formulation of new ideas, identify any fixation effects among the participants, and help them to circumvent these fixations and get rid of natural constraints. The facilitators could rely on the initial C tree of the initial C-K diagram to guide the exploration. The participants did not have access to this tree and the C-K theory was not explained to them. Participants' ideas were organized by the facilitators on a poster.

Finally, Phase P (Proposal) consists of the transformation of concepts into a project aiming at developing a roadmap on the implementation of certain paths explored previously. During phase P of the workshop, the participants, still in the same group, had at their disposal project sheets in order to detail how the ideas explored during phase C could be implemented in the form of a project. In order to select the ideas that the group would develop into a project, they proceeded by voting with stickers. They had two types of stickers available, stickers relating to the relevance of the project and stickers relating to the originality of the project. From the vote, they discussed the ideas they wanted to develop into projects. For each project, the participants had to develop the objectives to be achieved, the actors to be involved, the necessary means, the scale and the time of realization, and the points of vigilance. At the end of phase P, each group presented the result of their reflection during phases C and P to the other actors.

The turns to speak organized by the facilitators, the written materials allowing individual expression, and the effort of popularization of the knowledge by the researchers and the actors of the sector for the citizens allowed the participation of all the actors.

The participants gave their written consent to the use of their personal data and recorded exchanges for research proposes.

The participants distributed into homogeneous groups of citizens, residing in the district's municipalities, professional farmers in the district, and researchers synthesised the concepts around one or more key words. The keywords produced were analysed and represented in the form of a hierarchical graph. Figure 3.1 shows the 10 most recurring keywords of which the first five have a three-quarters weight of the total.

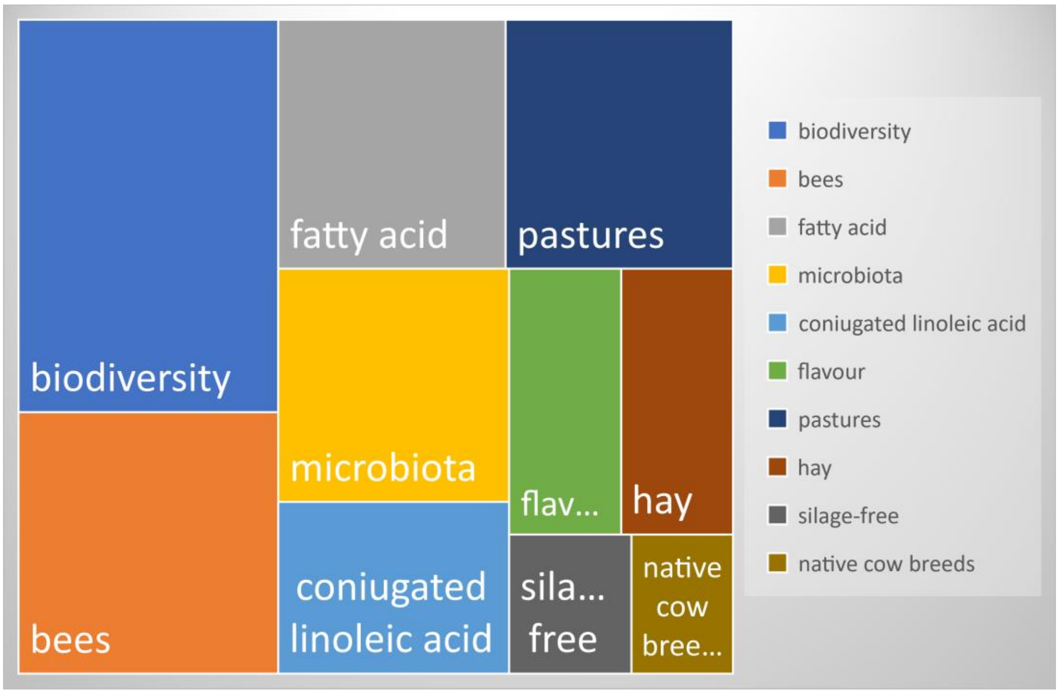

Figure 3.1 - Hierarchical chart of the keywords representing the proposals for innovations that emerged from the discussion between citizens, researchers and cattle breeding professionals. The comparison was based on the Knowledge, Concept and Proposal method.

The protection of biodiversity is the most recurring theme, which is declined in the form of the microbial, bee and pasture heritage. Immediately afterwards comes the nutritional value of the product with reference to the fatty acid profile.

3.2 Quantitative research literature analysis

Milk quality, animal welfare, sustainability and territorial identity represents the basis of renewed competitiveness for livestock farms in Alto Sannio area, by promoting the wholesomeness of dairy products and considering novel elements to be introduced into the food chain, with special regard to those feeds, that could enhance the link with the territory while respecting environment and animal welfare.

The linkage between animal primary production, wholesomeness of products, sustainability, and environmental protection is showed in Figure 3.2, obtained from a search in Scopus database processed with VOSviewer software tool (ver. 1.6.18, https://www.vosviewer.com/). A total of 936 articles and book chapters were found based on the following keywords: “milk”, “feed”, “dairy”, “forages”, “chain”, “environment”, “livestock”, “bacteria”, “diets”, “quality”, “nutrition”, “microbiology”. Successively, a network map was created and 6 main clusters, as a set of items included in a map and with a different colour, were identified. More in detail, the size of the label and the circle of an item is determined by the weight of the item, the colour of the item is determined by the cluster to which the item belong, and lines between items represent links (Figure 3.2).

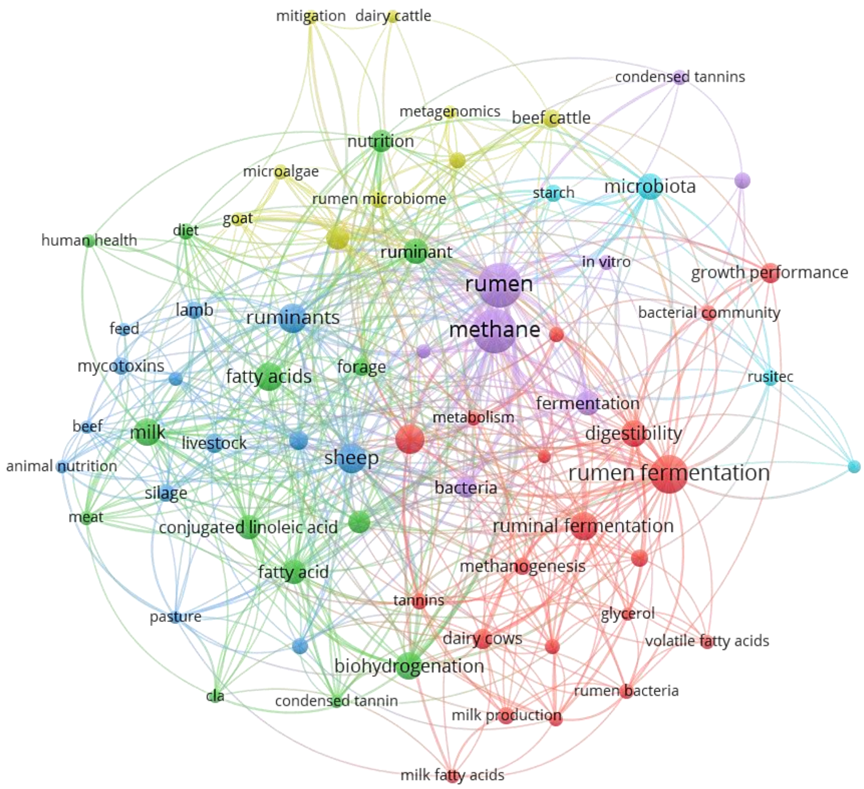

Figure 3.2 – Clusters relationship between the selected keywords processed in VOSviewer software.

The network visualization emphasizes, among others, rumen, methane, rumen fermentation, fatty acids, nutrition, microbiota, forage, milk, and conjugated linoleic acid. The comprehensive analysis of the targeted keywords, and the subsequent visualization of the links highlighted by the network, has suggested the possibility of studying the characteristics of Alto Sannio farms, including the introduction of innovations in animal diets, estimating the potential impacts of these innovations on environment.

The relationship between hay and dairy cows is obvious and abundantly investigated.

3.3 Development of the innovative idea: hay and bee heritage as a pivot for territoriality and one health issues

Based on the data that emerged from the analysis of the quantitative research literature and derived from the sharing of knowledge and experience through the Knowledge, Concept and Proposal method, the central part of the doctoral thesis was addressed to the development of an innovation based on hay management and the valorisation of the beekeeping heritage of Alto Sannio. Bees and hay in cattle feeding appear to be the two themes that best identify the concept of health in its broadest sense as well as the link to territoriality: hay alone or as a vehicle for microorganisms can significantly affect animal welfare and milk quality; pollinating insects provide an important ecosystem service that supports global biodiversity and environmental health (van den Oever et al, 2021).

Increasing attention is being paid to the use of hay in cattle feed, making it silage-free. It is no coincidence that the European Union has recognised 'hay milk' as a 'traditional speciality guaranteed' (TSG), defining strict criteria for milk production (e.g. silage must not be used as feed) (European Commission, 2016; European Parliament and Council of the European Union, 2012). Furthermore, hay milk is considered suitable for the production of hard cheese by the Austrian Food Codex (2011), so much so that approximately 15% of Austrian milk is produced on farms that meet hay milk standards. Recent studies have also shown that the adoption of dried fodder in the diet quantitatively and qualitatively improves milk production (Boettger et al, 2019).

However, it should be emphasised that the microbial input resulting from the use of silage significantly influences rumen activity and consequently milk quality. Scherzer et al (2019) showed that the use of silage, due to the bacterial input, significantly improves the fatty acid profile by increasing mono- and polyunsaturated fatty acids (MUFA, PUFA) and conjugated linoleic acid (CLA).

Therefore, combining the benefits of dry hay with those of the microbial population is an interesting auspice. The identification of a microbial biotype compatible with the forage essences of the district area as well as indicator of the environmental well-being of the area would fully meet the innovation proposals outlined in the previous paragraphs, providing an important ecosystem service by transferring pollen to crops and wild plants, thereby supporting global biodiversity and environmental health.

On this regard, bees represent one of the most interesting microbic bioreserves. In fact, in recent years, it has been shown that bees or their products, such as bee bread, harbor interesting microbial communities. In particular, ubiquitous microbial taxa such as *Apilactobacillus* or *Lactobacillus* have been examined in honey bees (Iorizzo et al, 2016; Nguyen and Rehan, 2023). The aim of this doctoral activity was to characterise and select strains of lactic acid bacteria isolated from bee material for use as bio-preservatives in the preservation of hay.

3.3.1 Materials and Methods

3.3.1.1 Isolation of lactic acid bacteria from natural bioreserves with a strong territorial connotation

Attention was paid to bioreserves of microorganisms that best represented the territory and its health characteristics. For this reason, particular interest was paid to bioreserves of bee origin, investigating the biodiversity and the characteristics of technological and functional interest of batteric isolates from bees on forage crops in Alto Sannio or from bee bread produced by them. The samples of honeybee foragers and of beebread were collected from *A. m. ligustica* hives within apiaries sites in Morcone. The LAB isolation was carried out by dissecting the social stomach and midgut of foragers and from the beebread. To dissect and separate the social stomach and midgut from bee samples, they were kept in the refrigerator for at least 5 min, devitalized by placing gentle pressure on the prothorax and then dissected, at room temperature, in a glass Petri dish containing sterile saline solution (9.0 g/L of NaCl). The dissection was carried out utilizing stainless-steel scissors for microdissection and a curved lancet (both washed in alcohol and sterilized with flame). The social stomach has been obtained by cutting the distal part of the esophagus and close to the proventriculus. The midgut was obtained by making a further incision at the level of the pyloric valve. The anatomical samples, of 5 foragers, were placed in the respective sterile glass tubes containing sterile saline solution for the subsequent isolation of the bacteria. Ten grams of each sample of beebread were placed in 90 mL of a physiological solution and serial decimal dilutions were obtained. The social stomachs, the mid guts and the bee bread, in sterile saline solution were homogenized and serial decimal dilutions were obtained. Briefly, LAB was enumerated and isolated by plating serial decimal dilutions on MRS agar medium (Oxoid Ltd., UK) adding 40 mg/L cycloheximide, on modified

MRS containing 10% fructose. Plates were incubated for 48–72h at 30°C under anaerobic conditions using an anaerobic system (Anaerogen, Oxoid, Milan, Italy).

Representative numbers (10%) of colonies randomly picked from each assayed medium have been purified by streaking on the suitable agar media. The colonies were randomly selected according to morphological differences (colony size and shape).

The pure isolates were tested for their Gram reaction, catalase activity and morphology. Gram-positive and catalase-negative were selected as presumptive LAB and were stored at − 80 °C in the corresponding liquid isolation medium, supplemented with 25% (*v*/*v*) of glycerol.

3.3.1.2 Genotypic Characterization

DNA extraction

Two millilitres of each overnight isolate cultures were centrifuged at 14,000g for 10 min at 4 °C and the pellet obtained was subjected to DNA extraction according to Querol et al (1992), with the addition of lysozyme (25 mg/mL, Sigma) and mutanolysin (10 U/ml, Sigma) for bacterial cell-wall digestion. Quantity and purity of the DNA were assessed by optical reading at 260 and 280 nm, as described by Sambrook et al (1989).

DGGE analysis

The DNA from each isolate was prepared for DGGE analysis by amplifying the V1 region of 16S rRNA using as primers: P1V1 (5′-GCG GCG TGC CTA ATA CAT GC-3′) (Cocolin et al, 2001) and P2V1 (5′-TTC CCC ACG CGT TAC TCA CC-3′) (Rantsiou et al, 2005). A GC clamp (5′- CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′) (Sheffield et al, 1989) was attached to the 5′ end of the P1V1 primer. PCR was performed in a Mastercycler gradient (Eppendorf, Hamburg, Germany). The reaction mixture, the amplification program and gel processing were performed as described by Testa et al (2014; 2020).

DNA sequencing and data processing

One representative isolate from each cluster, obtained by DGGE analysis, was amplified with primers P1 (50-GCGGCGTGCCTAATACATGC-30) and P4 (50-ATCTACGCATTTCACCGCTAC-30), as described by Klijn et al (1991), targeting 700 bp of the V1–V3 region of the 16S rRNA gene. After purification, (QIAquick PCR purification kit, QIAGEN GmbH, Hilden), products were sent to a commercial facility for sequencing (Eurofins MWG Biotech Company, Ebersberg, Germany). Sequences were aligned with those in

GeneBank utilising the Blast program (Altschul et al, 1997) to determine the closest known relatives, based on the partial 16S rRNA gene homology.

RAPD-PCR

The amplification products were separated by electrophoresis on 1.5 % (w/v) agarose gel (Sigma-Aldrich, Steinheim, Germany) in 0.5 x TBE buffer and then subjected to ethidium bromide staining. RAPD-PCR gels were digitally captured and analysed as described by Testa et al (2014).

3.3.1.3 Biochemical Characterization

The LABs have been assessed for their carbohydrate fermentation pattern using API 50CHL system kit and enzymatic patterns using API ZYM system kit, according to the manufacturer's instructions (bioMérieux SA, Marcy l'Etoile, France).

Detection of antimicrobial activity

The spot-on-the-lawn technique was performed against each indicator to detect growing cells (GC) of producers having inhibitory properties. The method used was described by Moraes et al (2010), and the presence of a distinguishable inhibition zone around the spots, evaluated after 24 h incubation at 28°C, was considered as positive antagonistic effect. The degree of inhibition was defined as low (5 mm $< \varnothing <$15 mm), moderate (15 mm $\leq \varnothing <$ 25 mm), strong (25 mm $\leq \varnothing <$ 35 mm), and very strong (35 mm $\leq \varnothing <$ 45 mm). A calibrated-densitometer (GS-800, Bio-Rad, Hermles CA, USA) was used for imaging acquisition and Adobe Photoshop CS6 Extended software was used for the measurement of clearing zones. Each experiment was carried out in triplicate.

Detection of ability to form Biofilm assay

The strains were screened in order to assess their ability to form biofilm in acid or ethanol stress conditions. For this purpose, each strain was cultivated in MRS broth at 28 °C until the early stationary growth phase was reached. The cells were recovered by centrifugation at 8500 rpm for 15 min, washed twice with PBS and resuspended in MRS broth (pH 6.2). Each strain was used to inoculate (final concentration of about 7 Log CFU/mL, corresponding to an Optical Density at 620 nm -OD_{620}- $\simeq$ 0.02) 96-well polystyrene microtiter plates (Thermo Scientific™ Nunc™) filled with 200 µL of MRS with 25% (NDF25) or 50% (NDF50) of neutral detergent fiber (Sigma Aldrich, Italy) or with MRS added with acid detergent fiber (Sigma Aldrich, Italy) at 25% (ADF25) or 50% (ADF50); wells filled with MRS at pH 6.2 were used as control. After 2 h and 24 h of incubation at 28 °C, the biofilm formation was detected using the crystal violet (CV) assay as reported by Merritt et al. [39]. Briefly, the medium was removed with a micropipette and the resulting biofilm was washed three times with 230 µL of phosphate buffered saline (PBS) to remove unattached cells. The biofilm was stained with 200 µL of CV (0.1% v/v) solution for 30 min. The excess of CV was removed, and the biofilm was washed three times with 230 µL of PBS. The dye attached to biofilm was dissolved with 200 µL of acetic acid (30% v/v) solution for 30 min, then 100 µL were transferred in a new microtiter plate and the OD_{620} was measured (Multiskan FC, Thermofisher Scientific). OD_{620} values from CV assay were used to determine the biofilm expression index. For this purpose, OD_{620} raw values were structured as numeric matrix, standardized, and elaborated by multivariate analysis. The method used for the biofilm expression is detailed in following paragraph (*Statistical analysis*).

Biolm-lifestyle: Cell survival in HMS

L. plantarum LpUM1 was chosen on the basis of its ability to produce biofilm on polystyrene plates. The ability to survive by *L. plantarum* LpUM1 in biofilm form was evaluated in a hay model system (HMS). MW was prepared as described by Caturano et al, 2023 and inoculated with cells.

At the time of the inoculum (time 0), after 3, 10 and 30 days of incubation, aliquots of samples were taken to enumerate cells and detached cells from biofilm.

As regards to cell enumeration, one g of HMS was taken from the batches, diluted in physiological solution (9 g/L NaCl) and the cells were enumerated in MRS agar after incubation for 24 h at 28 °C under anaerobic conditions using an anaerobic system (Oxoid).

Statistical analysis

Data were analyzed using the RStudio (v 3.5.0) environment (R Core Team, 2018). Regarding the screening experiments of biofilm assay (see par. 2.2.1), three independent tests were performed, and the mean value was used for data elaboration. The OD values, obtained from the CV assay, were first standardized using the R base function *scale* and subsequently were analyzed by statistical analysis based on a multivariate approach. In particular, for the data standardization, the OD values were reported in an electronic spreadsheet and organized in term of a numeric matrix with 68 rows and 5 columns. The rows represent the observations (*L. plantarum* strains) and the columns represent the experimental conditions. For each column, mean and standard deviation were calculated. The average value was subtracted from each value and then divided by the value of the standard deviation corresponding to the same column. Standardized data were processed with the aim to investigate the relationship between biofilm production (response variable) and the source of strains isolation, type and level of stress. For this purpose, the R package ComplexHeatmap (Gu et al, 2016) was used to process the heat-map and the package FactoMineR (Lê et al, 2008) was used for the Principal Component Analysis (PCA).

Fermentation scale-up and industrialisation of strains

The industrialisation actions of the strains involved carrying out activities, first on a laboratory scale and then on a pilot plant, aimed at understanding the kinetic parameters of growth and yield at the end of the process, including the freeze-drying operation. The industrialisation action was conducted for four strains, all related to *Lp. plantarum*, which from the screening data showed the best or most interesting antagonistic activities.

Laboratory-scale fermentation, bioreactor fermentation, cell concentration and finally freeze-drying were conducted in collaboration with the biotechnology company Mediterranea Biotecnologie srl, simulating all stages of the industrial process.

Growth kinetic parameters, such as maximum growth rate (max), duration of the lag phase, initial (y_0) and final (y_end) charge values were estimated by applying the equation of Baranyi and Roberts (1994), using the DMFit Web Edition software.

Evaluation of the viability of microorganisms in hay management

To assess the viability of bacterial strains for use as bioprotective cultures in hay management, three different forage species were used, *Hedysarum coronarium* L. (sulla), *Lolium perenne* L. (ryegrass) and *Trifolium* spp. (clover).

Each forage species was divided into two experimental plots, which were subjected to two different pre-harvesting operations in the oven, until a moisture level of 60% and 30% was reached, respectively. Each batch was inoculated with the bacterial suspension to reach a final concentration of about 10^6 CFU/g and stored under partially aerobic conditions.

At the time of packaging and after 7, 14, 21, 28, 35, 42 and 70 days, the presence of the strain used as starter was assessed by means of viable plate counts accompanied by the application of biomolecular techniques.

The microorganisms were counted on MRS plates (OXOID, Milan) after 72 hours of incubation at 37° C under anaerobic conditions (Gas Pack Anaerobic System). Analyses were conducted in duplicate. The results obtained were statistically analysed using OriginPro 7.5 software (OriginLab Corporation, Northampton, MA, USA).

3.3.2 Result and Discussion

Bacterial strains were identified as candidates for the fermentation of different types of forage and herbaceous essences from Alto Sannio. Attention was paid to bioreserves of microorganisms that best represented the territory and its health characteristics. For this reason, particular interest was paid to bioreserves of bee origin by investigating the biodiversity and the characteristics of technological and functional interest of batteric isolates from foraging bees on forage essences of the Alto Sannio or from bee bread produced by them.

3.3.2.1 Species Identification from bees bioreserves

The predominant species of lactic acid bacteria (LAB) from bee bread, stomach and intestine of foraging bees *Apis mellifera ligustica* were evaluated (Figure 3.3). In detail, for each compartment, species and biotype diversity was ascertained through multiple culture-dependent approaches, represented by the biomolecular technique PCR-DGGE, 16S rRNA gene sequencing and the biotyping technique RAPD-PCR. The study of a lactic acid bacteria community, performed with PCR-DGGE and sequence analysis targeting the V1-V3 region of the 16S rRNA gene (rDNA), revealed the presence of several species, including *Apilactobacillus kunkeei*, *Lactiplantibacillus plantarum*, *Fructobacillus fructosu*s, *Levilactobacillus brevis* and *Lactobacillus delbrueckii* subsp. *lactis*.

Considering a similarity level of 80% as the arbitrary threshold for the identification at species level, the isolates were grouped into clusters. According to the migration profiles, for each DGGE-gel, one strain from each cluster and all strains grouping alone were selected for subsequent genetic sequencing. A total of 45 isolated were sequenced and the results allowed the identification at species level. Combining these results with those obtained from the DGGE profiles cluster analysis, it was possible to identify 85 isolates as *Apilactobacillus kunkeei* (*Al. kunkeei*), 61 as *Lactiplantibacillus plantarum* (*Lp. plantarum*), 16 as *Fructobacillus fructosus* (*F. fructosus*), 6 as *Levilactobacillus brevis* (*Lv. brevis*) and 2 as *Lactobacillus delbrueckii subsp. lactis* (*Lb. lactis*). Our results highlighted a low inter-species variability in LAB community. In fact, the substantially predominance of *Al. kunkeei* and *Lp. plantarum* was detected.

The presence of these species agreed with other studies on honeybee microbiota (Dong et al, 2020; Feizabadi et al, 2020). *Lp. plantarum*, due to its ability to cope with different stress conditions, is a versatile and widespread microorganism found in different food-matrices and environments (Kleerebezem et al, 2003; De Leonardis et al, 2016; Iorizzo et al, 2016; Succi et al, 2017; Tremonte et al, 2017). *Al. kunkeei* colonizes fructose-rich niches (e.g. flowers, fruits, and fermented foods made from fruits) and is actually classified as fructophilic lactic acid bacterium (FLAB) (Endo and Salimen, 2013; Filannino et al, 2016). The levels of inter-species variability depend on the isolation sources: bee bread, honey stomach and mid gut. The highest variability was detected in bee breed which highlighted the presence of three predominant species, such as *Al. kunkeei*, *Lp. plantarum* and *F. fructosus*, as well as the presence of isolates belonging to *Lv. brevis* and *Lb. lactis*. Also, in honey stomach the presences of isolates belonging to four species was detected. However, respect to the bee breed, in honey stomach changed, even if slightly, the situation of predominant species. *Al. kunkeei* and *Lp. plantarum* represented the only predominant species, while isolates identified as *F. fructosus* as well as *Lb. lactis* were minority. Finally, the lower levels of inter-species variability were found in mid-gut, where *Al. kunkeei* represent the predominant specie. In fact, 56 isolates were identified as *Al. kunkeei* and only four isolates as *Lp. plantarum*.

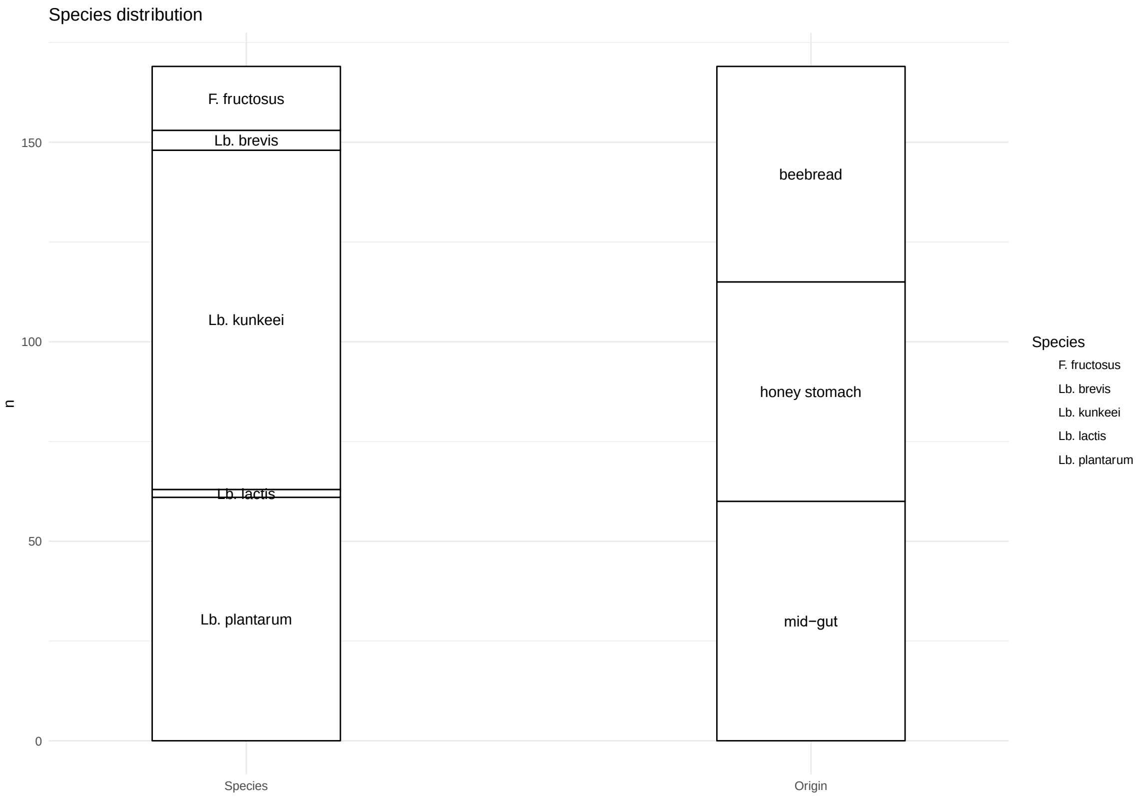

Figure 3.3 - Alluvial plot showing the frequency of strains belonging to the five species isolated from the beebread, honey stomach and mid gut.

The highest variability in species detected in bee breed is due to several factors including the host himself, the environment as well as the flowers (Aizenberg-Gershtein et al, 2013; Rokop et al, 2015; Donkersley et al, 2018). In particular, the high presences of fructophilic LAB (FLAB) species could be due to the specific acacia pollen characterized by a high frucotse/glucose ratio (Czipa et al, 2019; Thakur and Nanda, 2020). In fact, *F. fructosus* and *Al. kunkeei* could be considered usual FLAB species and *Lp. plantarum* has been described as a potentially new FLAB (Filannino et al, 2019). As also reported by other authors, it is reasonable to hypothesize that FLAB species could be considered members primarily associated with foraged foods and consequently transient member of the bee-associated gut (Rokop et al, 2015; Donkersley et al, 2018). In addition, the selective pressure of honey bees gastrointestinal tract (GIT) establish a great decrease in specie variability (Anderson et al, 2014; Kwong and Moran 2016). In fact, as clearly highlighted by results only *Al. kunkeei* was able to persist in mid gut. The high persistence of *Al. kunkeei* could be attributable to its ability to produce biofilms or to other metabolic and specific adaptability (Djukic et al, 2015).

3.3.2.2 Biotyping in Lactiplantibacillus plantarum specie

The target of the investigation was represented by *Lp. plantarum* species. For this purpose, the RAPD-PCR analysis was used to detect the distribution relation to the isolation from different environments. The RAPD-PCR enabled strain differentiation within species, according to the PCR patterns amplified randomly with specific oligonucleotides M13 and D8635. Considering the migration profiles, a similarity level of 85% was chosen to distinguish the different biotypes for each specie.

In Figure 3.4, the typing diversity among 61 *Lp. plantarum* strains isolated from bee bread (11), honey stomach (18) and from mid gut (56) was reported. In detail 10 clusters (Lpla.I - Lpla.X) were individuated and, respect to *Lp. kunkeei*, a lower relationship between the source of isolation and biotyping was ascertained. In fact, only three clusters (Lpla.I, Lpla.II, Lpla.VI) grouped strains come from a unique environment. The others seven clusters enclosed strains from multiple environments. More in detail, six cluster (Lpla.III-LplaV, Lpla.VII-VIII and Lpla.X) enclosed strains from bee bread and honey stomach. Finally, cluster IX grouped strains from all the isolation environments, four from mid gut, 6 from honey stomach and 1 from beebread. Based on the results, a high *Lp. plantarum* biodiversity was detected in bee bread and in honey stomach, while a specialization characterized the mid gut that was dominated by only one *Lp. plantarum* biotype.

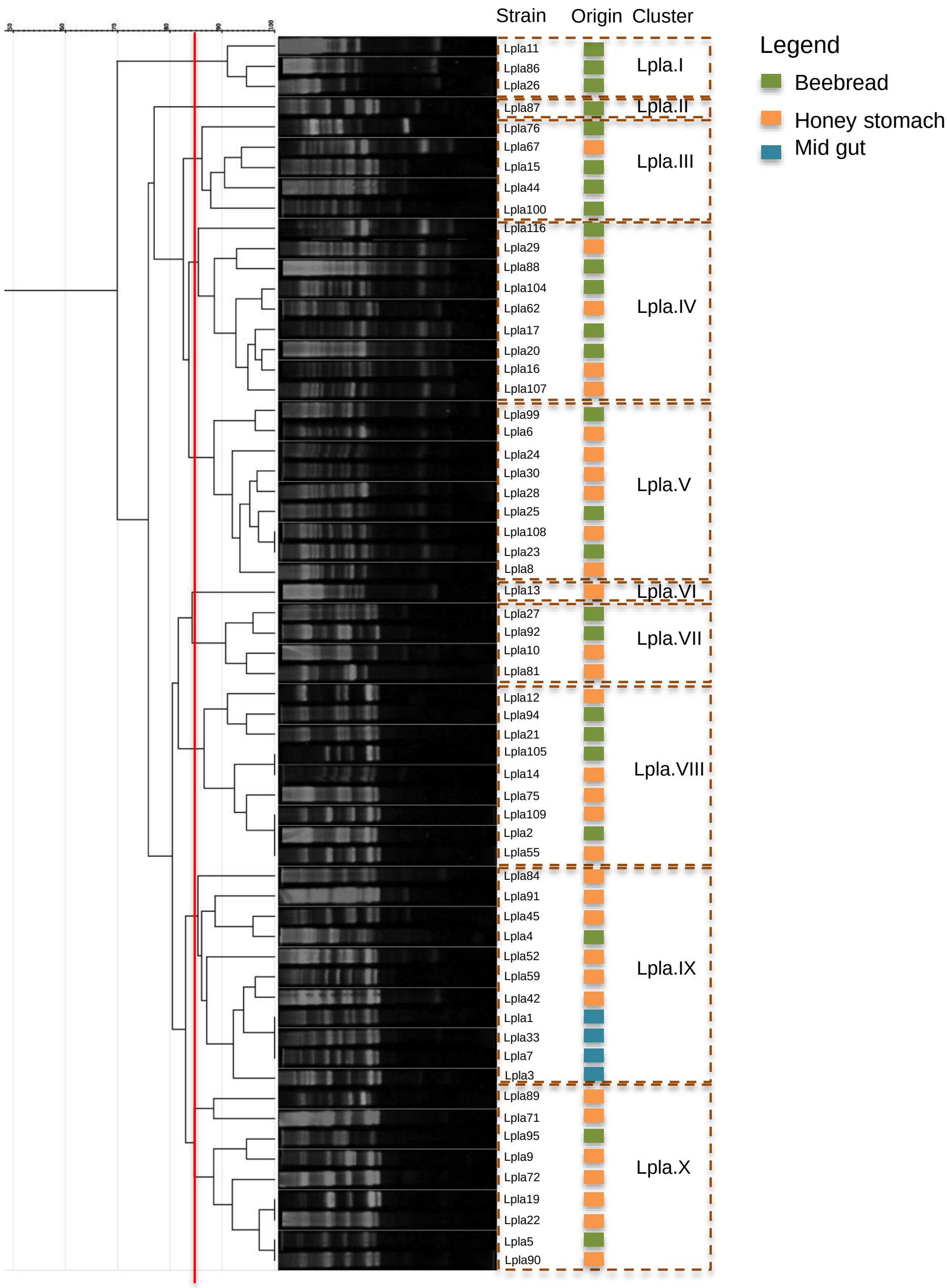

Figure 3.4 - Cluster analysis based on the RAPD-PCR profiles of the 61 strains belonging to the specie *Lactiplantibacillus plantarum*. Vertical red line indicates the threshold value (85 %).

The assessment of biochemical profiles attributable to different strains allowed the individuation of specific features as well as important differences related to the RAPD-biotypes. Interesting metabolic activities were found both in *Lp. plantarum*. A relationship between RAPD-biotypes and biochemical features was also detected for *Lp. plantarum* strains. Based on the strain-dependent biochemical features ten groups were identified. Three clusters collected the three RAPD-biotypes (Lpla.I, Lpla.II and Lpla.VI) detected exclusively in bee bread, exhibited several enzymatic activities including esterase, esterase lipase, α-glucosidase and β-glucosidase. RAPD-biotypes Lpla.I and Lpla.VI were also characterized by the ability to utilize raffinose, while the RAPD-biotype II metabolized the melezitose. Six different biochemical cluster grouped the 6 *Lp. plantarum* RAPD biotypes found in bee bread and in honey stomach. Final as regard *Lp. plantarum* strains, a specific biochemical cluster collected the RAPD-biotype Lpla.IX. The results showed that this biotype, detected not only in bee bread and honey stomach but also in mid gut, was characterized by the most complex biochemical profile. This last profile was able use metabolize the most common nectar sugar as well as the sugar described as toxic or poorly assailable to bees. Moreover, the RAPD-biotype Lpla.IX showed α-glucosidase, β-glucosidase activities and possess esterase and esterase lipase enzymes. Probably, it is reasonable to assume that this metabolic complexity makes the biotype Lpla.IX particularly versatile and adaptable to the different assayed environments. This biotype is particularly interesting playing a simultaneously role in the breakdown of complex polysaccharides and metabolize toxic sugars, the role of these *Lp. plantarum* strains in improving dietary tolerance as well maintaining the health of their hosts might be notable. Finally, also the RAPD-biotypes of *F. fructosus*, as reported in Figure 3.5, were characterised by specific biochemical profile highlighting a discrete biodiversity.

Of interest was the RAPD Lpla.IX biotype that showed α-glucosidasic, β-glucosidasic as well as esterase activity. Enzyme activities that could play an important role in conditioning the ruminal activity and consequently milk quality.

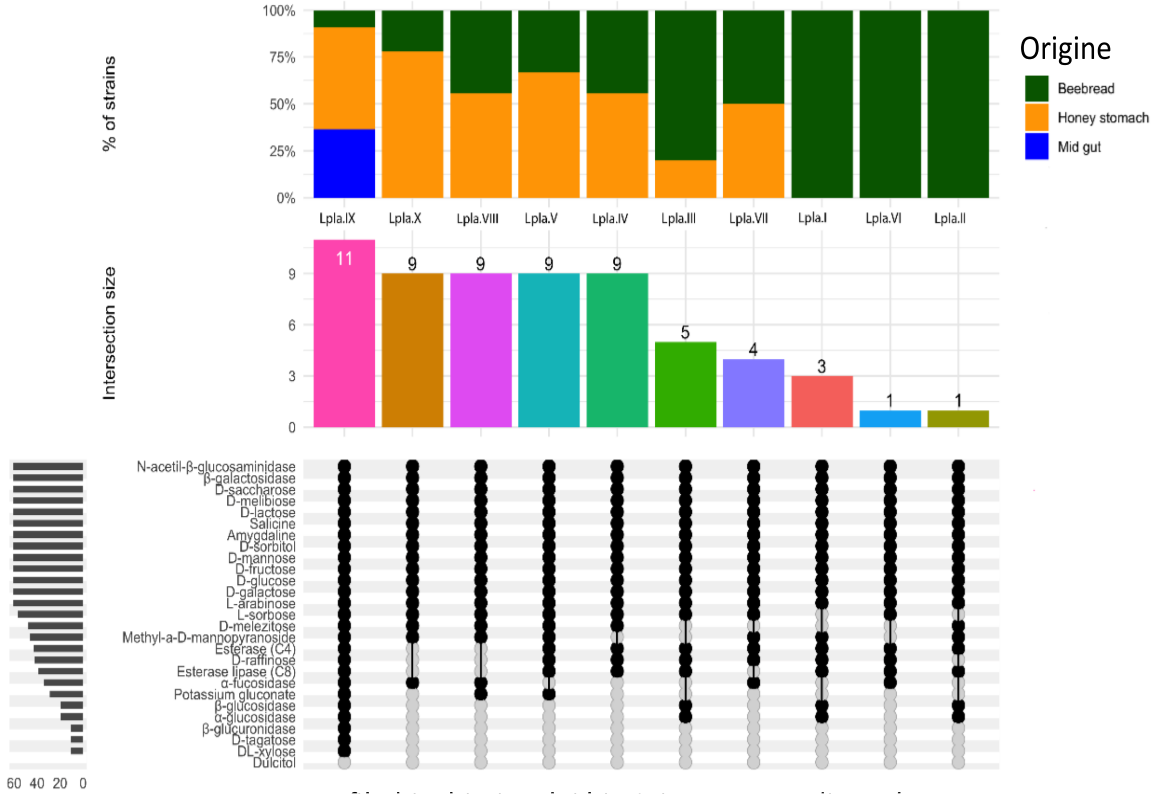

Figure 3.5 - Upset plot regarding the biochemical profile of the 10 RAPD_biotype groups belonging to the specie *Lactiplantibacillus plantarum*. Vertical barplot indicates the number of overlapping strains (intersection size) for each intersection represented by the connected dots. The intersection matrix is sorted in descending order. The grey horizontal barplots, reported as "Set size", indicate the number of strains showing enzymatic activity.

3.3.2.3 Antimicrobial activity expressed by Lactiplantibacillus plantarum strains

Evaluation of antimicrobial activity (Figure 3.6) shows a good ability of the strains to inhibit target microorganisms.

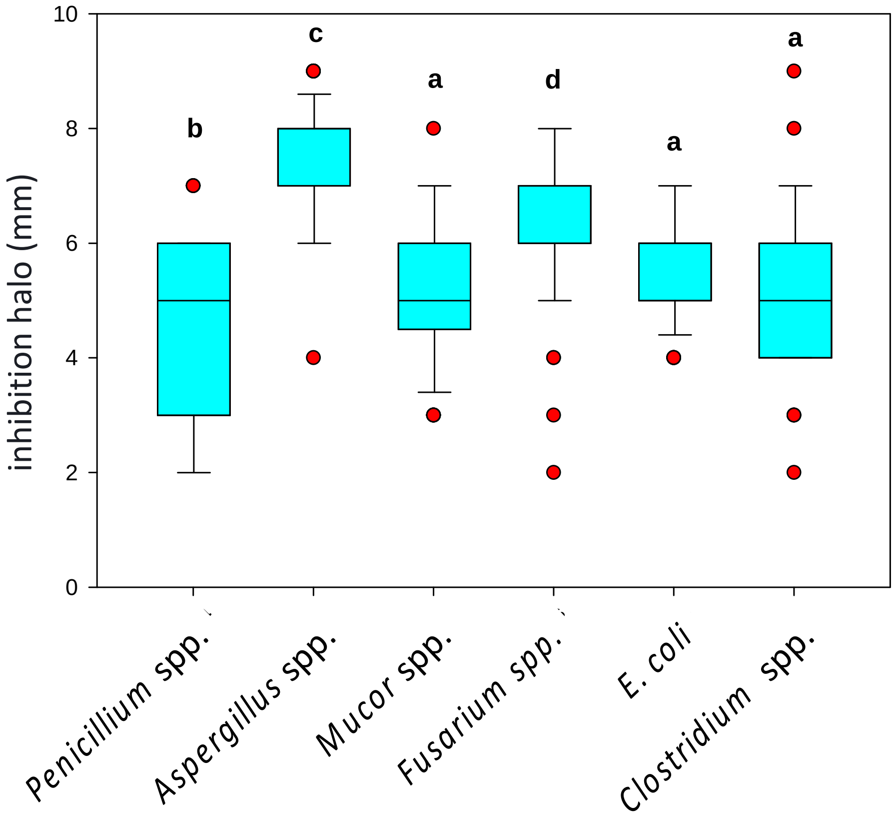

Figure 3.6 - Box plot showing the distribution of the producing strains in relation to the inhibitory activity exhibited against each indicator strain. Regardless of origin and species, all strains exhibited a medium to high inhibitory action against all indicator strains.

Figure 3.6 shows, for each indicator strain, the distribution according to their antagonistic capacity. The strains belonging to the species *Aspergillus* and *Fusarium* showed the greatest sensitivity, and the box plot analysis of the distribution of the producing strains shows a fairly close positioning, testifying to a high degree of homogeneity in behavior. An almost similar situation was also exhibited with regard to strains referable to *Mucor B. cereus* and *Mucor racemosus*. Only the behavior towards *Penicillium* appeared different, against which the producing strains showed a greater distance in distribution.

3.3.2.4 Ability to produce biofilm

The ability to produce biofilm in hard environmental conditions by *L. plantarum* strains isolated from different matrices was investigated. The results in Figure 3.7 are reported as biofilm expression (calculated as reported in previous paragraph) assuming values from – 2 to + 4. In particular, negative values correspond to raw values lower than the average values, while positive values indicate raw values higher than the average values. Moreover, the heat map also highlighted that the biofilm formation did not depend on the type and presence of cultural conditions. In fact, the strains grouped in cluster 3 were able to produce biofilm regardless to the presence of cultural conditions. This statement is in accordance with other studies who highlighted that some strains of lactic acid bacteria are naturally prone to form biofilm (Papadimitriou et al, 2016; Limanska et al, 2010). As for the effect of the variable "origin" on the biofilm, some authors evidenced that the biofilm formation is usually affected by various factors such as environmental features, nutrient availability and presence of specific matter (Fysun et al, 2019).

The ability to produce biofilms is of paramount importance as it would not only favour a higher survival capacity of the strains in different environments, but also ensure a better adhesion of the bacteria to the surface of the partially dried forage as well as adhering, once taken in with the diet, to the intestinal epithelium of the cattle.

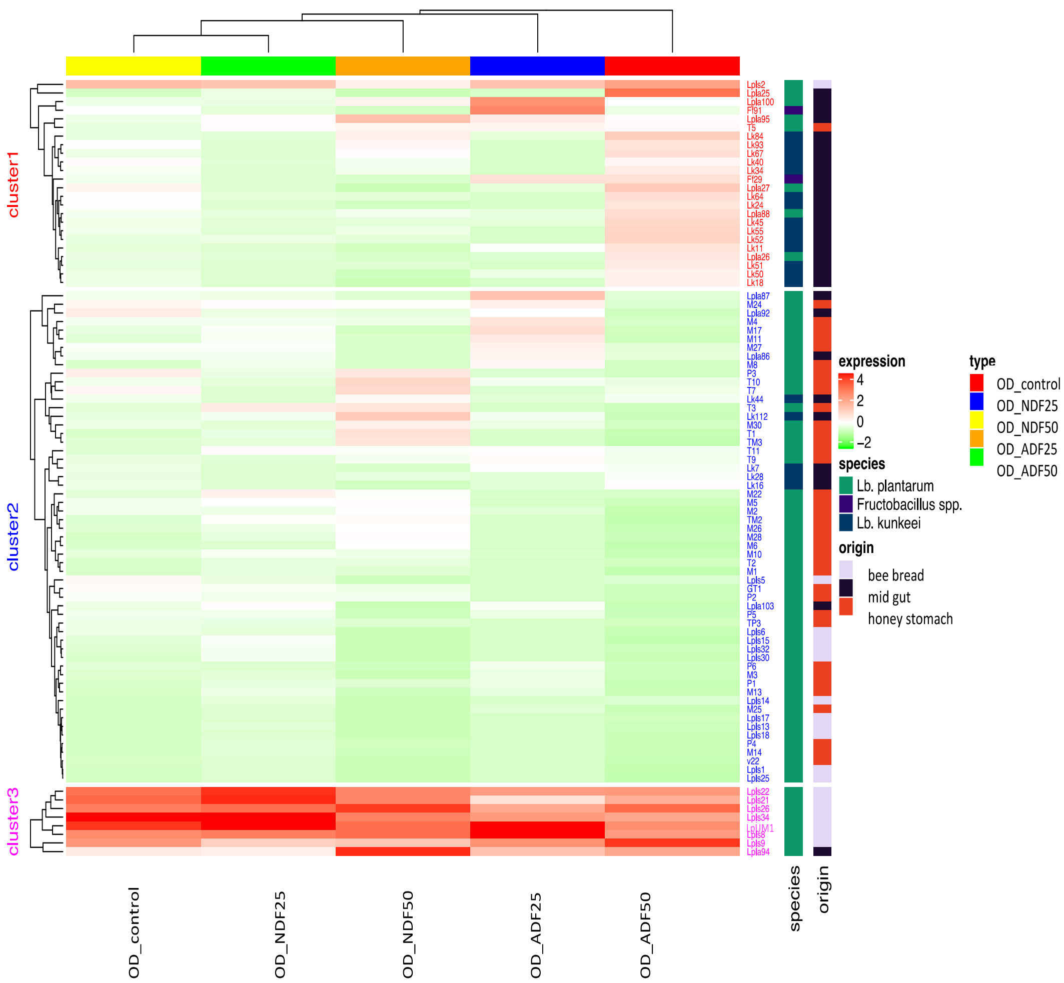

Figure 3.7 - Heat-map related to the production of biofilm (expression) determined on *LAB* strains from different bees origin. The strains were grown for 24 h at 28 °C under different conditions (type): in MRS broth (control), in MRS broth at with 25% (NDF25) or 50% (NDF50) of neutral detergent fiber or with MRS added with acid detergent fiber at 25% (ADF25) or 50% (ADF50).

3.3.2.5 Bioprotective cultures: industrialisation trials and scale up

Based on the results obtained from the screening investigations, four strains were identified, all referable to the species *Lp. plantarum*:

In co-operation with the company Mediterranea Biotecnologie srl, the identified strains were used in industrial process simulation trials that will be successful if they lead to a lyophilised preparation with a high and adequate microbial load. The results regarding the viable load levels of the strains were constantly monitored during the four phases of the industrialisation process: (i) Bioreactor fermentation; (ii) Cell concentration; (iii) Lyophilisation.

In detail, pilot-scale fermentation was conducted in a 5-litre bioreactor by applying the process specifications (agitation, pH control by basic use) stipulated in the company protocol. The development kinetics and related kinetic parameters are shown in Figure 3.8 and Table 3.1, respectively. The analysis of the kinetic parameters shows that the bioreactor fermentation conducted at constant pH resulted in an increase in the final level of cell accumulation.

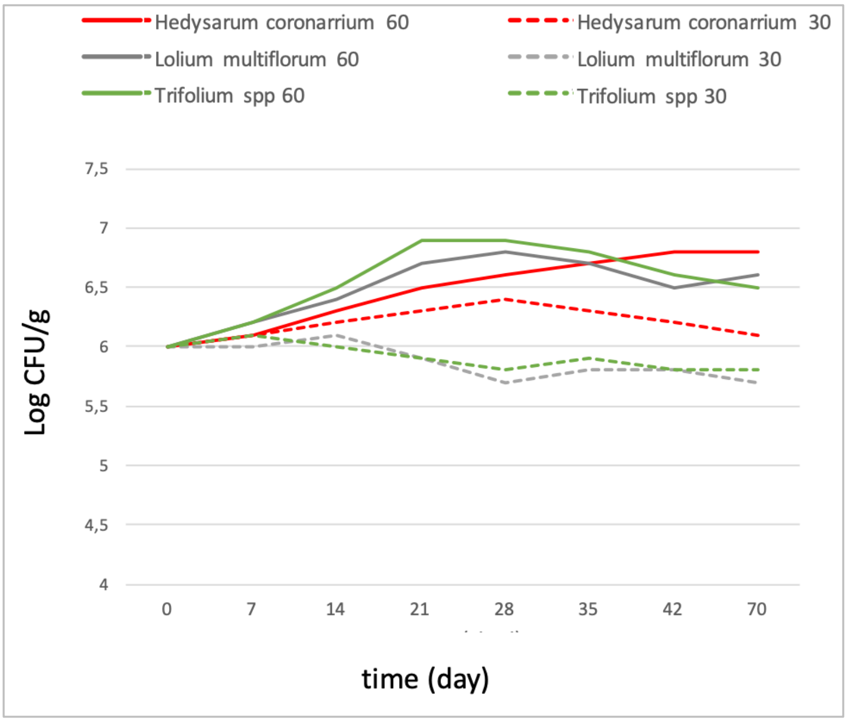

Figure 3.8 - *Lp. plantarum* LpUM1 cells in three forage species, *Hedysarum coronarium* (sulla), *Lolium perenne* (ryegrass), *Trifolium* spp. (clover) pre-dried until a moisture level of approximately 60% and 30%.

Table 3.1 - Growth kinetics parameters of the 4 strains of *Lb. plantarum* during pilot-scale fermentation under constant pH conditions.

ceppo	μ_{max} (h-1)	Lag (h)	Y_0 (Log ufc/mL)	Y_{end} (Log ufc/mL)	se(fit)	R^2
Lpls_22	0,13 (± 0,02)[a]	nd	8,0 (± 0,3)	10,1 (± 0,1)[a]	0,102	0,981
Lpls_9	0,14 (± 0,01)[a]	nd	8,0 (± 0,2)	10,3 (± 0,3)[a]	0,081	0,989
Lpa_94	0,19 (± 0,02)[b]	2,3 (± 0,2)	7,7 (± 0,4)	9,8 (± 0,5)[a]	0,072	0,992
LpUM1	0,18 (± 0,00)[b]	nd	7,8 (± 0,2)	10,2 (± 0,2)[a]	0,121	0,981

The highest growth rate was again shown by strain LpUM1, which was significantly similar to that exhibited by strain Lpls_22. Thus, the latter strain under constant pH conditions exhibited a lower reduction in growth speed. A reduction in maximum growth speed, compared to fermentation conditions at uncontrolled pH, was also appreciated for the Lpls_22 and Lpa_94 strains, which showed the lowest values.

Data evidenced that the best performance appears to be exhibited by the LpUM1 strain, which already exhibited the highest levels of charge significantly higher than those exhibited by the LpE_meb strain after 14 hours.

The concentration of the cells was preliminarily obtained by centrifugation at a temperature of 4°C. This operation allowed the separation of the biomass from the spent culture substrate. The product obtained, after addition of maltodextrins in order to exert a protective action against thermal stress, underwent lyophilisation treatment.

Examination of the data shows that both concentration and freeze-drying resulted in little if any cell damage. In fact, after the concentration process for all strains, no significant changes in cell number were appreciated. The freeze-drying process produced a significant decrease in cells for the Lpls_22_ and Lpa_94 strains. A reduction, even if slight was also appreciated for Lpls_9. While for the strains LpUM1 no reduction was appreciated.

3.3.2.6 Selection of the bioprotective culture for haymaking and validation on a laboratory scale

The results shown in Figure 3.8 highlighted an excellent performance of the strain when used in fodder dried to 60% moisture showed good viability, even showing significant growth in the first part of the storage period. Forage dried to 60% appears more hostile and does not allow an increase in the load of the intentionally added microorganism. However, even in this case, good strain viability should be noted.

3.3.3 Conclusions

Based on the results reported in this Chapter, the LpUM1 strain isolated from bee bread is not only an indirect marker of environmental quality, but also possesses important enzymatic, antagonistic and technologically interesting activities. In light of the results, the LpUM1 strain isolated from bee bread is not only an indirect marker of environmental quality, but also possesses important enzymatic, antagonistic and technologically interesting activities.

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Chapter 4

Biotechnological Innovation effect on animal welfare and milk quality

The high demand for milk and milk products, due to the nutritional value of milk itself as well as the growth of the human population, has led to a growing interest and concern about the quality and safety of milk products (Muehlhoff et al, 2013). Environmental factors (e.g. seasons, geographical origins) and nutritional factors (feeding systems) can cause variations in milk composition and yield. With the increasing consumer demand and expectation for milk with high nutritional value and the control of geographical origins and feeding schedules, they are becoming increasingly important.

4.1 Feeding and milk quality relationship, an overview.

Several environmental factors influence milk quality: for example, the yield and chemical composition (e.g. fat) of milk can vary over the seasons, especially with high temperatures in summer (Yano et al, 2014), and according to geographical characteristics such as different regions and altitudes (Pu et al, 2021). Indeed, environmental factors are closely related to nutritional factors, as forage composition can change according to geographical origin and seasons, affecting dairy animals and their milk production (Schwendel et al, 2015). From a nutritional perspective, feeding systems, such as conventional and organic treatments, are considered the main agents determining milk quality and have been studied by several groups (Magan et al, 2019; Manis et al, 2021; Mazzei and Piccolo, 2018; Rocchetti et al, 2020; Toso et al, 2002). Milk samples collected in summer season under high temperature contained a total of 53 differentiating metabolites including higher levels of carbohydrates (e.g. lactate, pyruvate, citrate, galactose-1-phosphate) and lower levels of lipids (phosphatidylcholines (PC), sphingomyelins (SM), monoglycerides (MG), diglycerides (DG), triglycerides (TG), etc.) and amino acids (isoleucine, glycine, proline, orotic acid) compared with those in spring season (heat-stress free), showing altered metabolic pathways of carbohydrates, lipids and amino acids in cattle under hot weather. In addition, the level of trimethylamine derived from gut microbiomes was found to significantly change under hot weather, implying high temperature may induce perturbation of gut microbiomes in cattle. Seasonal variations in lipid content in cattle milk were also confirmed by another group using LC-MS (Liu et al, 2017). In this study, polar lipids (PC, SM, phosphatidylethanolamines (PE), etc.) were shown to gradually increase over milking season (spring to autumn). However, it was not clear whether there was heat stress during the season. Metabolic difference in milk from buffaloes reared at different altitudes (low, medium and high) were evaluated using liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) (Pu et al, 2021). The milk from low and medium altitudes had higher abundance of amino acids (e.g. pyroglutamic acid, glutamine) and micro-nutrients (pyridoxine; vitamin B_6) than milk from high altitudes. The low altitude-derived milk contained relatively high levels of carbohydrates (mannose-1-phosphate (Man-1P), glucose-1-phosphate (Glc-1P), Gal-1P, etc.) involved in amino sugar, nucleotide sugar and galactose metabolism, and the high altitude-derived milk had relatively high levels of free fatty acids (dodecanoic acid, caprylic acid, capric acid, palmitic acid, myristic acid, etc.) associated with the fatty acid biosynthetic pathway. Results indicated low altitude-derived milk may be suitable for functional milk with ample nutrients (sugars, amino acids, vitamins, etc.), while high altitude-derived milk may be for raw milk to produce dairy products with abundant fatty acids. An NMR-based metabolomics was used to discriminate milk from different farms (large-scale distribution and local farms) (Tenori et al, 2018). Although a partial least square and canonical analysis (PLS-CA) statistical model provided some useful information to predict the type of diet adopted with respect to the metabolic profile further investigation may be required for a solid conclusion.

4.2 Feeding trial: administration of innovative hay to lactating cows

In our age dominated by climate changes, hay making is extremely vulnerable so that the propensity toward ensiling preservation of forages, legume in particular, has increased in the last decennials (Killerby et al, 2022). In addition to skepticism toward ensiling forages, quite common in inner areas of Centre-South Italy, silages are certainly less marketable than hay and their production implies the cost of specific machinery as well as of plastic film to wrap the dried forage. In hay making, however, additives can be used in order to limit dry matter losses occurring at harvesting and during storage (Killerby et al, 2022). Among them, chemical preservatives are reported to be generally more efficient in limiting spoilage than microbial additives even though efficiency depends on many factors, including type of forage (grass better than mixed hay and legume), dry matter content, and dose used. On the contrary, microbial inoculants are considered safe for workers and do not harm machineries (Killerby et al, 2022). In order to test the production and the use of innovative fodder in dairy cows' diet reared in the Alto Sannio area, sun-dried hay was produced during the spring-summer season within the introduction of biotechnological innovation.

The biotechnological innovation is linked to the use of microbial culture isolated and selected from the gastroenteric trait of honeybee foragers (*Apis mellifera ligustica* S.) with the main aim of enhance the relationship between dairy cattle farming, land cultivation, feed production and sustainability and biodiversity of Alto Sannio Area.

The isolated microbial strain is represented by *Lactiplanctibacillus plantarum* (LPUM1) whose characteristics have been presented and discussed in the previous chapters of this thesis work.

In the following paragraphs will be reported the preliminary data and results of the introduction of the innovative hay in dairy cows' diet, highlighting the effects of the use of this hay on some welfare traits of dairy cows and their nutritional profile.

4.2.2 Materials and methods

4.2.2.1 Innovative hay production

A total of 3 hectares of traditional sun-dried meadow biomass composed in average by 40% of *Hedysarium coronarium* (L.), 30% of *Trifolium* spp. (L.) and 30% of *Lolium perenne* (L.) was selected and used for the on-field trial.

In detail, forage with an appropriate moisture content (17%) was inoculated with a suspension containing the LPUM1 strain in order to reach a concentration of 10^5 - 10^6 CFU per gram of forage. The manual and intermittent distribution of the bacterial suspension on the naturally dried forage was carried out just before the baling operations. The average weight per bale was 350 kg.

To create a control batch, a similar portion of dried forage from the same land and with the same botanical composition was harvested without inoculum. After three months of storage, the bales were progressively opened and their state of preservation, as well as their chemical and microbiological quality, was assessed.

4.2.2.2 Feeding trial and animal data

In the chosen dairy farm (medium farm) lactating Simmental cows were selected and divided in two homogeneous groups named control group (CTR, n=4) and experimental group (EXP, n=4). Isonitrogenous and isoenergetic diets were formulated according to the nutritional needs of the animals (NRC, 2001). Cows were individually fed with a diet characterized by a ratio forage to concentrate on dry matter (DM) basis equal to 60:40. More in detail, all animals were fed with the same common mixture of concentrate (Table 4.1) while forage administered *ad libitum* varied according to the group, i.e., CTR group were fed with the traditional sun-dried hay produced in the selected farm, while in EXP group the traditional hay was partially (50%)

and subsequently totally (100%) replaced by the sun-dried innovative hay inoculated with LPUM1 strain.

Each lactating cows received daily an average of 9.0 kg DM of concentrate mixture divided in two meals, in morning (07:00 a.m.) and in the afternoon (05:00 p.m.).

Table 4.1 – Mixture of concentrate administered to CTR and EXP group.

	Concentrate mixture, %
Ingredients	
Corn meal	25.53
Barley meal	28.53
Commercial mixed feed*	14.26
Durum wheat bran	14.26
Commercial mixed feed**	12.38
Mineral feed[§§]	1.14
Buffer[§]	0.90

* Labelled ingredients: corn meal, wheat bran, wheat middling, sunflower meal, barley meal, dry corn grains, calcium carbonate, sugar cane molasses, sodium bicarbonate, sodium chloride, hydrogenated fats, soybean oil, dicalcium phosphate. Composition of compound feed (percentage, as fed): Protein: 15.0, Fat: 5.50, Crude Fibre: 7.50, Ash: 7.50, Sodium: 0.50.

** Labelled ingredients: dehulled soybean meal, dehulled sunflower meal, glutinate corn meal, wheat bran, wheat middling, molasses, sodium bicarbonate, hydrogenate palm fatty acids, calcium carbonate, barley, sugar cane molasses, sodium chloride, dicalcium phosphate, magnesium oxide. Protein: 34.0, Fat: 4.00, Crude Fibre: 8.69, Ash: 10.59, Sodium: 1.00, Magnesium: 0.55.

[§] Labelled ingredients: calcium and magnesium carbonate, mono-dicalcium phosphate, magnesium phosphate, sodium bicarbonate, barley meal, alfalfa protein concentrate, dried *Saccharomyces cerevisiae*. Composition of compound feed (percentage, as fed): Calcium: 17.0, Phosphorus: 3.2, Sodium: 7.5, Magnesium: 7.5.

[§§] Labelled ingredients: sodium bicarbonate, wheat bran, calcium carbonate, magnesium, common wheat. Composition of compound feed (percentage, as fed): Magnesium: 9.5, Sodium: 9.0, Calcium: 8.7.

The feeding trial lasted overall 48 days, including 13 days of adaptation to the experiment. More in detail, during the adaptation phase, the common hay administered to CTR group was gradually replaced by the inoculated hay until a replacement rate of 50% in the EXP group. However, for the following two weeks the same ratio conventional to inoculated (50 : 50) hay was replicated. After that, for the last 21 days of the feeding trial, 100% of conventional hay was replaced by 100% of inoculated hay.

4.2.2.3 Effect of innovative hay on animal welfare

During the feeding trial, at day 13, 27, 42 and 48, samples (n=20) of inoculated and traditional hay were collected and were subjected to microbiological and chemical analyses. According to the official methods (AOAC, 2000), dry matter (DM), crude protein (CP), ash, neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were investigated in both theses.

In order to evaluate the welfare status of the cows involved into the feeding trial, at day 0 and 48 all cows were scored for their body condition (using a scale from 1 to 5, 1=very thin; 5=very fat; Roche et al., 2009) and for the fecal consistency (using a scale from 1 to 5, 1=firm, hard feces; 5=liquid consistency; Hall et al., 2002). Moreover, at the end of feeding trial, during a routine veterinary check blood samples were collected by jugular venipuncture using EDTA-treated vacuum tubes. Plasma analyses were conducted by Istituto Zooprofilattico Sperimentale del Mezzogiorno (Salerno, Italy) following the standardized analytical methods (MP/SA/041 Rev.0 2021), to investigate uric acid, total cholesterol, triglyceride, creatinine, lipase, total protein, urea, iron, magnesium, inorganic phosphorus, potassium, sodium, aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) contents.

4.2.2.4 Effect of hay on milk quality

Milk samples

Milk samples, from the morning milking of the two groups (SFI and CTR) subjected to the experiment, were taken at different times (t1, t2, t3, t4) at regular 15-day intervals and placed in sterile falcons, refrigerated at optimal temperatures and transported to the DiAAA laboratories where they were analysed. The milk samples were subjected to microbiological analyses, aimed at assessing the presence of the main microbial groups of dairy interest, and chemical analyses, aimed at assessing the centesimal composition and fatty acid profile as well as estimating the different microbial populations.

Microbiological analyses

In order to assess the charge levels of the main microbial groups capable of colonising refrigerated raw milk, microbiological analyses were carried out on freshly collected milk samples transported to the laboratory. To this end, appropriate decimal dilutions were prepared using sterile physiological saline (NaCl 9 g/L) and the dilutions thus obtained were inoculated, to the extent of 1 mL, into selective substrates respecting the growth conditions.

In detail, the charge levels of lactic acid bacteria on MRS after incubation for 72 hours at 28 °C, those of total mesophils on PCA after incubation at 28 °C for 56 hours, those of total coliforms on VRBLA plates after incubation at 37 °C for 56 hours and those of yeasts on YPD after incubation at 28 °C for 56 hours were evaluated.

Milk chemical composition

- Ash

For the determination of ash, the milk sample (10 g) was dried, carbonised and calcined in a muffle furnace at 525 ± 25°C. The weight, constant values obtained after calcination represent the ash content expressed in percentage terms as follows

$$\% \, ash = \frac{\mathrm{M2 - m}}{\mathrm{M1 - m}} \, x \, 100$$

where:

m = weight of the capsule in grams;

M1 = weight in grams of the capsule + sample;

M2 = weight in grams of the capsule + ash;

The data obtained are acceptable if they have a repeatability of less than 0.02 g ash per 100 g product.

- Protein

For the determination of total nitrogen, the classical Kjeldahl method was used. It is based on the mineralisation of the nitrogen in the feed, i.e. its transformation into ammonia by heating the organic substance with concentrated sulphuric acid, distillation of the ammonia itself in an alkaline environment and its acidimetric determination. The distillation was carried out using a Kjeldhal steam distillation apparatus, through which approximately 50 mL of 50% sodium hydroxide was added. The distillate (about 200 mL) is collected in an Erlenmeyer flask containing 25 mL of 0.1N sulphuric acid to salify the ammonia gas. The excess sulphuric acid is titrated with 0.1N sodium hydroxide using a screened indicator (methyl red 0.2 g and methylene blue 0.1 g in 100 mL of methanol) with violet to green tint.

The percentage of total N is obtained with the following relation:

% N tot=14.01 ·(x-Y)·100/z

where:

X= mL of H_2SO_4 0.1N used for distillate collection;

Y= mL of NAOH 0.1N used for titration;

Z= weight in milligrams of milk (to be calculated knowing the density value).

The percentage of protein is obtained by multiplying the percentage of total N by the typical protein conversion factor for dairy products, which is 6.38.

% Protein=N tot·6.38

The data must have a repeatability of less than 0.05 g total protein per 100 g product.

- Sugars

The sugar content was determined by difference, and was expressed as g on dry weight.

- Fat

The fat content was determined by means of the Gerber method, which requires the use of an acidobutyrometer of the same name. It is based on the separation of milk fat by treatment with a mixture of concentrated sulphuric acid and isoamyl alcohol. The acid coagulates and then dissolves the proteins and, acting as a dehydrating agent, partially carbonises the same proteins and sugars, giving a brown-coloured solution. The fat, on the other hand, remains unchanged and undissolved and can thus be dosed after centrifugation. The Gerber butyrometer is a special glass tube with one end closed and the other fitted with a rubber stopper. The narrow (closed) end is graduated so that each division corresponds to 0.10 per cent fat in the milk.

The test is carried out in duplicate using two butyrometers, into each of which 10 mL of sulphuric acid, specific for Gerber determinations with a density of 1.82 at 15°C (90-91% w/w), is introduced by means of a graduated pipette, taking care not to wet the inner walls, and 11 mL of milk taken with a pipette, taking care that it stratifies on the acid without mixing. Immediately afterwards, 1 mL of isoamyl alcohol (density 0.815-0.818 g/mL; boiling point 124-130°C) is added. the butyrometers are closed with the appropriate rubber stoppers and shaken. Strong heating and browning of the mixture occur. If the coloration was violet, this would indicate the presence of formaldehyde added as a preservative (addition prohibited by law). The butyrometers are then placed in the centrifuge (previously heated) with the graduated part facing upwards. After 2 minutes of centrifugation, the percentage of fat is read directly on the graduated part. The result is expressed directly as g fat per 100 mL milk.

The repeatability is related to the scale of the butyrometer, which has ranges in the graduation of 0.1 g fat per 100 mL of product. The fat value by law must not be less than 3.2 % for whole milk. For semi-skimmed milk the value is between 1.0% and 1.8%, while for skimmed milk the limit is set at 0.5%.

- Fatty acid profile determination

Fatty acid composition was determined by high-resolution gas chromatography, analysing the fatty acid methyl esters obtained by trans-esterification of approximately 25 mg of each fat sample, previously dissolved in 2 mL of petroleum ether, and using 2 mL of BF_3-methanol reagent. Specifically, a Fisons model MFC800 gas chromatograph (Fisons, Milan, Italy) equipped with a 60 m x 0.32 mm i.d. fused silica capillary column and 0.5 µm Stabilwax film thickness (Restek, Bellefonte, PA, USA) was used. The following conditions were applied: (i) oven: 5 minutes at 170 °C, followed by heating (1 °C/min) to 220 °C, where the temperature was maintained for 30 minutes; (ii) carrier: helium 20 cm/s at 170 °C; (iii) injector: 250 °C, 1 µL; split 40:1; (iv) detector: FID, 250 °C. Results were expressed in mg/g sample.

4.2.2.5 Statistical analyses of the results

All data were subjected to descriptive statistic (Microsoft© Excel© ver. 1903 -11425.202429) and results are reported as mean ± standard deviation (SD). Data on plasma constituents were processed by analysis of variance considering the covariate effect "age at d 48" (GLM, ANCOVA).

4.2.3 Results and Discussion

4.2.3.1 Forages characterization

Lactiplanctibacillus plantarum (LpUM1) using during the production of inoculated forage was characterized by a ready growth in the hay, showing a charge level about 3 logarithmic cycle higher than those detected in control hay. Compared to inoculated hay, control hay was characterized by higher presence of both fungi and clostridia, respectively by 3 and 4 logarithmic cycles (Caturano et al, 2023).

As far as the composition of hays is concerned, inoculated hay was characterized respectively by 82.5 (±0.62, SD) g/100g of DM content, 13.1 (±0.6) g/100g DM of crude protein, 11.3 (±0.51) g/100g DM of ash, 51.0 (±1.72) g/100g DM of NDF and 40.9 (±0.59) g/100g DM of ADF contents. In Figure 4.1 is showed the comparison between chemical composition of inoculated and control hay, characterized by 87.0 (±0.67, SD) g/100 g of DM content. As reported (Figure 4.1) inoculated hay showed higher crude protein, ash and ADF content compared to control hay. However, DM content of both forages was lower than DM content (84.3 g/100g) reported for mature hay predominantly composed by legume plants (NRC, 2001).

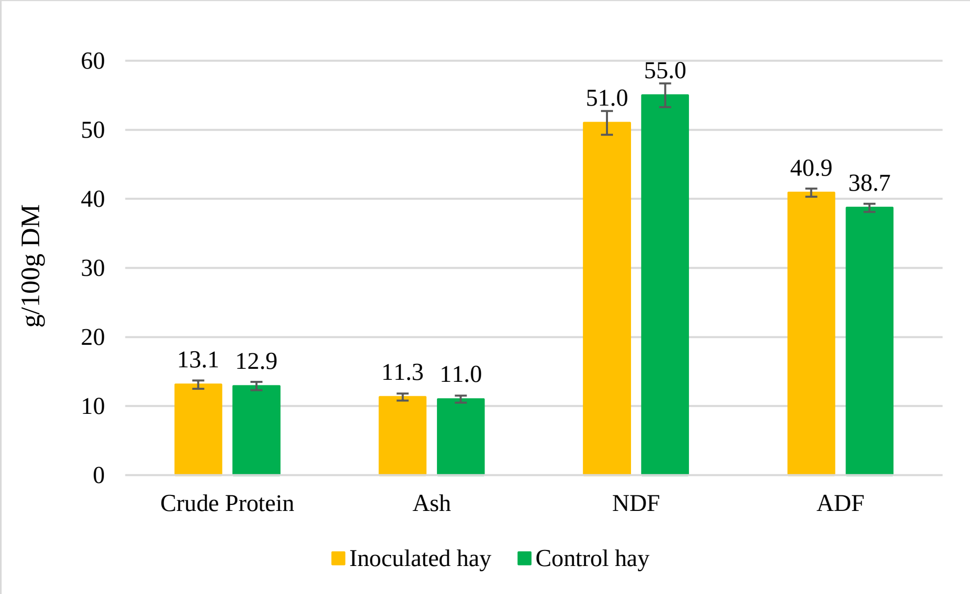

Figure 4.1 – Inoculated and control hay crude protein, ash, and fiber components (NDF, ADF) average values (± SD).

Considering the chemical composition reported for mature hay predominantly composed by legume plants (NRC, 2001) it should be noted that both inoculated and control hay were characterized by lower content respectively of crude protein (17.2 g/100g DM) and ADF (41.5 g/100g DM), and by higher content of ash (8.7 g/100g DM). Furthermore, inoculated hay and control hay showed respectively lower and higher level of NDF compared to literature data (53.6 g/100g DM) (NRC, 2001).

It is interesting noting that a higher NDF content was observed in legume than in grass inoculated hays as a possible effect of the buffering capacity of legume that counteracts the organic acids produced during fermentation (Killerby et al, 2022). The Authors however also report a higher preservation action of microbial additives, mainly lactic acid bacteria, when the dry matter content is lower than 20 g/100 g probably as a consequence of a low competition with inoculated bacteria and their production of metabolites.

4.2.3.2 Animal welfare and cows' nutritional profile

The effects of different hays on the welfare traits of lactating dairy cows are reported in Table 4.2.

Table 4.2 – Average values (±SD) of control and experimental group (n=4) body condition and fecal consistency of dairy cows.

	Control group	Experimental group
Body condition		
0 d	3.88 (±0.25)	3.81 (±0.24)
48 d	4.00 (±0.00)	3.75 (±0.29)
Fecal consistency		
0 d	3.38 (±0.48)	3.25 (±0.29)
48 d	3.75 (±0.29)	3.63 (±0.25)

At the end of feeding trial EXP group showed slightly lower body condition compared to CTR group, however both values were higher compared to value of 3.65 and 2.45 reported respectively for cattle after 0 and 72 days after calving (El-Kasrawy et al, 2020). It seems essential to highlight that body condition score in lactating animals is affected by the lactation period (days in milk after calving), however lactating cows generally shows the best productive and efficiency status when body condition score ranges between 2.5 and 4 (Carvalho et al, 2014; Zachut and Moallem, 2017).

The evaluation of fecal score could provide significant information about ruminant digestion processes and welfare status, allowing the diagnosis of early fermentation problems (Stallings, 1998; Hall, 2002) not detected during the experiment. However, control group showed a tendential higher fecal consistency compared to experimental group. Nevertheless, manure appeared formed by solid pat generally 3-4 cm in height.

Regarding the nutritional profile of the selected cows, plasma constituents showed in Table 4.3 were within or very close to the normal ranges reported in literature for adult bovine (Sako et al, 2007; Kaneko et al, 2008).

Significant differences between the two dietary treatments were observed only for cholesterol and inorganic phosphorus (Table 4.3). Cholesterol was higher in EXP group compared to CTR (4.60 vs. 2.92 mmol/L, $P<0.05$) but lower than 5.1 mmol/L, reported by Sako et al (2007). Similarly, inorganic phosphorus was higher ($P<005$) in EXP group (1.88 mmol/L) compared to CTR group (1.36 mmol/L) but within the normal range (minimum-maximum) of 1.81 – 2.10 mmol/L reported in literature (Kaneko et al, 2008).

Cholesterol concentration in bovine blood is mainly associated to diet, age, physiological state, and production attitude. Cholesterol constitutes one third of the lipids of the blood, is synthesized in the liver from acetyl-CoA and is excreted with the bile. Accordingly, there is a reciprocal relationship between cholesterol consumed and the hepatic synthesis activity; so that, the increase in cholesterol can related to a limited ability of the liver to remove and catabolize cholesterol (Kaneko et al, 2008). Moreover, it is the precursor of steroid hormones, vitamin D, bile acids and is a constituent of cell membranes (Kaneko et al, 2008). Cholesterol blood level depends on lactation phase, being higher in early lactation (Stefani et al, 2011).

Phosphorus represents part of inorganic substances of bone and cartilage tissue, and is important constituent of biologically active phosphorylate substances, as nucleic acids, phosphorylate proteins and ATP (Stefani et al, 2011). It regulates the acid-base balance of body fluids and is homeostatically balanced with calcium. Levels of inorganic phosphorous varied in bovine blood especially in relation to lactation phase, being higher in late than in early lactation, parity, being lower in multiparous cows, and season, being higher in winter than in summer (Stefani et al, 2011). An excess of phosphorus it could be related to diet, hormonal equilibrium, and kidney disease.

Table 4.3 – Average values (±SD) of plasma constituents for control and experimental cows.

Plasma parameters	**Unit**	**CTR**	**EXP**	**SEM**	**P-value**
AST	U/L	63.1	76.9	4.77	0.114, ns
ALT	U/L	51.4	60.1	9.19	0.560, ns
GGT	U/L	26.1	22.6	2.41	0.374, ns
Glucose	mmol/L	4.50	4.38	0.25	0.779, ns
Cholesterol	mmol/L	2.92	4.60	0.40	0.041, *
Triglycerides	mmol/L	0.13	0.08	0.03	0.355, ns
Total protein	g/L	92.0	77.3	4.60	0.089, ns
Amylase	U/L	319.3	373.9	38.13	0.387, ns
Creatinine	mmol/L	138.0	145.5	8.93	0.602, ns
PUN	mmol/L	8.43	6.92	1.66	0.575, ns
Uric acid	mmol/L	24.8	46.6	8.78	0.161, ns
Total bilirubin	µmol/L	0.42	0.69	0.36	0.649, ns
Ca	mmol/L	2.61	2.37	0.10	0.166, ns
P	mmol/L	1.36	1.88	0.13	0.046, *
Mg	mmol/L	1.23	1.28	0.06	0.586, ns
Fe	µmol/L	20.6	23.1	3.62	0.667, ns

CTR=control group; EXP=experimental group; AST=aspartate aminotransferase; ALT=alanine aminotransferase; ALP=alkaline phosphatase; GGT=gamma-glutamyl transferase. * $P < 0.05$; ns = not significant. All values are expressed as the means and pooled SEM.

4.2.3.3 Microbiological Quality

The analysis of the results regarding the microbiological quality of the different milks reveals a substantially good microbiological quality with regard to hygienic quality (Figure 4.2). The coliform load levels in both samples did not exceed 100 CFU/mL (data not shown).

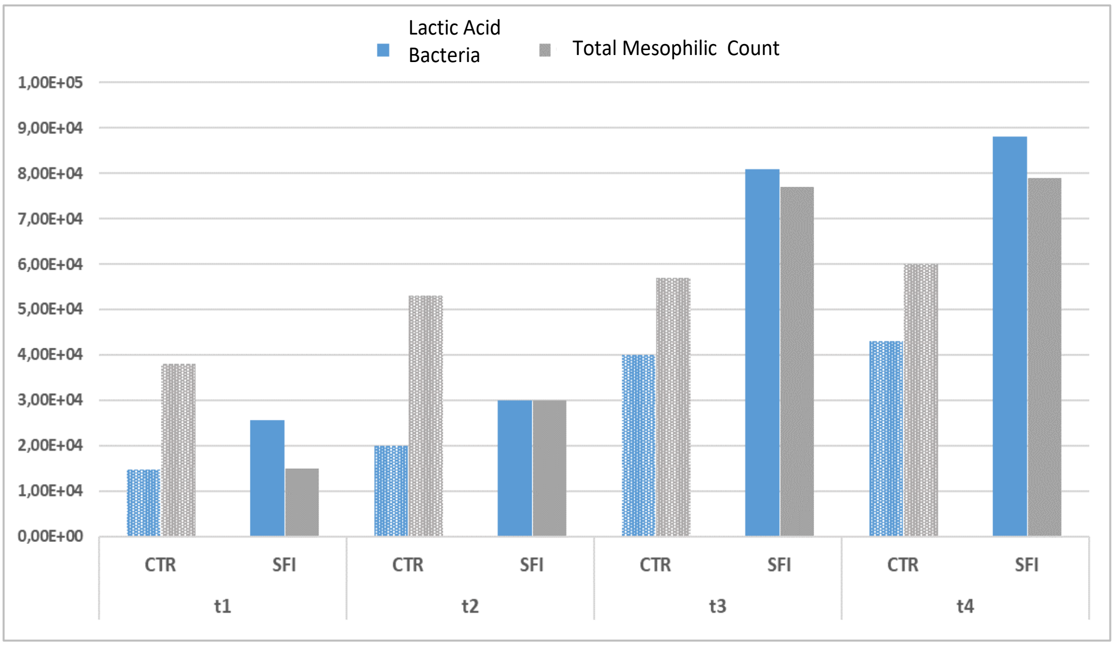

Figure 4.2 - Lactic acid bacteria count and total mesophilic count on CTR and SFI milk samples analysed at different times.

As shown in Figure 4.2, above, the milk samples from the control group (CTR) showed an initial lactic acid bacteria count of 1.47 E+04UFC/mL and a total mesophilic count of 3.8E+04 CFU/mL. The microbiological analyses conducted, with a 15-day repetition, showed values tending to increase slightly and then remaining more or less constant, both for lactic acid bacteria and total mesophilic count.

Analyses conducted on the SFI samples, on the other hand, showed that the lactic acid bacteria had an initial count (t1) of 2.57E+04 CFU/mL that increased over time almost by a logarithmic cycle. Similarly, the mesophilic bacterial load, starting from values of 1.50E+04 CFU/mL shows a slight increase in subsequent samplings, with higher values than in the control samples.

4.2.3.4 Milk chemical composition

Ash, fat and protein contents detected in the milk samples during the trail was reported in Figure 4.3. There are not differences between samples sampled and analysed at different time (t).

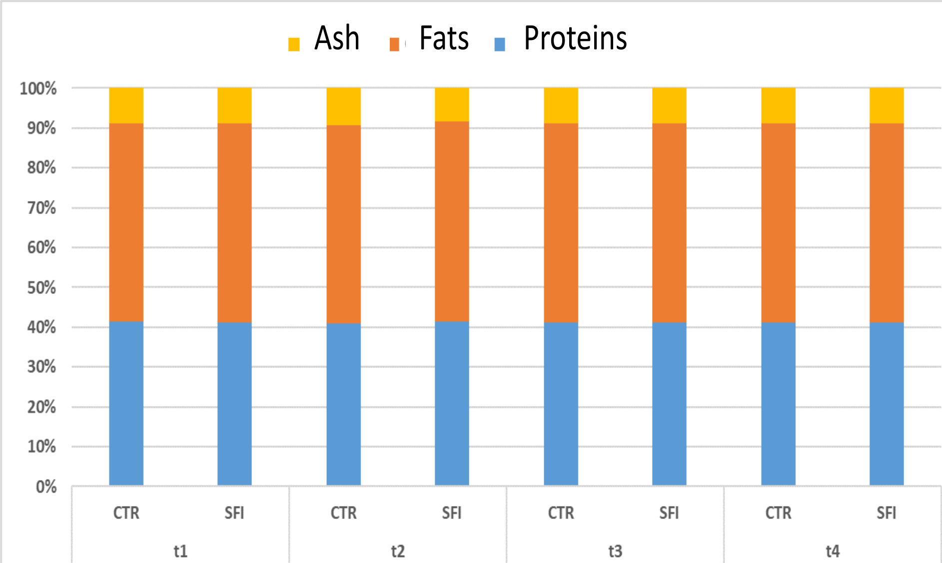

Figure 4.3 – Ash, fat and protein content of CTR and SFI milk samples analysed at different times.

4.2.3.5 Fatty acid profile

Results about the fatty acid profile of innovated produced milk (SFI) were reported and compared with values investigated for fatty acid profile of milk conventionally produced (Table 4.4).

Table 4.4 - Fatty acids in conventionally produced milk (CTR) and innovating produced milk (SFI).

Fatty Acid		t1		t2		t3		t4	
		CTR	SFI	CTR	SFI	CTR	SFI	CTR	SFI
C4:0	butirrico	1,95	2,32	2,35	2,64	2,22	2,89	2,04	3,22
C14:0	miristico	10,03	10,01	10,72	10,15	9,94	10,01	9,95	9,84
C15:0	pentadecanoico	0,62	0,53	0,57	0,52	0,32	0,57	0,39	0,59
C16:0	palmitico	30,21	29,16	30,18	28,31	30,60	27,47	30,62	26,41
C18:0	stearico	8,91	9,72	8,78	9,71	7,98	9,44	7,44	10,42
	SFA	**51,72**	**51,74**	**52,60**	**51,33**	**51,06**	**50,38**	**50,44**	**50,48**
C18:1	oleico	26,54	26,59	26,73	26,15	29,23	26,06	27,97	26,46
	MUFA	**26,54**	**26,59**	**26,73**	**26,15**	**29,23**	**26,06**	**27,97**	**26,46**
C18:2	linoleico	2,96	2,93	3,06	3,13	3,2	3,52	3,25	3,45
C18:3	a-linolenico	0,69	0,92	0,78	0,86	0,71	0,79	0,62	0,68
	PUFA	**3,7**	**3,9**	**3,8**	**4,0**	**3,9**	**4,3**	**3,9**	**4,1**

On the basis of the results acquired (Table 4.4), it can be seen that a diet based on fodder added with selected starters is able to bring multiple benefits to the final matrix, such as milk, which will in turn be of considerable importance for both the health and economic aspects. An important issue to focus on is the fatty acid content that can be found in the milk of cows fed with such fodder. In subjects that are fed a diet that includes fodder inoculated with

Lactiplantibacillus plantarum LpU1M, a considerable increase in fatty acids with different conformation, such as C18:2 and C18.3, polyunsaturated fatty acids that are very important for human nutrition and health, was recorded.

The fat content of bovine milk is greatly influenced by diet, breed, and lactation stage, and in the milk of healthy animals can vary from 2 to 5 per cent by weight. Most of the lipids in cow's milk (about 95%) are triglycerides contained in fat globules together with traces of other non-polar lipids. Triglycerides are glycerol esters, commonly composed of three fatty acid molecules attached to one glycerol molecule, which forms the backbone. The number of fatty acids esterified with glycerol can vary significantly in cow's milk, and more than 150 different fatty acids have been identified to date, making milk one of the most complex lipids among natural fats (Parodi, 2004). In the complex milk matrix, it is precisely the fat that is the fraction most susceptible to modifications at source (i.e., not caused by dairy technology). This is due to the fact that part of the fatty acids that form triglycerides are not synthesised in the mammary gland but are brought in from the blood and thus derive from the diet, following ruminal and animal metabolism. Important fatty acids for human health include: α-linolenic acid, which has been shown to have a preventive effect on fatal cardiovascular events (Hauswirth et al, 2004); conjugated linoleic acid isomers (CLA); branched-chain acids (branched; BCFA); oleic acid, as well as the aforementioned Omega-3 to Omega-6 ratio.

Several factors can influence the fatty acid profile of milk, most notably diet, breed, genetics, stage of lactation and animal health (Griinari et al, 1998; Elgersma et al, 2006). For many mammalian species, the fatty acid composition of milk reflects the dietary fatty acids composition. Ruminants are partly an exception, as dietary ingested fats are strongly modified by the metabolism of bacteria in the rumen and one of the major modifications concerns the biohydrogenation of polyunsaturated acids (Bauman and Griinari, 2003). However, as diet can markedly influence the bacterial population and ruminal microbiological processes, strong variations in fat content and fatty acid composition are also observed for ruminants in relation to diet. It follows that the fatty acids in bovine milk have two different origins: one originates from absorption from the blood circulation and the other from ex novo synthesis within the epithelial cells of the mammary gland (Bauman and Griinari, 2003). In particular, short-chain (4 to 8 carbon atoms) and medium-chain (10 to 14 carbon atoms) fatty acids derive almost exclusively from ex novo synthesis, whereas long-chain fatty acids (with more than 16 carbon atoms) derive from the uptake of lipids in the circulatory system. The exception is fatty acids with 16 carbon atoms, which can originate from both sources. Increasing the ratio of forages to concentrates in the diet tends to increase polyunsaturated fatty acids (PUFA) and Omega-3 fatty acids and decrease the proportion of saturated fatty acids (SFA) (Dewhurst et al, 2006). Feeding animals fresh green fodder greatly improves these trends and improves the fatty acid profile favorable for human health (Chilliard et al, 2007). Diets rich in starch, ingested by animals fed concentrates and maize silage, result in an increase in C18:1 trans-10 to the detriment of C18:1 trans-11, and also result in an increase in omega-6 fatty acids (particularly C18:2 n-6), thereby decreasing the omega-3/ omega-6 ratio (Griinari et al, 1998). This focus on the fatty acid profile of milk has recently led some dairy companies in various EU countries (e.g. France, Belgium, the Netherlands) to propose a price premium for cow's milk rich in fatty acids with a positive effect on human health (e.g. high concentrations of Omega-3 and PUFA, and low concentrations of SFA) (Borreani et al, 2013).

4.2.4 Conclusions

At the end of this study, we can state that the use of lactic acid bacteria in agriculture, at least as they were used in this case, succeed in improving the centesimal composition of the milk and having a much better fatty acid profile than standard milk, which is useful for its processing and healthier consumption.

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Chapter 5

Final consideration

The results of the study provide useful implications for both decision-makers and operators in the sector, as they contribute to the debate on the role of sectoral policies in supporting innovation in the agri-food chain with particular reference to the dairy one. The study demonstrates that collective planning tools play a crucial role in the creation of both horizontal and vertical synergies, positively influencing the creation of social capital, understood as a complex of social and relational networks. This type of capital favors the creation of bonds that go beyond the purely economic aspect and increase the level of trust and reciprocity. The restricted model is still current and highly performing if properly managed. The management of districts and the correct management of knowledge and experience models for the creation of new innovation proposals is essential. These elements, together with the quality of governance, are able to influence the outcome of innovation and sustainability processes.

A new financial model in the management of companies that takes into account ONE Health is urgent and fundamental. Innovations that take into account the central issues of global health from that of the environment to that of man via animal, plant and microbial biodiversity are capable of producing significantly beneficial effects.

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