Universidad de Oviedo

Official Doctorate Program in Chemical, Environmental and Bioalimentary Engineering

Programa Oficial de Doctorado en Ingeniería Química, Ambiental y Bioalimentaria

MicroRNA profile variations in cow's milk and dairy products according to the production system.

"Variaciones de los perfiles de microARN en la leche de vaca y derivados lácteos según el sistema de producción"

Doctoral Thesis

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Variaciones de los perfiles de microARN en la leche de vaca y derivados lácteos según el sistema de producción	Inglés: MicroRNA profile variations in cow's milk and dairy products according to the production system

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RESUMEN (en español)

La producción lechera en base a pastos mejora el bienestar animal, es más respetuosa con el medio ambiente y además mejora la calidad de la leche. Para proteger este tipo de producción, y con ello a los ganaderos y al ecosistema que forman los pastos plurianuales, se requieren mecanismos de autentificación que garanticen al consumidor el origen y la calidad de la leche que consume. La leche contiene miARN, moléculas que regulan la expresión de genes en eucariotas y sus perfiles podrían variar según factores como la dieta o el ejercicio. En este trabajo se estudiaron los miARN como marcadores de autentificación de la leche y sus posibles propiedades funcionales en la leche en función del sistema de producción.

La primera aproximación fue la secuenciación de los perfiles totales de miARN en leche cruda de tanque de explotaciones lecheras con sistemas de producción extremos, extensivo e intensivo, tanto en las fracciones de la grasa como de las células de la leche. Tras la validación por RT-qPCR, se confirmó que los perfiles de miARN cambian según el sistema de producción, y se identificó al *bta-miR-215* con niveles significativamente más altos en la grasa de la leche intensiva en comparación con su equivalente en extensivo.

En la Cornisa Cantábrica coexisten sistemas diferentes de producción de leche, como una línea continua desde lo más extensivo, representado por el pastoreo, hasta lo más intensivo, representado por granjas con estabulación permanente y gran contenido de silo de maíz y concentrados en la dieta. Para testar la potencial utilidad de los miARN para diferenciar entre los diferentes sistemas de producción de leche presentes en la Cordillera Cantábrica, se incorporaron al estudio nuevos miARN seleccionados de la bibliografía a los previamente obtenidos por secuenciación, y se muestrearon más de 100 ganaderías que, según su manejo, se agruparon en 4 categorías. Se identificaron dos miARN en la grasa de la leche, *bta-miR-155* y b*ta-miR-103*, asociados al pastoreo y al consumo de hierba fresca respectivamente. Teniendo en cuenta que el único sistema de producción con una regulación clara es la producción ecológica, se estudió si los miARN en leche eran capaces de diferenciarla, encontrándose que el *bta-miR-215* tiene niveles significativamente más bajos en las ganaderías ecológicas.

Como la leche cruda no puede ser puesta a la venta, se ha evaluado la resistencia de los miARN a los diferentes procesos tecnológicos de la leche. La pasteurización no afecta de forma significativa los niveles de miARN, sin embargo, el calentamiento en

microondas, y la producción de yogur y queso si afectan significativamente al contenido de miARN. Este hecho implica que los resultados de los contenidos de los miARN determinados en la leche cruda no son extrapolables al resto de productos lácteos.

Finalmente, y desde el punto de vista de la funcionalidad, se aislaron y caracterizaron exosomas de leche cruda de tanque de explotaciones con sistemas de producción extremos (pastoreo y estabulación permanente). La concentración de exosomas en la leche de las explotaciones lecheras de pastoreo era mayor y, además, estos exosomas contenían niveles más elevados de *bta-miR-451*.

Como conclusión, se ha demostrado que el sistema de producción en que se manejan las vacas en ordeño influye en el perfil de miARN de la leche cruda, y por lo tanto hemos señalado a los miARN como posibles biomarcadores para la autentificación del sistema de producción de la leche, concretamente de la producción de leche en pastoreo. Sin embargo, estos resultados no pueden ser extrapolados directamente para la autentificación de otros productos lácteos. Además, se han dado los primeros pasos para determinar que el sistema de producción puede modificar las propiedades funcionales de la leche.

RESUMEN (en Inglés)

Pasture-based dairy milk production improves animal welfare, is more environmentally friendly and also improves milk quality. To protect this type of production, and thus the farmers and the ecosystem formed by multiannual pastures, authentication mechanisms are needed to guarantee the origin and quality of milk intended for human consumption. Milk contains miRNAs, molecules that regulate gene expression in eukaryotes, and their profiles may vary according to factors such as diet or exercise. In this work, miRNAs were studied as markers of milk authentication and their possible functional properties in milk depending on the production system.

The first approach was to sequence total miRNA profiles in raw tank milk from extreme dairy farms, extensive and intensive production systems, both in the fat and cell fractions of milk. After validation by RT-qPCR, it was confirmed that miRNA profiles change according to the production system, and *bta-miR-215* was identified with significantly higher levels in intensive milk fat compared to its equivalent in extensive.

Different dairy milk production systems coexist in the Cantabrian coast, as a continuous line from the most extensive, represented by grazing, to the most intensive, represented by farms with permanent housing and high content of corn silage and concentrates in the cow diet. To test the potential usefulness of miRNAs to differentiate among the different milk production systems present in the Cantabrian coast, new miRNAs selected from the literature were added to those previously obtained by sequencing, and more than 100 farms were sampled and grouped into 4 categories according to their management. Two miRNAs were identified in milk fat, *bta-miR-155* and *bta-miR-103*, associated with grazing and fresh grass consumption respectively. Taking into account that the only production system with clear regulations is organic production, we studied whether miRNAs in milk were able to differentiate it, revealing that *bta-miR-215* has significantly lower levels on organic farms.

As raw milk cannot be offered for sale, the resistance of miRNAs to different milk technological processes has been evaluated. Pasteurization does not significantly affect miRNA levels, however, microwave heating, yogurt and cheese production significantly decrease miRNA content. This fact implies that the results of miRNA contents determined in raw milk cannot be directly extrapolated to other dairy products.

Finally, from the point of view of functionality, exosomes were isolated and

characterized from raw tank milk from farms with extreme production systems (grazing and permanent housing). The concentration of exosomes in milk from grazing dairy farms was higher and, in addition, these exosomes contained higher levels of *bta-miR-451*.

In conclusion, it has been shown that the production system in which milking cows are managed influences the miRNA profile of raw milk, and therefore we have identified miRNAs as potential biomarkers for the authentication of the diary milk production system, specifically grazing milk production. However, these results cannot be directly extrapolated to the authentication of other dairy products. Furthermore, the first steps have been taken to determine whether the production system can modify the functional properties of milk.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN _____

Agradecimiento

Que este trabajo sea testigo de mí respecto a:

- Mi director de tesis Luis Royo por su disponibilidad en cada momento, la paciencia infinita para ayudarme a desarrollar este trabajo, por ser el tutor ejemplar que siempre deja un margen de expresión a mis ideas y proposiciones, ya sean correctas o no, dejando la distancia justa y adecuada para desarrollar mi independencia en el trabajo y también por orientarme en la búsqueda de nuevas oportunidades. ¡Miles de gracias y no serán suficientes!
- El equipo del SERIDA, especialmente a Alfonso Carballal por su ayuda, aportando soluciones en temas de informática en todo momento. Roció Rosa, Adela Martínez Fernando Vicente y Mario Menéndez y el resto del grupo y el personal del SERIDA por estar siempre dispuestos a prestar ayuda.
- Todos los ganaderos que han participado en este estudio, por su predisponibilidad a ayudar a todo momento.
- El equipo de Hadassah Ain Karem en Jerusalém, especialmente Regina Golan, Shimon Raif y Mirit gracias por recibirme en vuestro laboratorio por un mes y toda la ayuda y el apoyo proporcionado durante y después de mi estancia.
- El equipo del INRA Jouy en josas, Fabienne Leprovost y Sandrine Le Guillou por aceptarme durante dos meses en su equipo, y toda la orientación y el apoyo proporcionado a todo tiempo.
- Todas las personas que han colaborado en este trabajo en las diferentes partes, gracias, Rafa Celaya del SERIDA, Bea Martínez, Ana Rodríguez del IPLA, Jorge Diaz y Jaime Alonso del Centro de Inteligencia Artificial de la Universidad de Oviedo, Eduardo Iglesia, Manuel Fernández Benjamín Fernández y Ana soldado de la universidad de Oviedo Marie Carmen López y Alberto Davalos del Instituto IMDEA Alimentación.
- El tutor Manuel Réndeles y la coordinadora de programa de doctorado Adriana Laca por resolver mis dudas administrativas a todo momento.
- Mi gratitud y mi estima a todos los miembros del jurado que van a examinar mi trabajo.
- Mis amigas, con quienes mi estancia en Asturias fue muy especial y las que están lejos de mi pero siempre están. Gracias Sanne, Salmi, Biti, Lidia, Nawel, Iyan, Patri, Hafsa, Saida, Hafida, Hajar, Anacelia, Vero, Swara, Naima, Todos los amigos.
- Mi Gran Compañero Andrés… y mi familia española.
- Y claro mi familia. Gracias por todo el apoyo, el sacrificio, el entorno afectuoso y propicio para mi bienestar y mis estudios. Ninguna palabra podría expresar mi respeto, consideración y profundos sentimientos hacia vosotros, esperando que siempre estéis orgullosos de mí. A mis hermanos Soukaina, Hasna, Younes, y sobre todo Amine.

La presente tesis doctoral ha sido desarrollada en el Servicio Regional de Investigación y Desarrollo Agroalimentario del Principado de Asturias (SERIDA) en el Área de Nutrición, Pastos y Forrajes y dentro del Grupo de Investigación de Nutrición y Sanidad Animal (NySA)

Trabajo financiado parcialmente por:

- Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) a través del proyecto RTA2014-00086-C03-02, y cofinanciado por el Fondo Europeo de Desarrollo Regional (FEDER).
- FICYT, Plan de Ciencia, Tecnología e Innovación de Asturias (PCTI) 2018-2022 (IDI2018-000237), y cofinanciado por el Fondo Europeo de Desarrollo Regional (FEDER).
- FICYT, Plan de Ciencia, Tecnología e Innovación de Asturias (PCTI) 2018-2022 (IDI2021-000102), y cofinanciado por el Fondo Europeo de Desarrollo Regional (FEDER).

Loubna Abou el qassim ha sido beneficiaria de un Contrato de Formación Predoctoral, Programa “Severo Ochoa” de ayudas predoctorales para la investigación y docencia del Principado de Asturias. BP17049. Consejería de Educación y Cultura. Gobierno del Principado de Asturias.

General Index

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Abbreviations

Acvr2a	Activin Receptor, Type-2A
Acvr2b	Activin Receptor Type-2B
AGO	Argonaute
AMPK	Adenosine Monophosphate-Activated Protein Kinase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APS	Ammonium Persulphate
BCA	Bicinchoninic acid
Bmpr2	Bone morphogenetic protein receptor type II
BOE	Boletín Oficial del Estado
bp	Pair of bases
Bta	Bos taurus
CD 4 / 9 / 81	Cluster of differentiation 4 / 9 / 81
cDNA	Complementary DNA
cGMP-PKG	Cyclic guanosine monophosphate- protein kinase G
CLA	Conjugated linoleic acid
CNSAT	Comisión de Normas Sanitarias para los Animales Terrestres
CO_2	Carbon dioxide
COPAE	Consejo de la Producción Agraria Ecologica
Ct	Cycle threshold
CV	Coefficient of variation
DAVID	Database for Annotation, Visualization and Integrated Discovery
DDW	Deuterium-depleted water
DGCR8	DiGeorge Syndrome Critical Region Gene 8
DIM	Day in milk
dL	Decilitre
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
dPI	Distribution polydispersity index
dsRNA	Double-stranded RNA
EU	European Union

EVs	Extracellular vesicles
FA	Fatty acid
Fabp3	Fatty acid binding protein 3
FAS	FA synthase
FDR	False discovery rate
FM	Fresh matter
g	gram
GLM	General linear model
GMO	Genetically Modified Organism
GO	Gene Ontology
GPS	Global Positioning System
GVC	Green vegetation coverage
h	Hour
ha	Hectare
HIV-1	Human immunodeficiency virus
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
Kg	kilogram
L	Litre
LSD	Lysergic acid diethylamid
LU	Livestock unit
m^2	Square meter
MAPA	Ministerio de Agricultura, Pesca y Alimentación
Mapk1	Mitogen-Activated Protein Kinase 1
MDR1	Multidrug Resistance Mutation
mg	Milligram
min	Minute
miRNA	microRNA
mL	Milliliter
MLG	Milk Lipid Globules
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
ncRNA	Non-coding RNA
ng	Nanogram

nm	Nanometer
NPN	Non-protein nitrogen
NTA	Nanoparticle tracking analysis
ºC	Degree Celsius
p	Probability
PCA	Principal component analysis
PI3Ks-Akt	Phosphoinositide 3-kinases- Protein kinase B
Pre-miRNA	Precursor-miRNA
Pri-miRNA	Primary miRNA
PV	Pairwise variation
PVDF	Polyvinyl difluoride
Ran-GTP	RAs-related nuclear protein- Guanosine-5'-triphosphate
Ras	Rat sarcoma virus
Rcf	Relative Centrifugal Force
RDA	Redundancy analysis
RIN	RNA Integrity Number
RIPA	Radio immunoprecipitation assay
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RPM	Reads per million
rRNA	Ribosomal ribonucleic acid
RT-qPCR	Quantitative real-time Polymerase Chain Reaction
s	Second
SADEI	Sociedad Asturiana de Estudios Económicos e Industriales
SD	Standard deviation
SFC	Spanish Food Code
snoRNA	Small nucleolar RNA
SNP	Single-nucleotide polymorphism
snRNA	Small nuclear RNA
t	tonnes
TBST	Tris-buffered salline
TGF-beta 4	Transforming Growth Factor beta 4
TRBP	Transactivation response element RNA-binding protein
tRNA	Transfer RNA

UDP-glucose	Uridine Diphosphoryl glucose
UTR	Three prime untranslated region
µL	Microliter
µm	Micrometer

Chapter 1

Literature review

Chapter 1: Literature review

1.1. Milk

1.1.1. Definition

According to the Spanish Food Code (SFC) "Natural milk is the whole product (unaltered nor adulterated) free from colostrum, obtained by the hygienic, regular, complete and interrupted milking of healthy and well-fed domestic female mammals". According to the Spanish legislation, the word milk refers only to cow's milk. Other types of milk are designated by the name of the species to which it corresponds (e.g. goat's milk or sheep's milk).

1.1.2. Types of commercial milk in Spain

On a large scale, milk is commercialized after having undergone various treatments that ensure its microbiological quality and durability, while responding to different types of consumer demand. Commercial milk is classified according to the technological treatment used during its production and the type of farm from which it comes. In this regard the SFC specifies four types of milk (SFC, 1967):

- Hygienized milk: raw milk subjected to an authorized technological process for the total destruction of pathogenic germs, without modification of its physical-chemical, biological and nutritional qualities (e.g. pasteurization).
- Certified milk: raw milk from farms where the production, packaging and distribution process follow a strict, official and sanitary control that guarantees the quality of the product (e.g. organic farms).
- Special milk: raw milk subjected to treatments that modify its characteristic composition, such as *concentrated milk* (concentrated to a fifth of its volume, removing a part of its water), *skimmed milk* (totally or partially deprived of its fat), *fermented or acidified milk* (under microbial action or lactic ferments), and *enriched milk* (supplemented with minerals and/or vitamins according to the legislation established for fortified foods or milk with added aromas and/or stimulants).

- Preserved milk: raw milk subjected to treatments (such as sterilization, condensation and dehydration) that ensure its safety for consumption for more than thirty days. In this type of commercial milk, the percentage of the different component changes depending on the applied treatment.

In 1990, the sale of raw milk in bulk was prohibited due to food safety issues. Twenty-eight years later, its small-scale commercialization was authorized. Aware of the risks involved in the sale of raw milk, productors/ sellers must meet new legal requirements described in the European regulation 853/2004. This change in the regulations, is a result of consumer demand for a better milk quality and flavor. On the other hand, the new legislation allows revaluation and diversification of farms (European Commission, 2004). Below the description will focus on raw milk components, synthesis, and the factors of variation of its composition.

1.1.3. Raw milk composition

The most abundant component in milk is water. Cow milk is made up of 88% water, which contains emulsified fatty globules, casein micelles in suspension (and cells), carbohydrates (mainly lactose), soluble proteins and dissolved minerals (Pendini, 2012).

1.1.3.1. Milk fat

Milk fat appears as microscopic globules, surrounded by a phospholipid membrane. Its content in milk is very variable according to breed, lactation stage and nutrition (De la Torre-Santos *et al.*, 2020; Lim *et al.*, 2020; Alothman *et al.*, 2019). The total fat average content of cow milk ranges from 3.3 to 4.4% (Djordjevic *et al.*, 2019). Milk fat consists of 97-98% triglycerides, 1% phospholipids, 0.5% cholesterol and 0.5% free fatty acids (FAs) (Pendini, 2012; Chilliard *et al.*, 2001). Triglycerides are made up of one glycerol and three FAs. In milk there are approximately 440 different FAs, either with a long carbon chain (>C18), medium carbon chain (C12-C16) or short carbon chain (C4-C10), and with different levels of saturation and structures (cis or trans) (Lindmark-Månsson, 2008). The percentage of each FA in milk depends on factors related to the animal and its environment (Chilliard *et al.*, 2001). Around 70% of milk FAs are saturated. The main ones are palmitic acid (C16: 0), myristic acid (C14: 0) and stearic acid (C18: 0). About 25% are monounsaturated, mainly oleic acid (C18: 1), 2.3% are polyunsaturated especially linoleic(C18:2) and linolenic(C18:3) acid and 2.7% are C18

FAs with one or more trans double bonds, essentially vaccenic acid (18:1, 11t). Milk also contains conjugated linoleic acid (CLA) (~ 0.4% of the fat fraction), mainly the rumenic acid isomer (cis-9, trans-11, CLA), that are synthesized in the mammary gland as a result of the action of the delta-desaturase on vaccenic acid (O'Callaghan *et al.*, 2019; Lindmark-Månsson, 2008; Lindmark-Månsson, 2003).

1.1.3.2. Milk proteins

The total percentage of nitrogen in milk is on average 3.5% (from 2.9% to 3.9%). The nitrogen in milk is contained in true protein (95%) and non-protein nitrogen (NPN; 5%). The most characteristic milk protein is casein (80%). This protein is present in three different variants: alpha, beta and Kappa. The remaining 20% of protein in milk corresponds to β-lactoglobulins (exclusive in ruminant milk), α-lactalbumin, serum albumin, immunoglobulins and lactoferrins. NPN substances are mainly urea (30-35%), creatinine, uric acid, amino acids and ammonia (O'Callaghan *et al.*, 2019; Akers, 2016; Salcedo-Díaz & Villar-Bonet, 2015; Pendini, 2012).

1.1.3.3. Lactose

Lactose is an exclusive carbohydrate to milk and also the most abundant in most species. It is a disaccharide composed of a glucose and a galactose molecule linked by a β1-4 glycosidic bond (Akers, 2016). Its percentage, around 4.8%, is stable under normal conditions. The quantity of lactose in milk can be affected by mammary gland infections (such as mastitis). When these infections occur the concentration of lactose decrease and the concentration of mineral salts increase (Mardones & Villagrán, 2020; Pendini, 2012).

1.1.3.4. Minerals

Milk contains a wide variety of minerals in low concentrations that range from 0.7 to 1.2% (Akers, 2016). Some minerals are bound to the fat globule membrane, such as calcium, copper, iron, magnesium, manganese, phosphorus and zinc (Akers, 2016). Likewise, a large part of calcium and phosphorus are bound to casein. On the other hand, there are free minerals in the form of ions in solution, such as calcium (Akers, 2016; Pendini, 2012). The concentration of the major minerals in milk is shown in **Table 1.1**.

Table 1.1. The concentration of major minerals in milk. According to Akers, 2016.

Mineral	Concentration (mg/dL)
Calcium	125
Magnesium	12
Sodium	58
Potassium	138
Chloride	103
Phosphorus	96
Sulfur	30

Milk also contains compounds in very low concentrations (less than 100 mg/L) like liposoluble vitamins (A, D, E, K), water-soluble vitamins, especially those of the group B, hormones, alcohols, ketones, gases, pigments, animal or microbial enzymes, genetic material (RNA, DNA and microRNAs described in detail later) (Akers, 2016; Pendini, 2012). Other elements can contaminate the milk after milking. For example, metal containers can be a source of copper, iron, nickel, or tin (Akers, 2016).

1.1.4. Synthesis of milk components

It is estimated that to produce one liter of milk, 500 liters of blood are required to circulate through the mammary gland. The circulating blood provides the precursors needed for synthesizing milk components (Akers, 2016). Once the precursors reach the mammary gland, some penetrate through the capillaries and pass directly from the blood to the milk without changes (minerals, some hormones, immunoglobulins ...). Others enter through specific transporters and once inside the epithelial cell each precursor is incorporated into the appropriate metabolic pathway. The elements provided by the blood are mainly glucose, amino acids, FAs, and mineral salts, in addition to β-hydroxybutyrate and acetate in ruminants (Akers, 2016).

1.1.4.1. Fatty acids

FAs circulating in the blood derived from diet or mobilization of FAs from adipose tissue as well as FAs from *de novo* synthesis within mammary cells are incorporated into the animal's milk. The FAs in milk that come from the diet are long

chain, medium and short chain FAs (C4 to C14) are synthesized *de novo* in the mammary gland (Akers, 2016). In the case of palmitic acid, it can be either synthesized in the mammary gland or supplied by the diet. (Akers, 2016; Palmquist, 2006).

De novo synthesis of FAs: The precursors of *de novo* FA synthesis are acetate (C2) and β-hydroxybutyrate (C4). Both come from the fermentation of dietary fiber in the rumen. Butyrate is converted to β-hydroxybutyrate during absorption in the rumen (Bauman *et al.*, 2011). They are transported in the blood and delivered into the epithelial cells of the mammary gland. ß-hydroxybutyrate can only be used as a precursor in FA synthesis but not in elongation, whereas acetate is the source of most of the other carbons used for FA formation (Bauman *et al.*, 2011). The process starts by the activation of the acetate by the enzyme acetyl-CoA synthetase resulting in acetyl-CoA. The acetyl-CoA is converted into malonyl-CoA (C3) by the enzyme acetyl-CoA carboxylase alfa (Akers, 2016). During elongation, the enzyme FA synthase (FAS) intervenes by adding acetyl-CoA carbons to the molecule. The first union is made between malonyl (C3) and acetyl (C2), releasing a CO_2, and forming a compound of four carbon atoms (C4). From there, in each cycle, 2 carbon molecules are added from acetyl-CoA thanks to the intervention of the enzyme FAS. Repeating the same reaction in seven successive cycles generates the palmitate. In most tissues that synthesize fat, under the effect of FAS, mainly palmitic acid is produced (Akers, 2016). When the palmitate (C16) is generated, the thioesterase enzyme releases the FA into the cytoplasm. In the mammary tissue of ruminants, and under hormonal control, the thioesterase II enzyme can interfere early, giving the possibility to release FAs of 4 to 12 carbon atoms in addition to C14 and C16 (**Figure 1.1**) (Akers, 2016).

Dietary FAs: Ruminant's ration mainly contributes to palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) (Doreau *et al.*, 2012). The main FA in fresh grass is linolenic acid (50%), followed by palmitic acid (20%), and linolenic acid 15%. Corn silage contains 2% of FAs, mainly linoleic (45%), oleic (20%), palmitic (15%) and linolenic (5%). In cereals the total of FAs is about 2 to 3%, of which 50% is linoleic. The fat supply in the diet of dairy cows, does not exceed 4%, however it varies according to the season. In temperate climates, fresh grass provides about 3% of dietary FAs during autumn and spring but only 1% during summer (Doreau *et al.*, 2012). The vegetative phase of plant development and the conservation mode of the forages also have a considerable effect on fat percentage, for

example, the hay making reduces the percentage of FAs, due to the loss of the leaves that are rich in lipids, and due to oxidation processes (Doreau *et al.*, 2012).

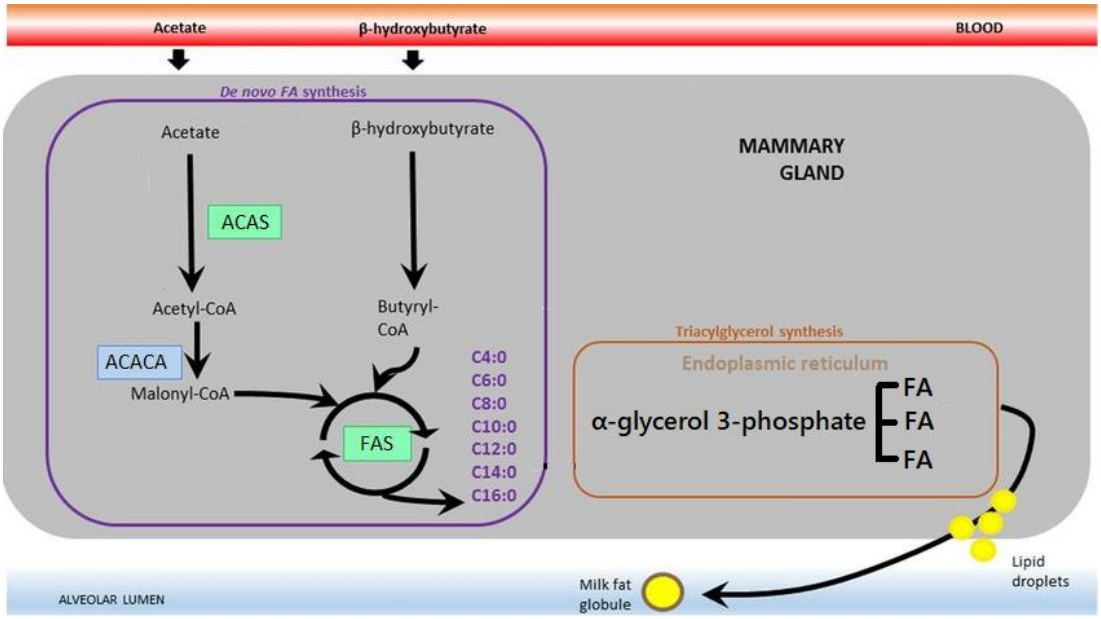

Figure 1.1. De novo FA synthesis cycle in the mammary gland, triglyceride synthesis and milk fat globule release. Modified from Knutsen *et al.* (2018).

At the rumen: Ruminal microorganisms act on ingested FAs when they get the rumen. FAs are released from both phospholipid triglycerides and glycolipids. Unsaturated FAs are subjected to hydrogenation (transform unsaturated FAs to saturated) and isomerization (modifications of their configuration to trans isomeration) (Chilliard *et al.*, 2001). A part of these FAs is used for the own metabolism of the microorganisms and the rest remains for the animal. After ruminal activity, ingested FAs differ from those absorbed by the intestine (Chilliard *et al.*, 2001). For example, linoleic acid is isomerized to rumenic acid, then it is hydrogenated resulting in trans vaccenic acid and finally the stearic acid (**Figure 1.2**) (Chilliard *et al.*, 2001).

In the mammary gland: Whether they come from diet or body reserves, FAs are transported in plasma, as non-esterified FAs or as lipoproteins. Upon reaching the mammary gland, the enzyme lipoprotein lipase extracts FAs from their transporters. Thus, the amount of FAs in milk depends on their concentration in plasma (Chilliard et *al.*, 2001). Under the effect of the enzyme delta-9 desaturase, stearic acid (C18: 0) is

converted into oleic acid (C18: 1 9-cis), reducing the C18: 0 / C18: 1 ratio, ensuring the fluidity of milk fat, facilitating its secretion (**Figure 1.2**).

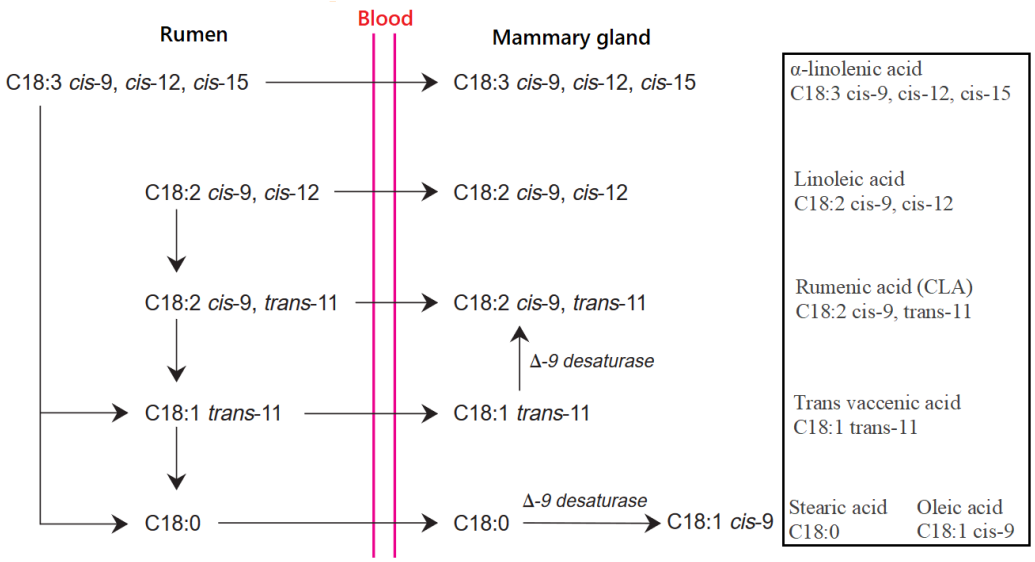

Figure 1.2. FAs modifications in the mammary gland. Modified from Chilliard *et al.* (2001).

The formation of triglycerol is carried out in the endoplasmic reticulum, using α-glycerol 3-phosphate as a carbon support for binding FAs. These are released as micro lipid droplets when they are less than 0.5 µm in diameter. During their transit, they can fuse with each other, generating progressively larger lipid droplets, called cytoplasmic lipid droplets. Finally, lipid droplets come into contact with the plasma membrane releasing milk lipid globules (MLG) by exocytosis, into the alveolar lumen (**Figure 1.1**) (Keenan, 2001).

1.1.4.2. Milk proteins

Specific milk proteins are synthesized from amino acids carried in the blood and those synthesized in the cells from different metabolic pathways. Protein synthesis in the epithelial cells of the mammary gland occurs in the endoplasmic reticulum and follows the same pattern known in other tissues: transcription of genetic information from DNA to messenger RNA, then translation, giving information from messenger RNA to ribosomal RNA and the transfer RNA that is responsible for bringing the specific amino acids of the anticodons according to the messenger RNA sequences, thus forming the

protein in question. Once synthesized, the secretory proteins are transported to the Golgi apparatus, where they are processed to be transported outside the epithelial cell (Aker, 2016).

1.1.4.3. Lactose and water

As mentioned before, water is the major component of milk, it reaches the mammary gland via the bloodstream (Aker, 2016; Pendini, 2012). Its entrance depends on the secretion of lactose and ions, which, thanks to their osmotic properties, draw water into the epithelial cells. (Shennan & Peaker, 2000). Two glucose molecules are needed to synthesize a lactose molecule. Glucose is taken up by the epithelial cells of the mammary gland, by different types of glucose passive transporters. These transports do not require energy, so the glucose passage rate is not limited by this factor, however, its levels in the cytoplasm regulate its entry (Mardones & Villagrán, 2020; Shennan & Peaker, 2000). Once inside the epithelial cell, one glucose molecule is first converted into UDP-glucose (Uridine Diphosphoryl glucose), then into UDP-galactose. From glucose and UDP-galactose, the enzyme lactose Synthase generates lactose in the Golgi apparatus of the epithelial cells in the mammary gland. Once lactose is synthesized in the Golgi apparatus, water is drawn to the lactose to balance the osmotic pressure, then transported through vesicles and released by exocytosis into the lumen of the alveoli (Akers, 2016).

1.1.5. Factors of variation in the synthesis of milk components.

The composition of milk determines its nutritional and industrial quality, which influences the profitability and competitiveness of dairy farms. The composition of the different milk components reflects the effect of several factors that may be modified or not by different management practices in the production system. These factors may be, on one hand, intrinsic to the animal such as genetics, physiological condition and health status, which are generally difficult to modify in the short term. On the other hand, the factors of variation may be extrinsic and highly dependent on management practices such as feeding and management of the farm (O'Callaghan *et al.*, 2019). The following is a review of the different factors that can affect the composition of milk.

1.1.5.1. Genetic factors

The variation of milk components is relatively subject to genetic factors, this variation can be observed both between breeds and within breeds between individuals (Kelsey *et al.*, 2003). If we look at the breeds, Jersey produces the highest percentages of fat, total protein and casein (Lim *et al.*, 2020). This breed is also characterized by a high ash percentage of about 0.83%, whereas the Hostein has an ash percentage of 0.74. Also, the highest levels of calcium and phosphorus in milk have been recorded in the Jersey (Cerbulis & Farrel 1976). As a result of the intensification of dairy farms, genetic selection has favoured the milk yield trait by maximizing the amount of milk produced per cow, which in turn has led to a reduction in milk fat and protein percentages (genetic traits negatively correlated with milk yield) (Brito *et al.*, 2021). This trend has been reversed by a system of payment to farmers based on total solids content in milk (Santiso et *al.*, 2003). The genetic parameters protein and fat percentage have a low to moderate heritability, which indicates that the environment of each animal and gene-environment interactions play an important role in the variation of these components (Bobe et *al.*, 2008; Soyeurt & Gengler, 2008). Therefore, a change in these percentages in the short term is not likely to be achieved only by genetic selection.

1.1.5.2. Stage of lactation

Throughout the lactation, milk production and composition vary (Bondan *et al.*, 2018). In early lactation, from calving to peak lactation (up to 35 days), milk production increases and the percentage of fat and protein decreases, with the highest percentages in the colostrum. During the mid to late lactation, milk production declines at a regular rate, accompanied by a slow increase in fat and protein percentages as lactation progresses (Bondan *et al.*, 2018). However, in tank milk from farms, the stage and the number of lactations do not affect the composition of milk in a relevant way, as the tank includes milk from cows at different stages and numbers of lactations.

1.1.5.3. Animal health status

Health status influences significantly both milk production and composition (Mardones & Villagrán, 2020; Malik *et al.*, 2018). Mastitis is one of the most common and studied health problems on dairy farms, it is an infection that usually disrupts milk production (Malik *et al.*, 2018). The bacteria act by affecting the selective function of the

alveolar membranes, allowing the passage of blood components such as soluble proteins, carbohydrates and salts. At the same time, the synthesis of casein, lactose and fat is altered, resulting in an abnormal milk composition. Mastitis also affects the minerals by decreasing potassium and calcium while it increases sodium and chloride. As a result, affected cows have lower milk yields and high somatic cell and bacterial counts (Malik *et al.*, 2018). The maximum values of somatic cell measurements established for the commercialization of milk are 400 000 cells/mL (BOE, 2007).

1.1.5.4. The climatic factor

Other factor that affects milk composition is climate. Climatic conditions affect milk composition both through the effect on forage composition, the animals' voluntary intake and their basal metabolism (Ouellet *et al.*, 2019). Independently of the diet, milk composition varies according to the ambient temperature, so that, the percentage of fat (Ouellet *et al.*, 2019) and protein (Ouellet *et al.*, 2019; Ng-Kwai-Hang *et al.*, 1982) in milk decreases in summer compared to winter. Also, temperature-humidity lead to a reduction in short- and medium-chain FAs and an increase in long-chain FAs (Liu *et al.*, 2017), however, lactoserum proteins are not affected (Kroeker *et al.*, 1985).

1.1.5.5. Feeding management

Feeding management has an important impact on milk components (Alothman *et al.*, 2019; Elgersma *et al.*, 2006). The milk component most affected by feeding is fat, so that it can vary according to diet by 0.1- 1%, while protein variations do not exceed 0.4%. These changes occur in about 7 to 21 days in the case of fat while, in the case of protein, variations can be seen from 3 to 6 weeks (Heinrichs *et al.*, 2016). Diet influences both the percentage of fat in milk and its FA composition (De la Torre-Santos *et al.*, 2020; Alothman *et al.*, 2019). As was mentioned before, the main precursors of milk fat are acetic and butyric acid derived from ruminal fermentation of fibres, dietary FAs and endogenous FAs. The fermentable carbohydrate supply reduces fibre digestion and generates propionic acid which generates lactic acid and glucose which activates insulin production resulting in the repression of free FA release from adipose tissue, thus the rumen propionate ratio can explain up to 60% of the variations in milk fat percentage (Sutton, 1985). This ratio is associated with the ratio of forage to concentrate in the diet, so that from a forage/concentrate ratio of 60/40, adding more concentrate to the diet

reduces milk fat (Sutton, 1985). Forage characteristics also affect milk fat percentage, so, depending on the size of the fibre, the fat percentage can vary, so finely ground fodder reduces the fat percentage of the milk (Woodford *et al.*, 1986). The degree of maturity and the storage mode of the forages also influence the fat percentage, so that the consumption of fresh grass through grazing increases the fat percentage compared to animals consuming silage and hay (Capuano *et al.*, 2013). Immature alfalfa hay in the diet provides more milk fat than medium or late flowering alfalfa hay (Kawas *et al.*, 1983). The effects of dietary protein on milk fat percentage within normal ranges are generally small and relate to milk yield rather than a direct effect on fat, however insufficient rumen degradable protein intake results in a failure of microbial digestion of fibre and hence reduction of fat percentage (Sutton, 1985). The supply of fat in the diet can result in a direct passage of fats through the rumen to the mammary gland and into the milk. However, FAs can also be saturated in the rumen by micro-organisms before reaching the milk (Chilliard *et al.*, 2001). It is important to note that an intake of more than 4 % fat in the diet can alter rumen fermentation and thus the percentage of fat in the milk (Sutton, 1985).

The protein yield of milk is related to ruminal fermentation, and the growth of the microorganism amounts in the rumen, which is affected by dietary energy and degradable protein (nitrogen availability) in the rumen (Russell & Rychlik, 2001; Cragle *et al.*, 1986). These factors determine the amount of microorganism protein synthesized in the rumen and dietary protein that reaches the intestines (Alothman *et al.*, 2019; Clark, *et al.*, 1992). Thus, the energy intake influences the percentage of milk protein by increasing ruminal microorganisms (Cragle *et al.*, 1986), when there is enough degradable protein (Russell & Rychlik, 2001). Therefore, the growth of rumen microorganisms' populations contributes to the increase of protein in the milk (Alothman *et al.*, 2019; Clark, *et al.*, 1992). The importance of the diet on the percentage of protein can be then observed. Alothman *et al.* (2019) reported that grazing increases the percentage of protein in milk due to the high content of the amino acid methionine (limiting factor for protein synthesis) in pasture compared to silage hay.

1.2. Dairy production systems

1.2.1. Dairy production system definition

A production system can be defined as the combination of production factors, the structure and the overall functioning of dairy farms (Leblanc, 2012). More concretely, dairy farming systems are any commercial livestock production system that includes raising, reproducing, and managing livestock; with the purpose of producing milk (CNSAT, 2014). The interest of characterizing production systems is to allow the grouping of farms with a similar production structure and similar operating methods, in order to assess the technical and economic performance of dairy farms (Leblanc, 2012) and also to facilitate comparative analysis between farms.

1.2.2. Classification of farms according to their level of intensiveness/extensiveness

There is a fairly wide range of production system possibilities, due to the multitude of factors involved. This makes characterization complex because the description of the implicated factors and their interrelationships is not sufficiently resolved (Marín, 1996). The classification depends on the reasons for which we want to distinguish types, also the number of system types for a determined classification depends on the level of precision and detail that is included (Brossier & Petit 1977). Dairy production systems can be presented according to the degree of extensiveness/intensiveness of the farms, which is represented by a continuous line (from 100% intensive industrial livestock farming to 100% extensive pastoral livestock farming). The classification of farms according to this criterion consists of setting thresholds or categories appropriate to different needs (Ruiz *et al.*,2017). Usually the established groups are intensive, mixed (or intermediate) and extensive livestock farming (Trillo, *et al.*, 2017; Ariza & Juaristi, 2008), which will be described below.

1.2.3. Description of Production systems

1.2.3.1. Intensive systems

Intensive free-stall farms are equipped with cubicles or warm bedding patio with free animals, but kept in permanent housing (Ariza & Juaristi, 2008). Intensive farms are characterized by being very sophisticated, with a high stocking density (around 4.75

animals/ha) and maximization of milk production per cow per unit area (Vicente-Mainar, *et al.*, 2013). Feeding is based on the use of silages and an increased use of concentrates per cow (Van Dijk *et al.*, 2015) which makes these farms dependent on external feeding (Álvarez-Pinilla *et al.*, 2007). In these farms the rations are made with the unifeed cart in many cases to elaborate total mixed ration, however, there are some cooperatives that make community rations that are transported daily to the members (Ariza & Juaristi, 2008). Genetic selection and the use of high-yielding breeds (Holstein Friesian) to increase productivity in dairy farming have been among the main promoters of dairy intensification (Steinfeld *et al.*, 2006). In the case of large herds, the animals are grouped according to their production and their physiological status and then various rations are formulated according to the group's average production (Ariza & Juaristi, 2008). In other cases, individualized nutrition is performed, where the cows receive additional supplementation to the unified based on their production. This is done through automatic dispensers that recognize animals electronically (Schwanke, *et al.*, 2022).

1.2.3.2. Grazing extensive systems

For a long time, the terms grazing and extensive farms have been used interchangeably to refer to systems with large areas of land. However, regardless of the territorial support of the exploitation, the denomination depends on factors and the production achieved (Marín, 1996). Extensive systems can refer either to extensivity itself, related to livestock management and exploitation, or to sustainability in socio-environmental terms. (Ruiz *et al.*, 2017). For this reason, the complete definition of extensive dairy farms cannot be based only on the measurement of animal access to grazing, but it should also consider other factors that would help for the quantification of the degree of extensiveness of a farm. These factors can be related to grazing: the duration of grazing per year, the duration of grazing per day, the proportion of feed obtained directly from grazing, the proportion of feed from on-farm harvesting (green grass, grass silage, hay…), and that coming from outside (Ruiz *et al.*, 2017). It is also important to consider the types of pasture used (natural, semi-natural or cultivated) (Ruiz *et al.*, 2017). There are other factors related to management and used inputs, such as the breeds, the reproductive management, the use of mechanization for harvesting, fertilizers, and chemicals (Ruiz *et al.*, 2017; Vicente-Mainar *et al.*, 2013). According to this, animals in extensive systems are mostly fed on pasture through grazing, taking advantage of the natural resources of the territory, with a low use of external inputs and a minimum supply

of concentrates. This provides a lower feeding system cost whose efficiency is based on high milk production per unit area. In general, this system is characterized using adapted breeds to the territory, and proper management of reproduction and genetic diversity of the herd (Ruiz *et al.*, 2017; Vicente-Mainar *et al.*, 2013).

1.2.3.3. Mixed Systems

In some cases, the two managements are combined, so the animals graze and their diets are completed with forages and concentrates (Ariza & Juaristi, 2008). In some farms, mainly located in areas of northern Spain, fresh grass is administered in the stable during spring and summer, while in winter the ration contains mostly grass and corn silages, combined with winter forages such as Italian ryegrass (Villar-Bonet & Quintana-Ruiz, 2021; Salcedo-Díaz, 2006).

1.2.3.4. Organic milk production systems

In contrast to extensive farming, which is currently not a formally characterized and recognized activity with a specific regulatory framework, nor is it clearly differentiated from other production systems (Ruiz *et al.*, 2017), organic milk production system is well characterized. In the European Union (EU), the legislation governing organic farming is the Council Regulation 2092/91 on Organic Agricultural Production. This law has undergone modifications such as the case of the Regulation 1804/1999 that incorporates animal productions, and the Regulation (EU) 2018/848 that establishes the standards for the production, processing, marketing, labeling and control of food, as well as the importation of organic food (European Commission, 2018). This regulation specifies the characteristics of organic livestock farming as summarized below. In this type of farms, animal welfare is primordial, without forcing their natural cycle, so breeds with a high degree of genetic diversity, high disease resistance, longevity and adapted to local conditions are used.

Feeding is based on the maximum use of pastures and feed produced on the farm. If the internal production of forages is not sufficient, it can be purchased off-farm, as long as it is organic. A maximum percentage of 5% of non-organic feed is allowed in the ration for the whole year. This percentage should not exceed 25% of the ration per day. The concentrate feed must be from certified organic raw materials, free of Genetically Modified Organism (GMOs) and products derived from GMOs. Growth factors and

synthetic amino acids are prohibited. The transformation of organic feed should preferably be using biological, mechanical and physical methods.

In organic production, the prevention and control of pests and diseases is crucial. In the case of insufficiency of preventive measures, chemically synthesized veterinary drugs, including antibiotics, may be used to avoid animal suffering. The stocking density must be a minimum of 6 m^2 per cow in the covered areas, and 4.5 m^2 per cow in the open area excluding pasture. In the pasture the minimum stocking density is 2 animals per hectare (ha). In these farms, fertilization is limited to organic fertilizers such as manure, compost, crop residues, slurry, green manure, or deep root plants (own, from organic farms and from other extensive farms if it fulfills the requirements). Mineral fertilizers should be used only when there are deficiencies, using rocks or minerals that have only undergone physical and non-chemical treatments.

1.2.4. Milk production systems in Asturias

The Cantabrian coast and Galicia are characterized by favourable climatic conditions to produce pastures and forages for milk production (Vicente *et al.*, 2013; Villar-Bonet & Quintana-Ruiz, 2021). However, due to the implementation of EU agricultural policies, the changing market situation and technological progress, among other reasons, the trend in milk production is towards intensification, which is accompanied with an increase in the average production per farm, a huge reduction in the number of farms and a more moderate reduction in the number of cows in lactation (Flores-Calvete *et al.*, 2016; Jiménez-Calderón *et al.*, 2015; Vicente *et al.*, 2013). Therefore, there are less small farms, and large farms increasingly resort to the use of external feed and rely less on their own production (more livestock unit (LU) /ha) (SADEI, 2017). The trend of this evolution in Asturias is illustrated in **Figure 1.3**.

According to the MAPA (Ministerio de Agricultura, Pesca y Alimentación) report 2016-2020 the Cantabrian coast concentrated in 2020 79% of livestock farmers in Spain, where Galicia has the largest number of farmers 56%, followed by Asturias 13%. Also, Asturias is the third Spanish community that contributes the most tonnes (t) to milk production. The total annual milk delivery of 1658 producers in Asturias were 570.872 (t), with farms of different production levels ranging between 50.000 kg to 8.000.000 kg

per year (**Table 1.2**), with an average production per lactating cow of 8149 kg per year (MAPA, 2020).

A study on feeding systems in dairy farms (sample of 55 farms) (Flores-Calvete *et al.*, 2016), revealed that in Asturias the total area (ha) dedicated to pluriannual herbaceous crops (meadows, prairies and pastures) was about 13.1 ha on average per farm. Also, the use of fresh grass in the diets of Asturian farms was around 74.20 %, both through grazing (48.30%) and by consuming harvested fresh grass indoors (59.10). Daily grazing hours vary according to the season of the year, the average duration in summer was 8.30 hours (h), in spring 5.70 h, in autumn 5.40 h and in winter 2.60 h. Another very important ingredient in the rations of these farms is grass silage, which was included in the diet of 87.60% of the farms. Whereas corn silage was used in 29.40% of the farms. The dominant forage conservation method is silage, so that 94.50% of farmers make silages, but only 27.90% make hay. According to the composition of the diet in dry matter, the ration included about 23.70% of fresh grass, 21.00% grass silage, 8.30% corn silage, 15.50% dried forages and 31.40 % concentrates. Corn silage is the main ingredient of the diet on intensive farms, whereas grazing is associated primarily with smaller farms (Jiménez-Calderón *et al.*, 2015). All these types of farm management in Asturias can be classified into 5 groups according to the main ingredient of the ration (Santiago *et al.*, 2017): Grazing, Corn silage, Grass silage, Dry forage and Concentrate.

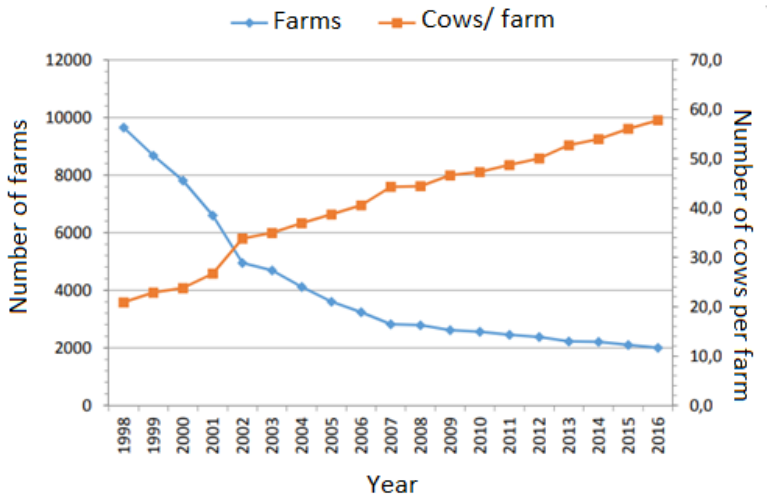

Figure 1.3. Evolution of the number of cows and farms between 1998 and 2016 in Asturias (Source: SADEI, 2017).

Table 1.2. Total milk deliveries by Asturian farmers according to different levels of production during 2020 (MAPA, 2020).

Milk deliveries by production level	Producers	Delivery (tonnes)
Total	1658	570.872
Up to 50.000 kg	202	5.457
From 50.000 to 200.000 kg	633	77.367
From 200.000 to 500.000 kg	490	163.704
From 500.000 to 1.000.000 kg	232	162.971
From 200.000 to 3.000.000 kg	96	141.780
From 3.000.000 to 8.000.000 kg	5	19.594

1.2.5. Implications of production systems based on grazing

Several studies have shown the positive externalities of pasture-based production systems on milk quality, animal welfare and health, the environment, the farmer's lifestyle and the economy, concluding that pasture-based production systems have several advantages compared to intensive production.

1.2.5.1. Environment impact

Intensiveness is supported by a wide use of inputs (fertilizers, pesticides…) and thus reinforce the environmental impacts like greenhouse gas emissions, acidification, water contamination, soil degradation etc (Groot & van't Hooft 2016; Steinfeld 2016; Vicente-Mainar *et al.,* 2013). Dairy systems based on grazing are characterized by the minimal use of external inputs, such as the utilization of organic residues generated on the farm, thus increasing the organic content of the soil and increasing the level of fertility (Demanet *et al.*, 1999). Morais *et al.* (2018) reported that for every kilogram of milk, an average total greenhouse gas emissions of 0.83 kg CO_2 were produced, and grazing farms can reduce emissions by up to 32% than other production systems, explaining this reduction with the lower consumption of concentrate in these farms. Thus, pasture-based dairy farms adopting appropriate grazing strategies, are considered less damaging to the environment than intensive systems (Nehring *et al.*, 2009).

1.2.5.2. Milk quality

As previously mentioned, feed management has a noticeable effect on milk composition, on both component yields and its subcomponents (Alothman *et al.*, 2019; Elgersma *et al.*, 2006). Grazing has a clear effect on the composition of milk fat in such a way that it decreases the proportion of saturated FAs and increase the content of unsaturated FAs, known to have a positive effect on human health such as vaccenic acid, CLA, and omega-3 FA (Santa *et al.*, 2022; De La Torre-Santos *et al.*, 2021). Grazing has also a positive an effect on milk antioxidant content, and antioxidant capacity, as lutein and β-cryptoxanthin (Santa *et al.*, 2022; De La Torre-Santos *et al.*, 2021). It has been shown that grazing leads to a better protein: fat ratio with higher β-lactoglobulin and lactoferrin, constituents of whey protein, which improves the bioactive status of pasture-based milk (Alothman *et al.*, 2019).

1.2.5.3. Animal welfare and health

Pasture is the normal habitat of cow as a naturally grazing animals, which is supposed to achieve a higher level of animal welfare (Smid *et al.*, 2020), and consequently a low predisposition to certain diseases, such as lameness and mastitis (Crump *et al.*, 2021). Grazing is reported to contribute for a better functioning of the immune response oxidative status in the animals thanks to the high levels of antioxidants in green forage and the physical exercise during the grazing activity (Di Grigoli *et al.*, 2019). On the opposite, the intensification, in many cases, lead to negative effects on cow health, for example, increasing the farm size increases the risk of lameness, and the risk of mastitis is affected by milking frequency (Ma *et al.*, 2020) reducing cattle resilience (Berghof *et al.*, 2019).

1.2.5.4. Economic, cultural and social advantages

The highest cost in dairy farming is usually the cost of feed (Finneran *et al.*, 2012). Considering that in temperate zones fresh grass is the most economical feed source for milk production (Finneran *et al.*, 2012), grazing farms reduce one of the most important costs. Regarding the use of concentrates in feed, less is generally used on pasture farms, which further contributes to this reduction (Vicente-Mainar *et al.*, 2013). Labour requirements vary among farms and represent a considerable cost, e.g., depending on calving management labour cost varies, in the case of spring calving systems the net profit

per farm is higher than farms adopting less seasonal systems such as intensive farming. (Geary *et al.*, 2014). Farm efficiency is linked to farm mechanization, which leads to high capital investment, as a result, start-up costs of grazing farms are reduced compared to intensive farms (Dillon *et al.*, 2005). In grazing farms, the use of fertilizers, pesticides and veterinary drugs can be reduced, resulting in lower variable costs (Dillon *et al.*, 2005). Thus, despite low milk production, compared to intensive farms, grazing farms can be as profitable or more profitable than indoor systems (Dillon *et al.*, 2005).

Villar-Bonet & Quintana-Ruiz (2021) have mentioned some of the advantages of this type of livestock farming in northern Spain: preserving traditional forms of milk production, enhancing the culture of pasture management, which is being neglected, sustaining of the rural landscape, and thus of tourism and cultural heritage and avoiding the abandonment of the small producer's activity thus maintaining the rural population.

1.3. The need for differentiation of an extensively produced milk

1.3.1. The situation of pasture-based milk in Spain

Despite these advantages and positive externalities, dairy farming is at serious risk of abandonment in northern Spain, reflecting a process of intensification of dairy farming across Europe (Villar-Bonet & Quintana-Ruiz, 2021). Except for some regional or sectoral initiatives, no national regulations clearly define this dairy production systems (Villar-Bonet & Quintana-Ruiz, 2021; Ruiz *et al.*, 2017). This makes it difficult for extensive farms to certify the advantages of their milk to consumers who demand socially and environmentally responsible products (Stampa *et al.*, 2020; Croissant *et al* 2007). In this context, Villar-Bonet & Quintana-Ruiz (2021) refer to the concept of "leche de pasto" and "leche de pastoreo" and define the first as "all milk from lactating cows where green forage is a major component of their diet either grazed or in the stable where the green forage is mowed and fed to the cows". Whereas "leche de pastoreo "is restricted to milk from lactating cows grazing outdoors". These two products are seasonal as they would be on the market for the duration of grazing and the availability of green forage (Villar-Bonet & Quintana-Ruiz, 2021). As mentioned before, these two denominations are still irregular, as there is no legal text defining them and their concrete definition is still pending, i.e. to define how many days per year and how many hours per day the lactating

cows must remain in grazing in the case of the “leche de pastoreo”. In the same way for the “leche de pasto” a threshold for green fodder consumption in relation to the total dry matter intake in the diet should be set up (Villar-Bonet & Quintana-Ruiz, 2021).

1.3.2. Importance of product quality certification

There is a growing consumer demand for products including a new definition of quality concept that includes traditional attributes related to nutritional value, flavor, aroma, and color, together with new indicators related to ethical aspects, such as animal welfare and environmental impact of the production system (Rychlik *et al.*, 2017; Luykx *et al.*, 2008). Consumers assume that products from grazing cows are more natural and better to meet animal welfare demands than those from the cows raised under intensive farms (Stampa *et al.*, 2020). Hence, the importance of certification strategies that aim, on one hand, to support local producers and prevent the abandonment of such farming practices, and on the other hand, to help the consumers to make fully informed purchasing decisions (Valenti *et al.*, 2013; Coppa *et al.*, 2012). Thus, certification marks the added value of products, and therefore they have higher prices (Coppa *et al.*, 2012) that consumers are often willing to pay (Tempesta & Vecchiato 2013). Therefore, to guarantee the authenticity of certified products for consumers, the application of reliable traceability methods is necessary.

1.3.3. Traceability

Traceability enables a product to be monitored through all stages of production, processing and distribution until its consumption, with the aim of justifying its added value and price surcharge, guaranteeing its origin. In the case of milk produced under extensive systems, it is required to show that the milk comes from a farm associated with pasture consumption and/or grazing activity or that the milk has differential components (Villar-Bonet & Quintana-Ruiz, 2021). There are several traceability systems that can be used, for example, GPS collars for locating and monitoring animals, their location and behaviours, digitising the results, thus guaranteeing grazing performance (Villar-Bonet & Quintana-Ruiz, 2021). The FA profile in milk can be an indicator of the diet of the animals (Vicente-Mainar *et al.*, 2017) and the management system (extensive, semi-extensive, intensive) (Morales-Almaráz *et al.*, 2011), so its use as a tool for milk origin authentication was proposed (Capuano *et al.*, 2014). Similarly, compounds such as

carotenoids, phenolic compounds are potential tracers in milk, from animal diets (Prache, 2003), because their appearance in animal products or tissues is unequivocal due to the feed they have eaten (Prache, 2003). Here the use of MicroRNAs (miRNAs) as biomarkers of milk production systems will be detailed.

1.4. MicroRNA

1.4.1. Definition and nomenclature of miRNAs

MicroRNAs (miRNAs) are endogenously initiated non-coding RNAs of 21-25 nucleotides that perform a variety of functions within cells, including extensive coordination of gene expression regulatory networks (Sohel, 2016; He & Hannon, 2004), adding further complexity to gene regulation. The miRNAs are denoted miR as opposed to their precursor annotated mir (Griffiths-Jones *et al.*, 2006). The organism of origin is indicated by three letters in the prefix: bta for *bos taurus*, hsa for *homo sapiens*. Depending on the position of the miRNA on the precursor-miRNA (pre-miRNA), the addition of 5p or 3p is specified in the suffix, for example *bta-miR-XX-5p* or *bta-miR-XX-3p* (**Figure 1.4**). In general, miRNAs belong to families where each member shares a common "Seed" region which is by definition the recognition site of the target mRNA, and which is typically located between nucleotides 2 to 8, while the rest of the nucleotides may be different (**Figure 1.5**), this difference is indicated by the addition of a letter in the suffix: *miR-XXa* or *miR-XXb*. The difference in the chromosomal region of the miRNA is expressed by adding a numerical suffix: *miR-XXa-1* or *miR-XXa-2* (Griffiths-Jones *et al.*, 2006). This nomenclature is not valid for plants, and the case of the *let-7* and *lin-4* families (see 1.4.2 Discovery of miRNAs) (Griffiths-Jones *et al.*, 2006). Each miRNA is defined by a unique sequence, but there are variants of different lengths called isomiRs (**Figure 1.6**) (Neilsen *et al.*, 2012) that are the result of imprecise cleavages during biogenesis (Lee Y. *et al.*, 2009).

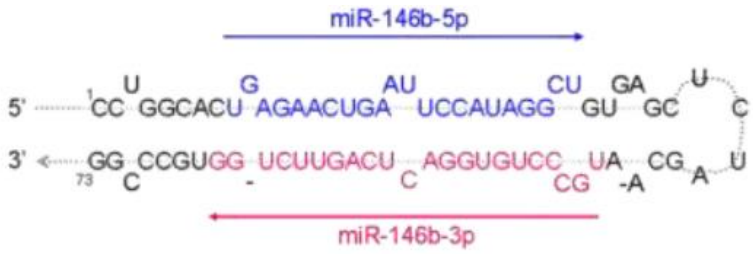

Figure 1.4. Structure of a miRNA precursor *pre-mir-146b*. Extracted from (Mobuchon, 2015)

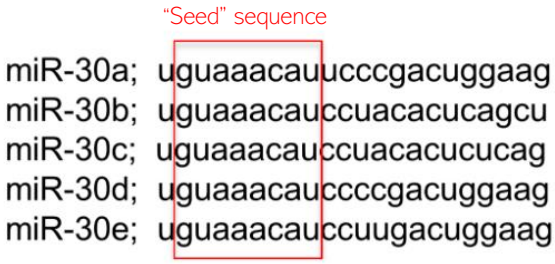

Figure 1.5. Example of a miRNA family, the human *miR-30* family, showing the "Seed" region. Extracted from (Ketley et al., 2013)

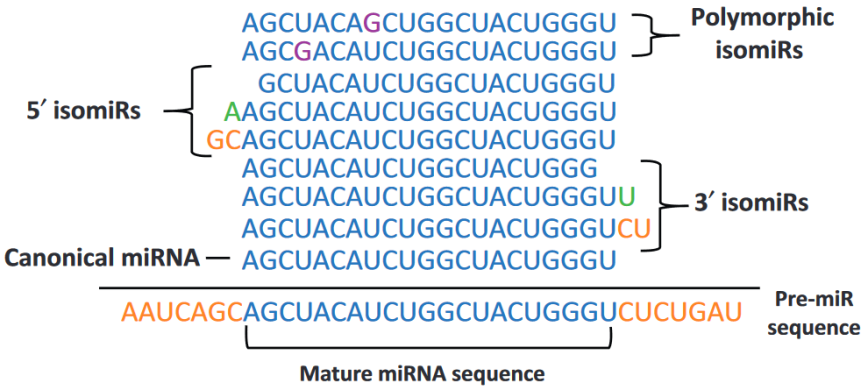

Figure 1.6. Representation of the different types of isomiRs. Human *miR-222* isoforms show an example of the heterogeneity of isomiRs. The sequence of the canonical isoform is shown in blue. The 5' and 3' isomiRs are variants of the miRNA that differ at their 5' and 3' ends, respectively. The modifications corresponding to the precursor (orange) and the one that differs from the precursor (green). the polymorphic isoforms with distinct nucleotides (purple) within their sequence. Extracted from (Neilsen *et al.*, 2012)

1.4.2. Discovery of miRNAs

Lin-4 was the first member of the miRNA family discovered in *Caenorhabditis elegans* (four different larval stages L1–L4), which is involved in directing the post-embryonic proliferation and the stem cell differentiation in the worm (Lee *et al.*,1993; Ambros, 1989). This was discovered by the isolation of a mutation in the *lin-14* gen causing a failure in the development of a larval stage (Chalfie *et al*., 1981). The same thing happens when the miRNA *lin-4* is downregulated, which can indicate that *lin-4* could negatively regulate *lin-14* (Lee *et al.*, 2004). *Lin-4* negatively regulates the translation of its target *lin-14* (Wightman & Ruvkun, 1993), which, in turn, encodes a nuclear protein, whose downregulation involves the transition from the L1 to L2 larval stage (Lee *et al*., 1993). The opposite, a mutation in *lin-4*, interrupt *lin-4* regulation, continuing the L1 specific cell-division pattern (Chalfie *et al*., 1981). The same occurs with the target *lin-28,* which initiates the developmental transition between the L2 and L3 stages (Moss *et al.*, 1997). However, *lin-28* has fewer *lin-4* binding sites compared to *lin-14* (Moss *et al.*, 1997). *Let-7* was the second miRNA to be discovered in the same worm. It controls the transition from the L4 stage into the adult stage (Reinhart *et al.*, 2000). These findings point to a new mechanism of gene regulation in worm development and suggest the possibility that miRNAs are present in species other than nematodes (He & Hannon, 2004). In recent years, the number of miRNAs discovered has been steadily increasing. Currently, 38589 miRNAs in 271 species are listed in miRbase (version 22.1, 2022).

1.4.3. Canonical pathway of miRNA biogénesis

miRNAs can be mainly of intergenic and intragenic origin (Godnic *et al.*, 2013; Issabekova *et al.*, 2012). Intergenic miRNAs genes are located between genes and their transcription is mainly performed by RNA polymerase III. These miRNAs have been highly conserved throughout evolution (Ozsolak *et al.*, 2008). Intragenic miRNA genes are located in the exons or introns of protein-coding genes and are transcribed by RNA polymerase II (Lee *et al.*, 2004) (**Figure 1.7**).

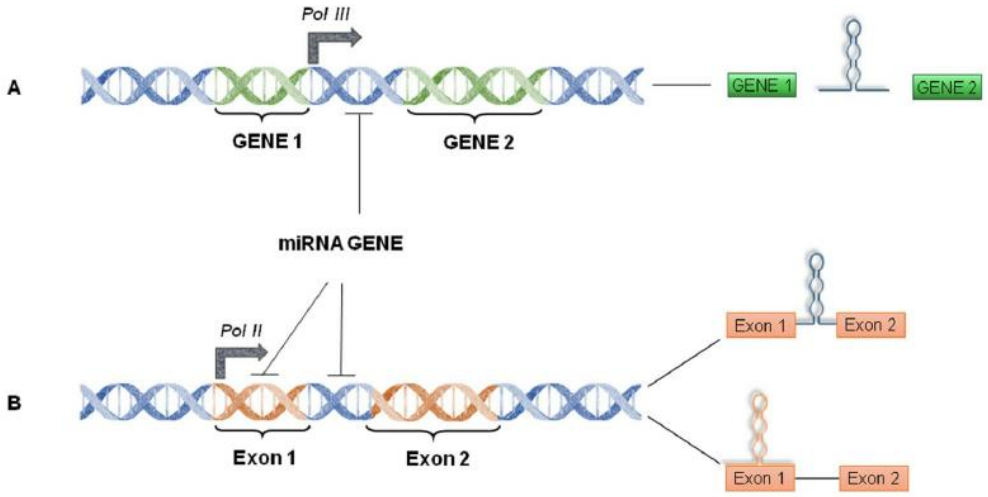

Figure 1.7. Genomic organization of miRNA genes. (A) intergenic miRNA gene; (B) exonic/intronic miRNA gene. Extracted from (Tomaselli *et al.*, 2013)

Initially, miRNAs are transcribed from DNA, Thus, primary miRNA (Pri-miRNA) molecules are generated, which have at the 5' end 7-methyl-guanosine and at the 3' end a poly adenine tail. The enzyme Drosha and DGCR8 act on the Pri-miRNA, cleaving its ends, resulting in a Pre-miRNA consisting of an imperfect stem-loop structure with a size of ~ 70-bp (Ha & Kim, 2014). After initial cleavage by Drosha, the pre-miRNA is transported from the nucleus to the cytoplasm by Exportin 5 (Exp5) together with a Ran-GTP protein (Yi *et al.*, 2003). Once inside the cytoplasm, the pre-miRNA is cleaved by the RNaseIII Dicer/TRBP enzyme and generates a small imperfect duplex: dsRNA, which contains both the mature miRNA strand and its complementary strand (miRNA *) (Ha & Kim, 2014). The miRNA is immediately incorporated into the RNA-induced silencing complex (RISC) Argonaute (AGO) protein, which unwinds the miRNA duplex and removes the complementary strand from the guide strand, so that only the guide strand is incorporated (Ha & Kim, 2014) (**Figure 1.8**)

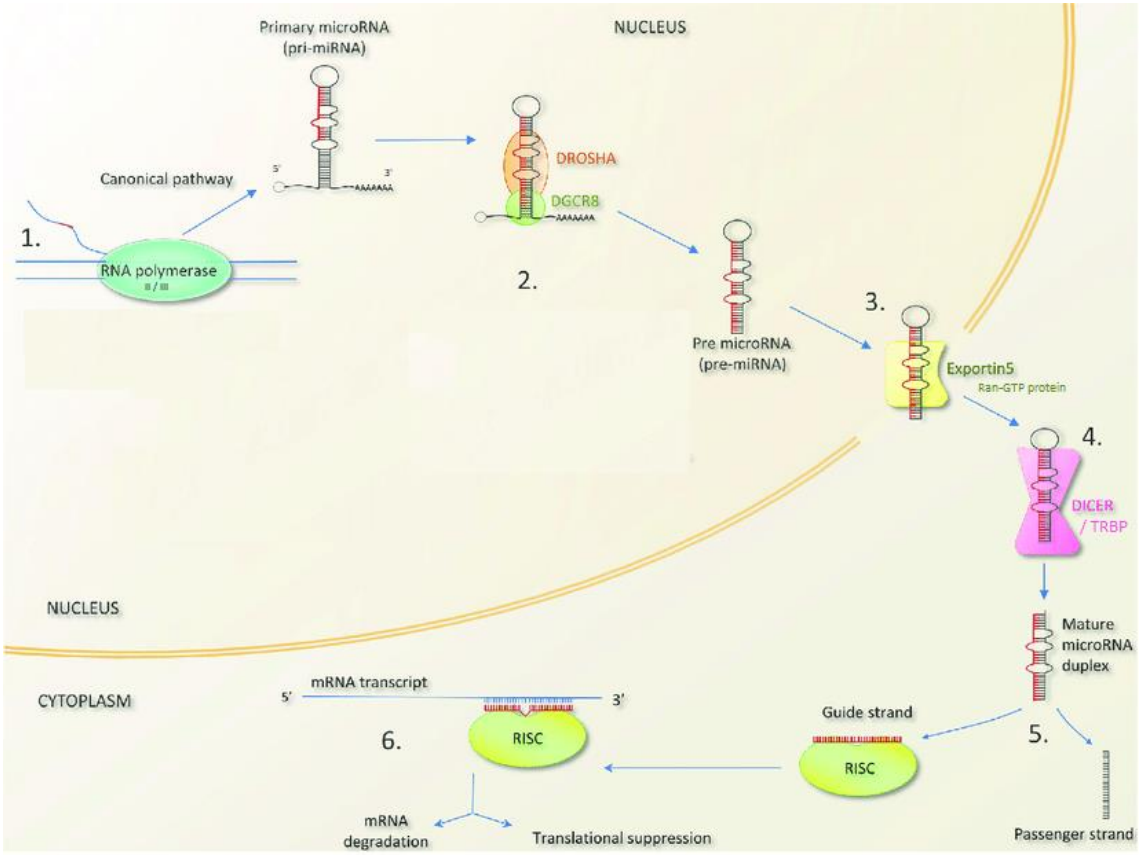

Figure 1.8. miRNA biogenesis and modes of action. Extracted from (Drury *et al.*, 2017).

Promoter genes of miRNAs are often located in clusters, i.e. close to other miRNA genes, and may therefore be co-transcribed, resulting in a correlation of their expression patterns for several genes (Baskerville & Bartel, 2005).

1.4.4. Mode of action of miRNAs

miRNAs bind their targets by complementarity to their "Seed" region (2-7 nucleotides) (Bartel, 2009). The reduced number of nucleotides for complementation allows one miRNA to target many mRNAs and one mRNA to be targeted by more than one miRNA (Lim *et al.*, 2005). The consequence of binding a miRNA to a target mRNA is usually repression of target gene expression, where the RISC complex could interfere with translation initiation. Binding of a miRNA to its target mRNA, could also generate a degradation of the mRNA initially targeting at the poly-A tail that causes the destabilisation of the mRNA (**Figure 1.9**). Furthermore, miRNAs may also act on ribosomal moieties responsible for polypeptide chain synthesis by degrading

polypeptides or by binding complementarily to the 3′UTR (three prime untranslated region) of target mRNAs, inducing premature ribosome dissociation (Valinezhad Orang *et al*, 2014). Other studies have shown that miRNAs can also activate gene expression directly or indirectly in the presence of different cofactors or in response to different cell types and conditions (Vasudevan, 2012). Nevertheless, mammalian miRNAs generally act to repress target mRNA (Guo *et al.,* 2010). Most animal miRNAs repress target translation, however in plant, miRNAs act by causing cleavage in their mRNAs targets, since in plants, miRNA-target mRNA base pairing is almost perfect, and the complementary site is not limited to the 3' UTR (**Figure 1.9**) (He & Hannon, 2004).

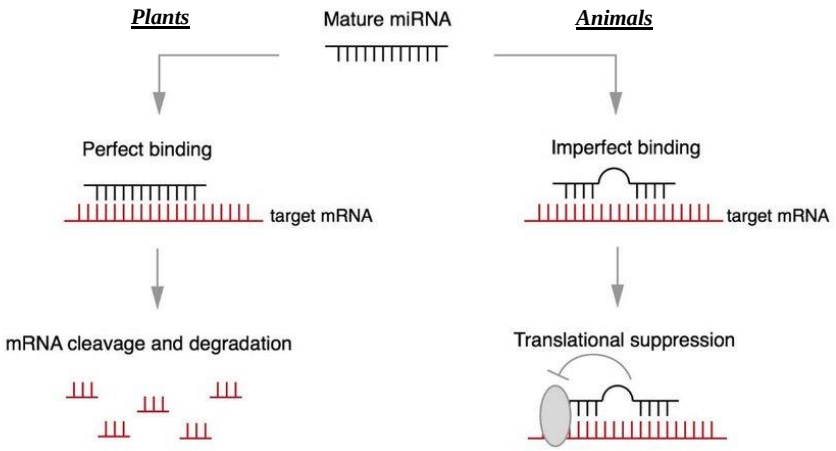

Figure 1.9. Basis of interaction between miRNAs and their mRNA targets in animals and plants. Extracted from (Teixeira *et al.*, 2014)

1.4.5. Functionality of miRNAs.

miRNAs are involved in the epigenetic regulation of gene expression in several ways: miRNAs expression can be regulated by various epigenetic mechanisms, they are also capable of repressing the expression of epigenetic factors; miRNAs and epigenetic factors can interact to modulate common targets (Bianchi *et al.*, 2017). As mentioned above, miRNAs regulate hundreds of targets, and it is estimated that most protein-coding genes are under their control (Friedman *et al.*, 2009). This implies that practically all biological processes are subject to miRNA-dependent regulation. The basis for a complete understanding of the regulatory function of miRNAs is the identification of the targets that regulate miRNAs and pathways in which they are involved (Pian *et al.*, 2020). The physiological functions of miRNAs have been deduced mainly through miRNAs

overexpression studies in animals and cell cultures or by using antisense molecules to disrupt their pairing with their targets and then deduce their functions (Vidigal & Ventura 2015). Several computational approaches have also been implemented for miRNA target gene prediction. These approaches are based on predicting the degree of sequence complementarity between a miRNA and its target (Lim *et al.*, 2003). Several tools and databases have been proposed so far: DIANA-miRPath (Vlachos *et al.*, 2015), TargetScan (Agarwal *et al.*, 2015), there are even databases only devoted to ruminants, such as RumimiR (Bourdon *et al.*, 2019). Some of them include experimentally supported targets in different species, such as TarBase, where the information is collected manually from the literature (Sethupathy *et al.*, 2006).

miRNAs can regulate cell differentiation, cell proliferation, cell death, fat metabolism, neuronal patterning, etc. (Wahid *et al.*, 2010). Some of the biological functions of these molecules are related to viral infection and immune response. This response is explained by the presence of a sequence in the 3' UTR in all retroviral mRNAs corresponding to the *miR-32* that represses viral expression (Lecellier *et al.*, 2005). miRNAs can also have a pro-viral function as in the case of *miR-122* which is necessary for hepatitis C virus to be efficiently expressed (Jopling *et al.*, 2005). These molecules have a role in tumor suppression or may also function as oncogenes (Esquela-Kerscher & Slack, 2006). Beyond the functions they exert in the cells that produce them, miRNAs can also be transferred to other cells, in protein complexes or through EVs, such as microvesicles and exosomes (Chen *et al.*, 2012).

1.4.6. Genetic and epigenetic regulation of miRNAs synthesis

To generate a given miRNA sequence, a number of prerequisites must be met, as mentioned in the section on biogenesis. The source of variation may be related to the coding sequences of the miRNAs, which may influence the expression levels and profiles of the miRNAs. However, it has been shown that miRNA genes have lower SNP densities (Saunders *et al.*, 2007). Genetic variations may also affect miRNAs transcription and maturation. These variations can lead to altered transcription rates, aberrant expression patterns, disruption of miRNA maturation by changing the binding affinity of miRNA to biogenesis enzymes and proteins (Cammaerts *et al.*, 2015). The transcription of miRNAs is also controlled by epigenetic factors (Bianchi et *al.*, 2017). For example, the decrease in *miR-31* expression in triple-negative breast cancer cell lines is linked to the

hypermethylation of its promoter (Augoff *et al.*, 2012). Another study shows that the loss of *miR-124a* is attributed to the activation of cyclin D kinase 6, an oncogenic factor, and phosphorylation of the tumour suppressor gene retinoblastoma (Lujambio *et al.*, 2007).

1.4.7. Environmental regulation of miRNAs synthesis

Research on miRNAs, particularly those related to human diseases, has made enormous progress. The increased risk of breast cancer leads in particular to a deregulation of *miR-125b, miR-145, miR-21,* and *miR-155* expression (Iorio *et al.*, 2005). Viral infections also have an impact on miRNAs expression. For example HIV-1 infection significantly decreased the levels of *miR-28, miR-125b, miR-150, miR-223* and *miR-382* in activated CD4+ T cells (Huang *et al*., 2007).

The expression of miRNAs is also governed by factors related to the organism's environment. The organism's response to stress can underlie the variation in miRNAs expression. For example, it has been shown that *miR-10a* and *miR-21* expression in blood is regulated by psychological stress (Beech *et al.*, 2014). As well, another study indicates that stress levels in students were negatively correlated with the expressions of *let-7b* and *miR-21*, whose expressions decreased from periods of low to high stress (Gidron *et al.*, 2010).

Exercise induces specific miRNA profiles. Fernández-Sanjurjo *et al.* (2020) show in a study about miRNA profiles in response to acute endurance exercise in male amateur runners that different doses of exercise induce specific circulating miRNA (see section 1.4.9) profiles comparing a 10 km race, a half marathon and a marathon.

Environmental pollution may also exert an influence on the expression of miRNAs. In fact, several studies show that exposure to some heavy metals has an effect on the expression of miRNAs. Arsenic exposure resulted in decreased expression of *miR-210* expression and increased expression of in *miR-22, miR-34a, miR-221,* and *miR-222* (Marsit *et al.*, 2006). Another study reported that when human hepatoma HepG2 cells were exposed to cadmium, differential expression of *let-7* miRNA family members was observed (Fabbri *et al.*, 2012). A downregulation of *miR-302* and an upregulation of *let-7, miR-125b,* and *miR-132* were observed in mercury-treated neuronal/glial cell cultures (Pallocca *et al.*, 2013).

Variations in miRNAs expression can be affected by dietary factors. Ferreo *et al.* (2021) show that nutrients influence miRNA profiles, *miR-23a-3p* expression was positively correlated with dietary sodium but negatively correlated with lipids and vitamin E. Also, vitamin D intake was negatively correlated with *miR-1277-5p* and *miR-144-3p* expression.

1.4.8. Environmental regulation of miRNAs synthesis in cattle

This part will focus on environmental factors that cause miRNAs variation in cattle. Several studies have shown that different diets result in different miRNAs profiles in bovine muscle (Muroya *et al*., 2016) subcutaneous muscle fat (Romao *et al.*, 2012) and serum (Quan *et al.*, 2019). A recent study shows that bovine rumen epithelium undergoes massive changes on miRNAs level due to dietary transition (Pacífico *et al.*, 2022). Even the inclusion of a single ingredient in the diet may be behind theses variations in milk. As in the case of sunflower oil supplementation that affects the expression of *bta-miR-20a-5p* and *bta-miR-142-5p* (Mobuchon *et al.*, 2017), and the same happens after linseed oil supplementation (Li R. *et al.*, 2015) that also impacts other miRNAs in the bovine mammary gland. Not only the quantity and components of the feed affect miRNAs, but also the quality of the feed may be an important factor in these variations. Low-quality forage diets such as corn stover and rice straw, affect miRNAs such as *bta-miR-99b* in rumen, *bta-miR-2336* in duodenum, *bta-miR-652* in jejunum, *bta-miR-1* in liver, and *bta-miR-181a* in mammary gland (Wang *et al.*, 2016). Feeding restriction also have an effect on miRNAs expression of holstein cow's mammary gland, whereas no significant changes in miRNAs were observed in Montbéliarde cows (Billa *et al.*, 2021).

Grazing also affects plasma miRNA profiles, as shown by Muroya *et al.* (2016) comparing indoor grain feeding and grazing systems in Japanese black cattle. This study has shown that *bta-miR-10b* is high in grazing animals. However, the effect of green grass consumption through grazing *vs.* grain feeding and the effect of grazing as an exercise factor *vs.* indoor housing are not disassociated here. Another study evaluated the effect of grazing as an exercise factor (Muroya *et al.*, 2015) in the same breed, showed variations in miRNAs between housed and grazing animals. The *bta-miR-451* stood out for its high level in both plasma and biceps of grazing animals.

The adaptability of animals to different conditions can also be reflected in the variation of the miRNA transcriptome. It has been shown in a study comparing yak and cattle, taking into account that their genomes are similar, that the adaptation of yak to high altitudes results in a different miRNome than that of cattle (Guan *et al.*, 2017). At the farm, animal management may be at the origin of miRNAs variations. For example, the relocation of animal groups, potentially a spontaneous source of stress, has resulted in high expression in milk exosomes of *bta-miR-2284z* and *bta-miR-146* in animals subjected to relocation (Colitti *et al.*, 2019). Other studies have demonstrated the response of Holstein cows to stress and its effects on miRNAs expression. When analyzing the expression of miRNAs in the mammary gland, differential expression of some miRNAs has been observed between heat stressed and normal cows (Li *et al.*, 2018). Lee *et al.* (2020) found that the differentially expressed miRNAs in blood under summer heat stress were *bta-miR-19a*, *bta-miR-19b*, *bta-miR-30a-5p*, and several miRNAs of the *bta-miR-2284* family, indicating that pregnant cows are more susceptible to heat stress (Lee *et al.*, 2020). The health status of cows leads to variations in miRNAs in different tissues. Inflammation due to mastitis upregulates the expression of *bta-miR-21, bta-miR-146a, bta-miR-155, bta-miR-222,* and *bta-miR-383* in milk (Lai *et al.*, 2017).

The expression of miRNAs is dynamic and can also vary according to the physiological state of the cow. In the study by Li *et al.* (2012), changes in mammary gland tissues miRNAs expression were found depending on whether the cow was lactating or not. A total of 56 miRNAs in the lactating mammary gland showed differences in expression compared to the non-lactating mammary gland (Li *et al.*, 2012). Another study by Wang *et al.* (2012) examined the expression of several miRNAs 30 days prepartum, 7 days postpartum and 30 days postpartum. This study showed that with the exception of *bta-miR-31,* the expression of all miRNAs studied increased between the dry and colostral period.

1.4.9. miRNAs in cow's milk

Body fluids contain miRNAs, including colostrum, seminal fluids, tears, and plasma (Weber *et al.*, 2010) as well as milk (Chen *et al.*, 2010), which has been shown to be a rich source of miRNAs (Carrillo-Lozano *et al.*, 2020). The cellular origin of miRNAs in milk is still under debate. Comparison between serum and milk in humans has concluded that most milk miRNAs were not provided by the blood circulation (Alsaweed

et al., 2016), but originate from their biogenesis (described above) in mammary alveolar epithelial cells (Hata et *al.*, 2010). The secretion process of the different milk components differs, resulting in different profiles and concentrations depending on the milk fraction (Li et *al.*, 2016). Fat is released as MLG by exocytosis into the alveolar lumen (Huston & Patton, 1990), which explains why the miRNome of milk fat and mammary gland tissue are highly correlated (Li *et al.,* 2016). Munch *et al.* (2013) reported that human milk fat had the highest miRNAs content. This makes the fat miRNome a good alternative for assessing the miRNome profile of mammary gland tissue (Li *et al.,* 2016). The milk cell fraction is more heterogeneous, being a mixture of immune cells and exfoliated epithelial cells that are poured into milk from the udder (Alhussien *et al.*, 2018). Alsaweed *et al.* (2016) thus showed that miRNAs in milk cells are mainly derived from epithelial cells. However, epithelial cells may not reflect the true metabolic state of mammary gland cells as they are generally dead cells (Krappmann *et al.*, 2012). In whey most miRNAs were present in exosomes (described in the next section) (Izumi *et al.*, 2015). The fat and cell fractions showed a higher amount and diversity of miRNAs than whey (Alsaweed *et al.*, 2016; Li *et al.*, 2016), however the miRNAs profiles of milk fat and whey was similar compared to those of cells (Li *et al.*, 2016). Whey had the lowest miRNAs content of the three fractions (Alsaweed *et al.*, 2015).

1.4.10. Milk circulating miRNAs and its bioactive functions.

Most miRNAs are found in the cellular environment, although many of these molecules are known as extracellular or circulating miRNAs, usually observed in body fluids (Sohel, 2016). These miRNAs are characterized by the fact that they are quite stable and resistant to adverse conditions (Izumi *et al.*, 2012); as they are enveloped by membrane vesicles such as exosomes, microvesicles (**Figure 1.10**) (Sohel, 2016), thus enabling cell-to-cell communications (Hwang, 2013). Exosomes and microvesicles are nanospheres with a diameter between 30 nm and 1 μm (Benmoussa *et al.*, 2020). The larger ones, called ectosomes or microvesicles, are usually produced by budding from the plasma membrane. Exosomes are smaller and are released upon invagination of multivesicular bodies and fusion of the latter with the plasma membrane (Sohel, 2016). Apart from the cited vesicles, miRNAs can be released into the extracellular environment through high density lipoprotein (lipid transporter) (Lee H. *et al.*, 2009) and AGO 2 protein complex (one of the major components of RISC complex) (Turchinovich *et al.*,

2011) (**Figure 1.10**), which also provide protection from harsh extracellular conditions (Sohel, 2016).

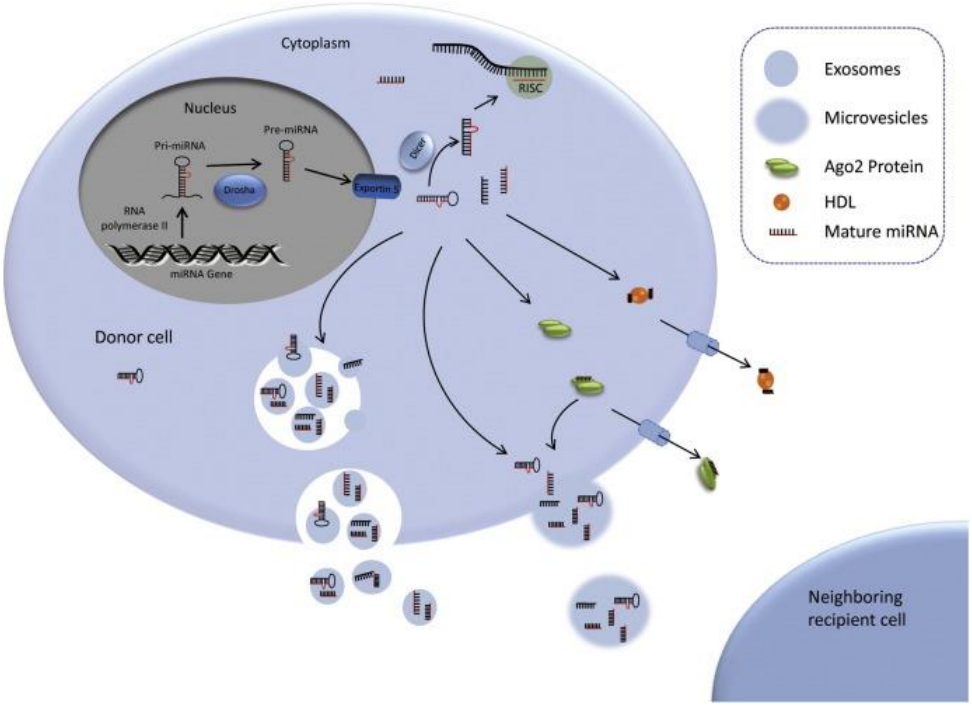

Figure 1.10. Modes of miRNA release into the extracellular environment. MiRNAs can be released into the extracellular environment via exosomes or coupled to the Ago2 protein; they can be wrapped in microvesicles or attached to high-density proteins. Extracted from (Sohel, 2016).

In view of this, some studies have challenged the dogma that miRNAs are endogenous regulators, suggesting that dietary miRNAs can be absorbed and bioavailable in the recipient organism. Furthermore, these miRNAs can regulate gene expression in host cells of different organisms. It has been shown that humans and mice can take up *miR-168a* from rice and that this miRNA alters low-density lipoprotein receptor adapter protein 1 expression in humans and mice liver (Zhang *et al.*, 2012). Similarly, miRNAs in sow's milk are absorbed by newborn piglets (Gu *et al.*, 2012). In humans and mice, an increase in plasma miRNAs *miR-29b* and *miR-200c* has also been observed due to their uptake in cow's milk. The regulatory role after their consumption has also been demonstrated (Baier *et al.*, 2014). Several studies confirmed that milk miRNAs contained in exosomes can be transferred into human cells after milk consumption and that these

miRNAs are able to regulate gene expression in the recipient cell (Benmoussa *et al.*, 2020; Reif *et al.*, 2019; Samuel *et al.*, 2017), thus conferring a functional character to milk (Zempleni *et al.*, 2015).

1.4.11. The use of miRNAs as biomarkers

miRNAs fulfil several criteria that make them good biomarkers in different fields of study. MiRNAs vary according to various factors related to diseases, diet, stress, physiological state, etc. This means that a given miRNA profile (presence/absence) or the differential level of a given miRNA may be indicative of a particular state, highlighting the specificity of miRNAs, and their ability to differentiate between specific conditions (Pacífico *et al.*, 2022; Fernández-Sanjurjo *et al.*, 2020; Mobuchon *et al.*, 2017; Moruya *et al.*, 2016; Holloway *et al.*, 2012; Huang *et al.*, 2007). They also showed a high sensitivity varying in the same way as the studied criterion (Condrat *et al.*, 2020). Also, their profiles and levels in the body fluid can be representative of the tissue of interest, for example, miRNAs levels and profiles in milk fat represent those of the mammary gland (Li *et al.*, 2016). The use of miRNAs from body fluids such as milk confers ease of sampling, through non-invasive methods, and low cost. The miRNAs found in milk are highly resistant to acidic pH and to enzymes such as RNases (Izumi *et al.*, 2012), which implies that they are very stable and resistant to industrial treatments (Chen *et al.*, 2010; Zhang *et al.*, 2012). Their levels are quantifiable by various techniques, such as PCR.

The use of miRNAs as biomarkers has been attempted in several areas, especially for detecting diseases (Condrat *et al.*, 2020). Regarding their use as biomarker for cattle and milk production, Chen *et al.* (2010) identified miRNAs that could serve as biomarkers to distinguish poor quality or "manipulated" milk from pure raw milk, in addition to assess quality control of commercial dairy products, such milk and powdered formula milk. In another study, the miRNA *bta-miR-451* were identified plasma as molecular grazing markers in Japanese Shorthorn cattle (Muroya *et al*., 2015). Also, *bta-miR-223* and *bta-miR-142-5p* levels in skim milk were used as early diagnostic biomarkers of bovine mastitis (Tzelos *et al.*, 2022).

Chapter 2

Hypothesis, justification and objectives

Chapter 2: Hypothesis, justification and objectives

The region of the cantabrian coast is characterized by edaphoclimatic conditions with great potential for the production of feed for dairy cattle, either pastures or forages. The model of dairy production has changed in Spain: the number of cows and farmers has decreased and milk production has been maintained through genetic improvement. This implies an intensification of production with an increase in inputs that is not parallel to the increase in price and quality (Vicente *et al.*,2013).

On the Cantabrian coast, these intensive systems coexist with systems based on grazing. The product of each system has characteristics that differentiate it from the others (Vicente *et al.*, 2017). Pasture and forage based dairy production (i) confers important positive environmental externalities (e.g. carbon sequestration) (ii) is related to a higher content of functional nutrients in the milk produced, (iii) improves the economic margin per-litre of milk produced and (iv) is more respectful of animal welfare, an issue that is becoming increasingly important to consumers.

Growing consumer concern about food characteristics has created a new concept of quality, particularly for animal products, which includes the traditional attributes of nutritional value, flavour, taste and colour as well as indicators related to more ethical aspects such as animal welfare and environmental impact (Luykx & Van Ruth, 2008). Consumers assume that pasture-based cow diets are more natural and conform better to ethical requirements compared to more intensive silage- and cereal-based farming (Croissant *et al.*, 2007). For these reasons, the possibility of accrediting the production system is an increasingly relevant element that conditions the choice of consumers at the time of purchase within their economic possibilities (Valenti *et al.*, 2013).

A useful way to develop the potential of pasture-based farms is to differentiate them by establishing certification mechanisms for the final product and its production system, based on the traceability of this product from the farm to the fork. For this purpose, it is important to define a traceability system through the use of molecules that are present in milk in different amounts depending on factors that differentiate and characterize the production system, such as diet, exercise during grazing. In this sense, miRNAs have been proposed.

miRNAs are small non-coding RNAs of 21–25-nucleotide endogenously initiated, that are involved in the post-transcriptional regulation by binding to specific targets in messenger RNA (He & Hannon, 2004). Their expression varies according to its genetic context (Cammaerts *et al.*, 2015) and on environmental factors (Colitti *et al.*, 2019; Lai *et al.*, 2017; Muroya *et al.*, 2015). Especially milk have been shown to be a rich source of miRNAs (Carrillo-Lozano *et al.*, 2020). Furthermore, miRNAs present in milk are highly resistant to both acidic conditions and enzyme treatment (Zhang *et al.*, 2012), implying that they are stable and resistant to industrial treatments (Chen *et al.*, 2010; Izumi *et al.*, 2012). Changes in diet (Bauman *et al.*, 2011), exercise (Padovani *et al.*, 2009) and stress (Tao *et al.*, 2011) have been shown to affect mammary gland gene expression. Considering all this and the above-mentioned characteristics of miRNAs, it seems reasonable to think that milk production in one production system or another may lead to changes in the levels and profiles of milk miRNAs.

This work is framed within the smart specialization strategy for Asturias S3, in the field of Agri-Food, and the Sustainable Development Objective 12, which seeks to ensure responsible food consumption and production, that uses energy and resources efficiently, that takes into account animal welfare, that limits its impact on the environment, as well as the creation of decent jobs in the sector and thus contribute to rural development and the maintenance of the population in the face of the demographic challenge. The general objective of this work is to identify a set of miRNAs whose levels in raw milk and/or dairy derivatives vary according to their origin, and therefore allow the accreditation of milk from animals managed under a sustainable production system based on grazing, feeding with forages from the own farm and a low consumption of concentrates. To meet this objective, the following specific objectives were pursued:

1- Investigate whether miRNA levels vary according to the milk production system and identify candidate miRNAs as biomarkers for the production system from which the milk originates.
2- Study the variations of miRNA content in cow raw milk depending on the dairy production system.
3- Assess miRNA levels in raw milk from organic farms versus non-organic grazing farms.
4- Evaluate the resistance of the studied miRNAs to milk technological and dairy product manufacturing processes in:

a. Pasteurized milk

b. Microwaved milk

c. Yogurt

d. Cheese

5- Study of the functionality:

a. Through bioinformatic analysis of miRNAs

b. Exosomes characterization

Chapter 3

Do microRNA levels in raw cow's milk vary according to the dairy production system?

Chapter 3: Do microRNA levels in raw cow's milk vary according to the dairy production system?

3.1. Introduction

Consumers' growing concern about food characteristics has contributed to the creation of a new concept of quality, particularly for animal products. This concept includes traditional attributes related to nutritional value, flavor, aroma, and color, together with new indicators related to ethical aspects, such as animal welfare and environmental impact of the production system (Luykx & Van Ruth, 2008). Consumers assume that products from cows raised under pasture are more natural and better to meet animal welfare demands than those from cows raised under the cereal-rich diets typical of intensive production systems (Stampa *et al.*, 2020; Schwendel *et al.*, 2017). Intensive systems are considered less sustainable than production based on pastures, particularly because they have a larger ecological footprint and because they divert cereals from human consumption (Stampa *et al.*, 2020; Vicente-Mainar *et al.*, 2013). These considerations highlight the need to be able to determine whether agri-food products come from one production system or another, so that consumers can make fully informed purchasing decisions. (Valenti *et al.*, 2013).

miRNAs have been proposed as being useful as biomarkers in different areas (Pacífico *et al.*, 2022; Fernández-Sanjurjo *et al.*, 2020; Mobuchon *et al.*, 2017; Moruya *et al.*, 2016; Holloway *et al.*, 2012; Huang *et al.*, 2007). Therefore, in this work, the ability of these molecules to differentiate milk according to its origin was explored, by assessing their levels in relation to feeding and farm management (e.g. exercise through grazing, stocking rate...), as these factors allow characterizing the type of milk production system.

Given that the characterization of production systems as intensive or extensive is not always straightforward due to the lack of regulatory definitions and the complexity of the factors involved (Ruiz *et al.*, 2017; Marin, 1996), and that this work explores for the first time how miRNAs vary according to milk production systems, thus, it was necessary to select farms with the maximum contrast in the production system (extensive *vs.* intensive), in order to theoretically maximize the differences in miRNA levels between

the studied farms. Intensive systems were defined as those without grazing and with high amounts of concentrate in the ration, and extensive systems as those based on grazing and with low amounts of concentrate in the ration.

Therefore, the aim here was to assess the ability of miRNA levels to vary between intensive and extensive farms, then bioinformatics was used to predict metabolic pathways and target genes that may be regulated by miRNAs whose levels differ between the two production systems for a complete understanding of their context.

3.2. Material and methods

3.2.1. Sample collection and preparation

For sequencing, six farms were sampled at the end of spring season (May 2016). On three farms (extensive production), animals grazed for at least for 12 hours each day and ate a diet based on fresh grass, with a small amount of concentrates and no corn silage. On the other three farms (intensive production), animals did not graze, instead eating a diet based on conserved feed and concentrates. Sampling has been carried out on farms with totally opposite management, leaving aside farms with mixed management, in order to maximize possible differences in milk composition. Farm characteristics and details of the diets are described in **Table 3.1**. All animals on all farms were Holstein cows.

Table 3.1. Characteristics and diet of farms where milk was sampled for miRNA sequencing.

Production system	Number of cows	Milk production (L/cow/d)	Grazing (h/d)	Grass silage (kg FM /cow/d)	Hay (kg FM /cow/d)	Corn silage (kg FM /cow/d)	Concentrate (kg FM /cow/d)
Intensive (3 farms)	51	30.00	0	17.00	2.00	10.00	10.00
	65	28.00	0	10.00	10.00	15.00	11.00
	90	29.00	0	16.00	2.00	20.00	12.00
Extensive (3 farms)	24	21.00	20	10.00	2.00	0.00	7.00
	14	31.00	12	14.00	6.00	0.00	6.00
	15	29.00	18	15.00	3.00	0.00	6.00

L/cow /d: liter per cow per day, h/d: hour per day, kg FM /cow/ d: kilogram of fresh matter per cow per day

For the validation by quantitative real-time Polymerase Chain Reaction (RT-qPCR), twenty farms were sampled during autumn 2017 and spring 2018. Ten dairy farms applied an extensive system in which animals grazed and consumed a diet based on fresh grass without corn silage and a low amount of concentrates (4-8 kg/cow/day), while the other ten applied an intensive system in which the animals were housed and fed a diet

containing corn silage (16-30 kg/cow/day) and high amount of concentrate (more than 10 kg/cow/day) (**Table 3.2**).

Table 3.2. Characteristics, diet and sampling season of farms where milk was sampled for RT-qPCR validation.

Production system	Sampling season	Number of cows	Milk production (L/ cow/d)	Grazing (h/d)	Grass silage (kg FM /cow/d)	Hay (kg FM /cow/d)	Corn silage (kg FM /cow/d)	Concentrate (kg FM/ cow/d)
Intensive (10 farms)	Autumn	124	37.40	0	8.00	0.80	30.00	11.50
	Autumn	116	37.00	0	10.00	0.00	30.00	10.50
	Autumn	90	29.00	0	16.00	3.00	20.00	12.00
	Autumn	240	36.00	0	10.00	2.50	16.00	12.00
	Spring	250	38.00	0	12.00	0.90	30.00	12.30
	Spring	37	27.00	0	14.00	0.00	28.00	10.50
	Spring	110	30.00	0	16.00	0.00	20.00	11.00
	Spring	72	28.00	0	15.00	2.50	20.00	12.00
	Spring	118	36.00	0	16.00	0.00	22.00	10.50
	Spring	124	37.00	0	11.00	4.50	20.00	12.00
Extensive (10 farms)	Autumn	20	21.00	6	10.00	6.80	0.00	6.50
	Autumn	24	21.00	20	12.00	0.00	0.00	7.00
	Autumn	12	26.20	22	0.00	6.00	0.00	4.00
	Autumn	8	18.80	21	0.00	4.60	0.00	4.10
	Autumn	35	19.50	20	14.00	0.00	0.00	4.70
	Autumn	15	29.00	18	33.00	0.70	0.00	6.20
	Spring	30	27.00	18	0.00	4.00	0.00	8.00
	Spring	7	20.00	22	6.00	0.00	0.00	5.00
	Spring	16	23.00	21	0.00	0.00	0.00	5.00
	Spring	22	24.00	20	0.00	0.00	0.00	6.00

L/cow/day: litre/cow/day, h/d: hour/day, kg F/cow/day, kg of fresh matter/cow/day

From each of farm, bulk tank milk after an even number of milkings (to avoid differences due to afternoon and morning milk composition) was sampled. Samples were maintained at 4 ºC and immediately transported to the laboratory for processing. Milk was well mixed, and 50 mL of each sample were centrifuged at 1900 × *g* for 20 min. The fat in the upper phase was transferred to a new 50 mL RNase-free tube. Then 7.5 mL of QIAzol lysis reagent (Qiagen®, Crawley, U.K.) was added, and the emulsion was vigorously mixed until the fat was well dispersed. The pellet (cellular fraction) from the initial centrifugation was washed twice with phosphate-buffered saline (PBS) (Gibco, Life Technologies Australia), then homogenized with 1 mL of QIAzol lysis reagent. All samples were stored at -80 °C until RNA extraction.

3.2.2. Total RNA isolation

For each sample, total RNA was extracted from 2 mL of milk fat in QIAzol lysis reagent and from 1 mL of cell pellet resuspended in QIAzol lysis reagent. RNA was extracted using the miRVana miRNA isolation kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The protocol basically consists of adding 200 µL of miRNA Homogenate Additive, mixing well and incubating the mixture on ice for 10 min. Next, 2 mL of Acid Phenol: Chloroform (5:1 ratio) was added, mixed and the mixture was centrifuged for 5 min at a maximum speed of 10 000 × g, at room temperature, to separate the aqueous phase from the organic phase. The supernatant was transferred to a new tube without disturbing the interface. To this supernatant a volume of 2.5 mL of 100 % ethanol (equivalent to 1.25 of the initial volume of the fat+ QIAzol) at room temperature was added and the lysate/ethanol mixture was passed through the columns by centrifugation. After filtering the entire mixture (lysate/ethanol), the column was washed twice with miRNA wash Solution. Finally, the column was transferred to new tubes, and 100 µL of Nuclease Free Water preheated at 95°C was added to elute the RNA from the column by centrifugation for 20 to 30 s. The total RNA collected was stored at -80°C.

The concentration and integrity of RNA (RIN: RNA Integrity Number) for sequencing were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA concentration and purity (A260/280 ratio) for RT-qPCR was assessed by the Nano-Drop spectrophotometer (ND-1000, Thermo Scientific, Waltham, MA, USA)

3.2.3. Search for miRNAs candidates from sequencing

3.2.3.1. RNA sequencing

In order to identify miRNAs differing between the two production systems, 12 libraries corresponding to the fat or cellular fractions of milk from six samples of bulk tank milk (three per production system) were prepared and sequenced using Illumina platform (Illumina, San Diego, CA, USA) as 50 bp single reads. The raw sequence data were processed for quality control and low-quality reads were removed from raw data using CASAVA 1.8 based on chastity. Then adaptors were trimmed, and sequences with read length between 15 to 40 nucleotides were mapped to the bovine genome (bostau 7) and miRNA database (miRBase, release version 21) to identify the konwn miRNAs. Expression levels of each miRNA were estimated based on the frequency of reads, and results were normalized to the number of reads per million (RPM) using the following formula:

$$\mathrm{RPM} = \frac{\text{specific miRNA reads number}}{\text{total mapped miRNA reads per library}} \mathrm{x}\ 10^6$$

3.2.3.2. Identification of reference miRNAs for RT-qPCR normalization.

To select the miRNAs to be used as candidates for normalization in RT-qPCR, we chose those miRNAs with more stable expression among samples for each milk fraction, i.e. the miRNAs with the smallest coefficient of variation (CV).

$$\mathrm{CV} = \frac{\text{standard deviation (SD)}}{\text{mean}}$$

3.2.3.3. Identification of miRNAs whose levels differed between production systems

To identify those miRNAs whose levels differ between production systems, the results from miRNAs sequencing were analyzed using three statistical tests. One test was

the ratio of the difference between the means to the sum of the standard deviations between the two production systems (strict Cohen's d).

$$\mathrm{d} = \frac{|(\bar{x} - \bar{y})|}{(\mathrm{SDx} + \mathrm{SDy})}$$

This ratio indicates how many sums of deviations fit between the means, the higher the ratio is, the greater the difference between the means (Cohen, 1988). Another test was Student's t test, for which lower t values indicated greater differences between the means for the two production systems (Student, 1908). The third test was the absolute value of the correlation coefficient (r), the higher this value is, the greater the difference between the two systems (Pearson, 1895).

$$r = \left| \frac{\Sigma(xi - \bar{x})(yi - \bar{y})}{\sqrt{\Sigma(xi - \bar{x})^2 \, \Sigma(yi - \bar{y})^2}} \right|$$

$\bar{x}$: mean miRNA level in intensive farms
$\bar{y}$: mean miRNA level in extensive farms
xi: miRNA level in an intensive farm (i)
yi: miRNA level in an extensive farm (i)
SDx: standard deviation intensive farms
SDy: standard deviation extensive farms

After that, to choose the miRNAs whose levels differed between production systems, they were ranked first according to each statistical test and then, according to the average of the classification of the three tests.

3.2.4. Validation of candidate miRNAs using RT-qPCR

3.2.4.1. RT-qPCR analysis

A set of miRNAs identified in *section 3.2.3.3* and determined to differ significantly between the two production systems were validated by RT-qPCR in milk

samples from twenty dairy farms managed under extensive or intensive production systems (**Table 3.2**).

Total RNA was used for cDNA synthesis using the TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). This protocol consists of 4 steps that will be described hereafter. During the first step "The poly(A) tailing reaction" the mature miRNA is modified adding a poly(A) tail at the 3' end by a poly(A) polymerase. This consist of adding 0.5 µL of 10× Poly(A) Buffer, 0.5 µL of ATP, 0.3 µL of Poly(A)Enzyme, and 1.7 µL of RNase-free water to 2 µL of total RNA. Incubation was carried out in a thermal cycler (Labcycler SensoQuest, Syngen, Germany) with the following program: 37 ºC for 45 min, 65 ºC for 10 min, and maintained at 4 ºC. After adding the poly(A) tail at the 3' end, an adapter is added at the 5' end. The adaptor acts as a binding site for the Primer during the second step "miR-Amp Reaction". For this 3 µL of 5× DNA Ligas Buffer, 4.5 µL of 50% PEG 8000, 0.6 µL of 25× Ligation Adaptor, 1.5 µL of RNA Ligas and 0.4 µL of RNase-free water were added to 5 µL from the previous step. The mixture was incubated according to the following program: 16 ºC for 60 min and maintained at 4 ºC. During the Reverse Transcription (RT) Reaction, the Primer (Universal RT primer) binds to the 3' poly(A) tail, and the reverse transcription of the miRNA is activated, by adding 6 µL 5× RT Buffer, 1.2 µL dNTP Mix (25 mMeach), 1.5 µL 20× Universal RT Primer, 3µL of 10× RT Enzyme Mix and 3.3 µL of RNase-free water. The content of each tube was incubated according to the following cycle: 42 ºC for 15 min, 85 ºC for 5 min, and maintained at 4 ºC. The fourth stage "The miR-Amp Reaction" aims to increase the number of cDNA molecules by adding 25 µL of 2× miR-Amp Master Mix, 2.5 µL of 20× miR-Amp Primer Mix and 17.5 µL of RNase-free water to 5 µL of the previous step and incubated according to the following standard cycle: 95 ºC for 5 min, 95 ºC for 3 s and 60 ºC for 30 s in 14 cycles, then 99 ºC for 10 min, and maintained at 4 ºC. The resulting cDNA was stored at -20 ºC until use.

Levels of miRNAs were determined using RT-qPCR (TaqMan Advanced miRNA Assays; ThermoFisher Scientific, Waltham, MA, USA) in a StepOne thermal cycler (Applied Biosystems, Foster City, CA, USA). The final reaction solution contained 10 µL of 2×TaqMan Fast Advanced Master mix (ThermoFisher Scientific), 1 µL of 20x TaqMan Advanced miRNA Assay (ThermoFisher Scientific), 4 µL of RNase free water, and 5 µL of cDNA (diluted 1:10). The thermocycling program was set at 95 °C for 20 s, followed by 40 cycles at 95 °C for 1 s and 60 °C for 20 s. All PCR reactions were

performed in duplicate and a maximum of 0.5 threshold cycles were permitted between duplicates. Negative controls lacking cDNA were included in all experiments.

3.2.4.2. Selection of stable reference miRNAs and geNorm analysis.

Normalization is an essential component of a reliable qPCR assay. geNorm (Vandesompele *et al.*, 2002) is one of the most popular algorithms to find stable reference genes from a set of tested candidate reference genes in a given experimental condition. GeNorm determines a stability value M which is inversely proportional to the expression stability of the miRNAs in question. It also establishes the pairwise variation (PV) that defines the minimum number of genes suitable for reliable normalization.

We used geNorm to find the optimal number and choice of reference miRNAs for normalization, using miRNAs identified in *section 3.2.3.2* in 22 tank milk samples representing all the experimental variation of dairy production systems existing in the area of study (Abou el qassim, 2017).

3.2.4.3. miRNAs levels normalization and estimation.

Levels of miRNAs were normalized based on the geometric mean of the reference miRNAs selected by geNorm, then estimated using qbase+ 3.1 software (Hellemans *et al.*, 2008) and expressed in base $\log^2$. Unless otherwise noted, results were reported as mean ± SD.

3.2.4.4. Statistical analysis

For statistical analysis of the RT-qPCR results, the statistical software R-Commander (version 2.7-1 Boca Raton, FL, USA) was used. The normality of the data and the homogeneity of the variances were checked a priori using the Shapiro and Levene tests, respectively. For data that did not meet normality or homoscedasticity, the Kruskal-Wallis test was performed. Otherwise, miRNAs levels between extensive and intensive production systems were compared using t-test. Significance was defined as $p < 0.05$.

For comparative analyses between sequencing samples (3 *vs.* 3), a Kruskal-Wallis test was performed because we are working with small samples ($n < 10$) for which it is not known whether it is valid to assume normality of the data.

3.2.5. Prediction and functional analysis of genes targeted by miRNAs

The Target Scan 7.2 bioinformatics tool (Agarwal *et al.*, 2015) was used to predict bovine mRNA targets of candidate miRNAs. Pathway analysis of targeted genes was performed using Panther bioinformatics tool version 16.0 (http://www.pantherdb.org/) based on Gene Ontology (GO) classification (Mi *et al.*, 2021).

3.3. Results

3.3.1. miRNAs levels in fat and cellular fractions of milk

3.3.1.1. RNA quality

From the fluorescence plots and the RIN values (**Figure 3.1**) the RNA quality was assessed. The RIN value was 2.53 ± 0.15 for RNA from milk fat and 6.67 ± 0.21 for RNA from milk cells. Based on the results of these analyses, the use of the miRVana miRNA isolation kit was validated for the extraction of total RNA from the rest of the samples.

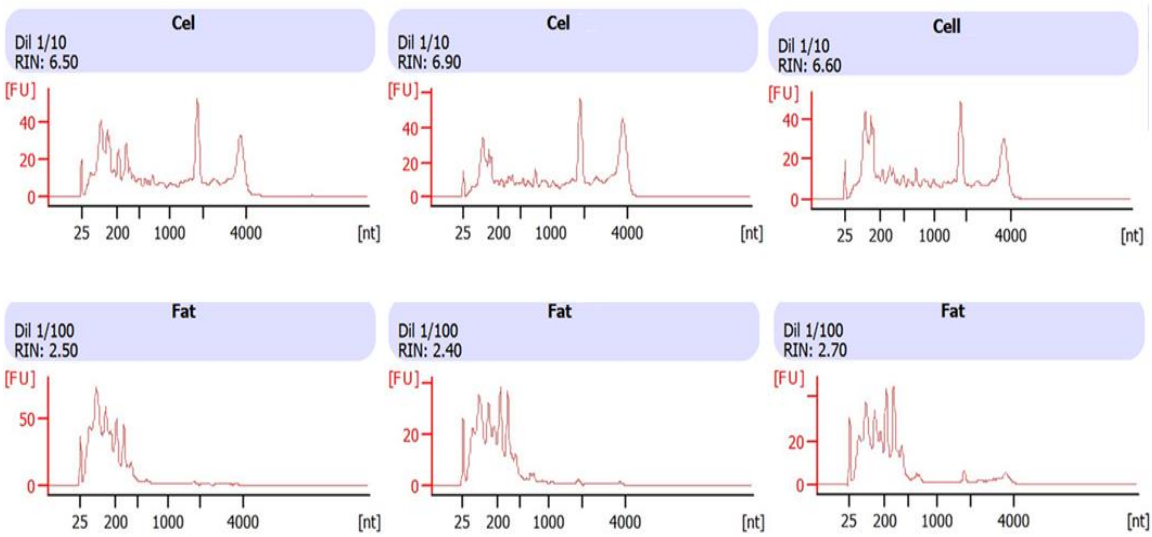

Figure 3.1. Fluorescence plots indicating the integrity of miRNAs, and the RIN value in the cell phase and their corresponding values in fat.

To assess the quality of the samples used for RT-qPCR validation, RNA concentration and purity (A260/280 ratio) was assessed by the Nano-Drop spectrophotometer. The total mean RNA concentration was 192.40 ± 37.40 ng/µL in milk fat and 30.60 ± 15.80 ng/µL (mean ± SD) in milk cells.

3.3.1.2. Sequencing

The six libraries from the cellular fraction of milk yielded a mean of 24,017,290.17 reads, significantly more than the 6,964,122.33 reads ($p = 0.004$) from the six libraries from the fat fraction (**Table 3.3**). Almost half the reads came from small

RNAs, of which the most abundant were transfer RNAs (tRNAs) in the cellular fraction and non-coding RNAs (ncRNAs) in the fat fraction. Significant differences were found between the fat and cellular fractions for percentage of all small RNAs except small nuclear RNAs (snRNAs) and miRNAs. Ribosomal RNAs (rRNAs), small nucleolar RNAs (snoRNA), and ncRNAs were more abundant in the fat fraction than in the cellular fraction, while the converse was true for tRNAs. The levels of miRNAs in the fat fraction differed significantly between extensive and intensive systems ($p = 0.040$, **Table 3.4**). Extensive production was associated with higher miRNAs levels.

We identified 518 known miRNAs in the cellular fraction of milk and 477 in the fat fraction. Most of these miRNAs (454) were present in both fractions (**Figure 3.2**)

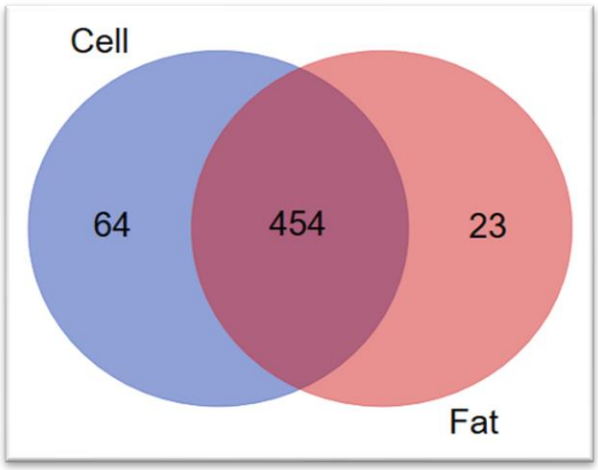

Figure 3.2. Venn diagram showing specific and common miRNAs identified for cell and fat fractions.

Table 3.3. Read mapping statistics.

		Total reads	Small RNA reads	Small RNAs (%)						
				Total	rRNA	snRNA	snoRNA	tRNA	miRNA	ncRNA
Cellular fraction	Intensive farms	32,560,190	16,693,358	0.51	12.68	3.33	5.69	44.99	2.23	31.08
		26,678,803	13,626,331	0.51	8.32	2.04	4.69	64.25	0.88	19.82
		23,616,528	11,871,084	0.50	16.55	3.67	5.50	37.56	1.96	34.76
	Extensive farms	19,944,125	9,753,050	0.49	13.61	3.13	5.55	51.53	1.44	24.72
		21,166,161	9,650,109	0.46	14.93	3.85	6.60	45.40	1.39	27.83
		20,137,934	10,053,989	0.50	15.94	3.93	6.05	36.84	2.34	34.89
Fat fraction	Intensive farms	7,620,977	3,867,636	0.51	20.63	3.44	6.07	29.79	1.71	38.36
		7,209,138	3,139,312	0.44	21.89	3.70	6.64	25.26	1.92	40.59
		6,180,232	3,115,110	0.50	26.02	3.76	6.04	15.75	1.57	46.85
	Extensive farms	7,056,743	2,599,438	0.37	19.39	3.69	8.39	24.80	2.21	41.53
		6,682,264	3,279,814	0.49	25.00	4.07	7.13	16.93	2.35	44.51
		7,035,380	3,854,280	0.55	25.92	4.26	6.97	19.20	2.25	41.40

rRNA: Ribosomal ribonucleic acid; snRNA: Small nuclear RNA; snoRNA: Small nucleolar RNA; tRNA: Transfer RNA; miRNA: microRNA; ncRNA: non-coding RNA

Table 3.4. Differences in abundance of small RNA classes depending on milk fraction and production system.

Small RNA class	*p* value based on Kruskal-Wallis test		
	Cellular *vs*. fat fraction*	Cellular fraction: extensive *vs*. intensive	Fat fraction: extensive *vs*. intensive
rRNA	0.004	0.513	0.827
snRNA	0.196	0.268	0.184
snoRNA	0.024	0.127	0.050
tRNA	0.004	0.827	0.513
miRNA	0.260	0.825	0.040
ncRNA	0.004	0.827	0.513

*Both intensive and extensive production systems together, rRNA: Ribosomal ribonucleic acid; snRNA: Small nuclear RNA; snoRNA: Small nucleolar RNA; tRNA: Transfer RNA; miRNA: microRNA; ncRNA: non-coding RNA.

3.3.1.3. Validation of miRNAs whose levels differed between intensive and extensive production

The first 10 miRNAs in the fat fraction whose levels differed between the two production systems are ranked in **Table 3.5**, and those in the cellular fraction are ranked in **Table 3.6.** Levels of the first five miRNAs from each fraction were validated using RT-qPCR and milk samples from another 20 farms. The following miRNAs in the fat fraction were subjected to validation: *bta-miR-215, bta-miR-369-3p, bta-miR-6520, bta-miR-7863*, and *bta-miR-133a*. The following miRNAs in the cellular fraction were subjected to validation: *bta-miR-574*, *bta-miR-3432a, bta-miR-2285e*, *bta-miR-197*, and *bta-miR-2284y*. Six miRNAs in the fat fraction and five in the cellular fraction were chosen as candidates for normalization (**Table 3.7**).

Table 3.5. Ranking average of the first ten differentially expressed miRNAs in the fat fraction, the three applied tests values, and their rankings.

miRNA	Tests values			Ranking according to			Average ranking
	Test 1[a]	Test 2[b]	Test 3[c]	Test 1	Test 2	Test 3	
*bta-miR-215**	3.180	0.010	0.960	1	1	1	1.00
*bta-miR-369-3p**	1.760	0.020	0.900	3	2	2	2.33
*bta-miR-6520**	1.360	0.030	0.850	4	3	4	3.67
*bta-miR-7863**	1.970	0.080	0.860	2	7	3	4.00
*bta-miR-133a**	1.300	0.040	0.840	5	5	5	5.00
bta-miR-532	1.260	0.040	0.840	6	4	6	5.33
bta-miR-148a	1.210	0.120	0.780	7	13	7	9.00
bta-miR-138	1.000	0.070	0.770	22	6	8	12.00
bta-miR-450a	1.190	0.140	0.760	8	18	10	12.00
bta-miR-6527	1.010	0.090	0.770	21	8	9	12.67

* These miRNAs were validated using RT-qPCR, [a] strict Cohen's d test, [b] Student's *t* test, [c] correlation coefficient test.

Table 3.6. Ranking average of the first ten differentially expressed miRNAs in the cellular fraction, the three applied tests values, and their rankings.

miRNA	Tests values			Ranking according to			Average ranking
	Test 1[a]	Test 2[b]	Test 3[c]	Test 1	Test 2	Test 3	
*bta-miR-574**	5.770	0.000	0.990	1	1	1	1.00
*bta-miR-3432a**	5.520	0.010	0.980	2	3	2	2.33
*bta-miR-2285e**	2.540	0.010	0.950	5	2	3	3.33
*bta-miR-197**	1.970	0.010	0.920	6	4	5	5.00
*bta-miR-2284y**	2.750	0.020	0.940	3	8	4	5.0
bta-miR-219	1.740	0.010	0.910	9	5	7	7.00
bta-miR-2397-3p	1.770	0.020	0.900	8	7	8	7.67
bta-miR-2308	2.560	0.050	0.910	4	14	6	8.00
bta-miR-2419-5p	1.620	0.020	0.890	11	6	9	8.67
bta-miR-2409	1.790	0.040	0.890	7	12	10	9.67

* These miRNAs were validated using RT-qPCR, [a] strict Cohen's d test, [b] Student's *t* test, [c] correlation coefficient test.

Table 3.7. Ranking of the most stable miRNAs according to the coefficient of variation.

Milk fraction	miRNA	Coefficient of variation
Fat	*bta-miR-532*	0.060
	bta-miR-151	0.070
	bta-miR-27b	0.090
	bta-miR-103	0.090
	bta-miR-30a-5p	0.090
	bta-miR-99a-3p	0.090
Cellular	*bta-miR-103*	0.080
	bta-miR-107	0.090
	bta-miR-181a	0.090
	bta-miR-28	0.100
	bta-miR-345-3p	0.100

According to the stability value M (inversely proportional to the stability of miRNAs expression) the decreasing order of miRNAs expression stability in fat was *bta-miR-30a-5p, bta-miR-151, bta-miR-103, bta-miR-532, bta-miR-99a-3p and bta-miR-27b* (**Figure 3.3**). The decreasing order of miRNAs expression stability in cells was *bta-miR-103, bta-miR-107 bta-miR-28, bta-miR-181a and bta-miR-345-3p* (**Figure 3.4**). The optimal number of miRNAs for normalization was determined based on the PV analysis, considering that the values below the threshold of 0.15 do not indicate the need to include other miRNAs for the calculation of the normalization factor. In fat, the PV value indicates that the optimal number of miRNAs control for normalization was two (**Figure 3.5**), therefore, the optimal normalization factor can be calculated as the geometric mean of *bta-miR-151 and bta-miR-30a-5p*. However, in cells fraction, after geNorm analysis, the PV value was not below the 0.15 threshold (**Figure 3.6**), despite that, geNorm recommended selecting the three most stable miRNAs: *bta-miR-103 bta-miR-107 and bta-miR-28.* Following geNorm, normalization of levels in the fat fraction was optimal using the geometric mean of *bta-miR-151* and *bta-miR-30a-5p*. In the case of cellular fraction, geNorm recommended the use of the geometric mean of the three most stable miRNAs (*bta-miR-103, bta-miR-107, bta-miR-28*).

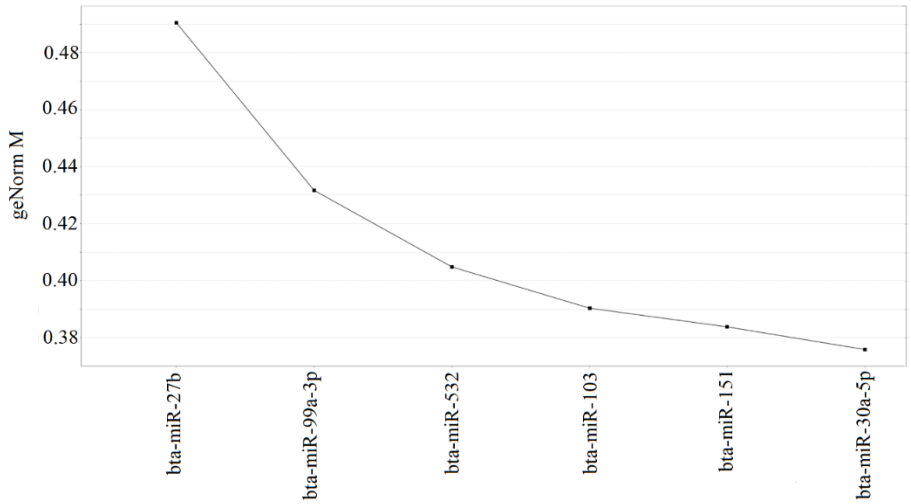

Figure 3.3. M-stability value of candidate normalizers miRNAs in milk fat

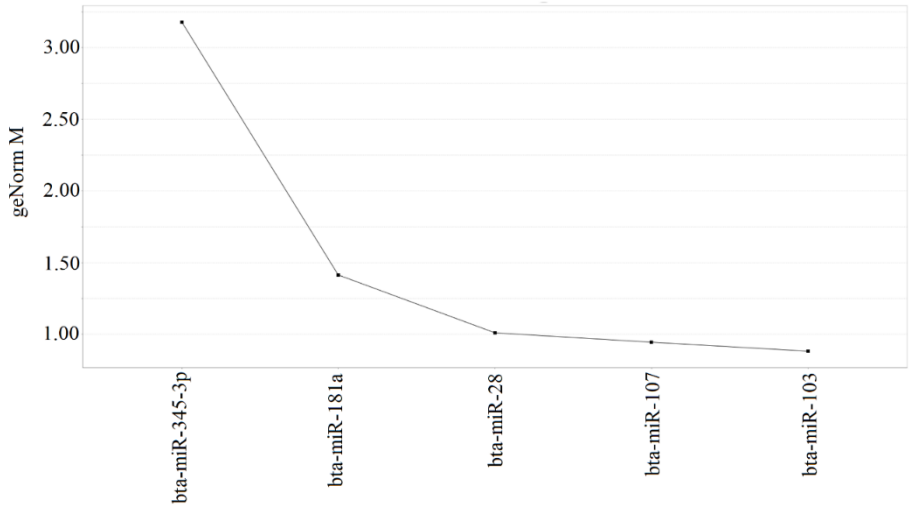

Figure 3.4. M-stability value of candidate normalizers miRNAs in milk cells

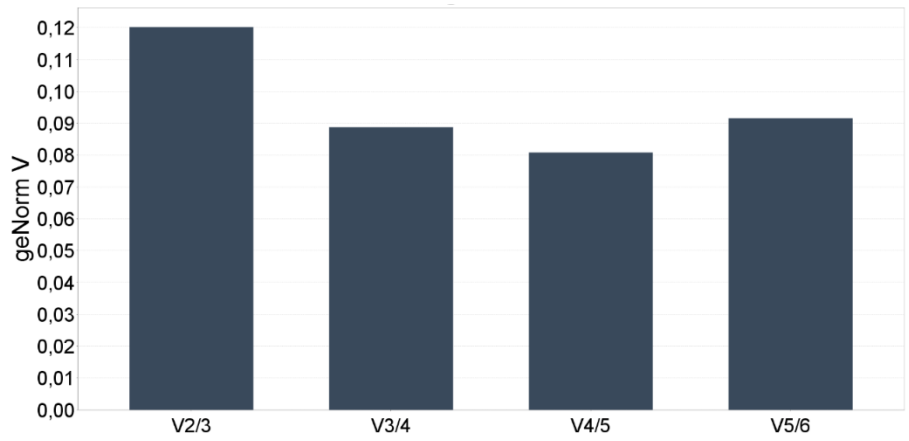

Figure 3.5. Determination of the optimal number of normalizers based on pairwise variation in milk fat

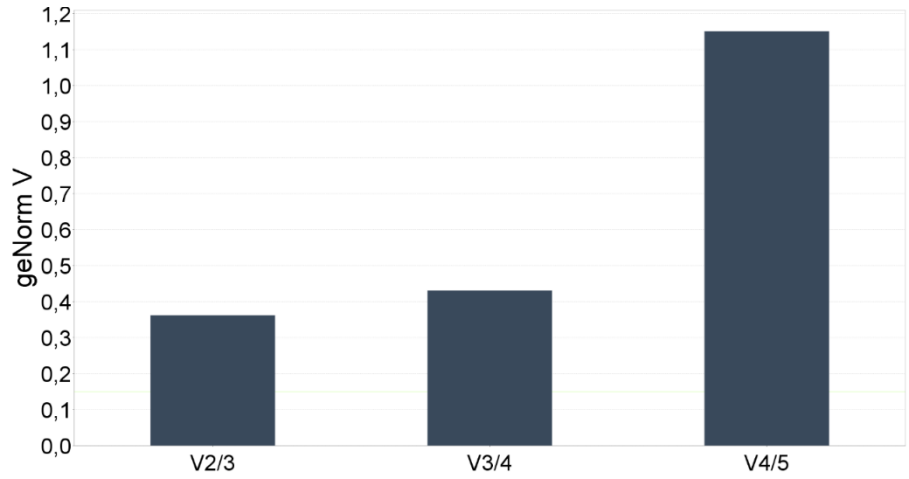

Figure 3.6. Determination of the optimal number of normalizers based on pairwise variation in milk cells

Based on analysis of the normalized miRNAs levels (**Figures 3.7** and **Figure 3.8**), the only miRNA in the fat or cellular fractions that differed significantly between intensive and extensive production systems was *bta-miR-215* in the fat fraction. This miRNA was significantly more abundant in the fat fraction of milk from intensive production ($p = 0.030$). The miRNAs *bta-miR-2284y* and *bta-miR-2285e* in the cellular fraction showed a trend towards lower levels in milk from extensive production, but the differences did not reach statistical significance.

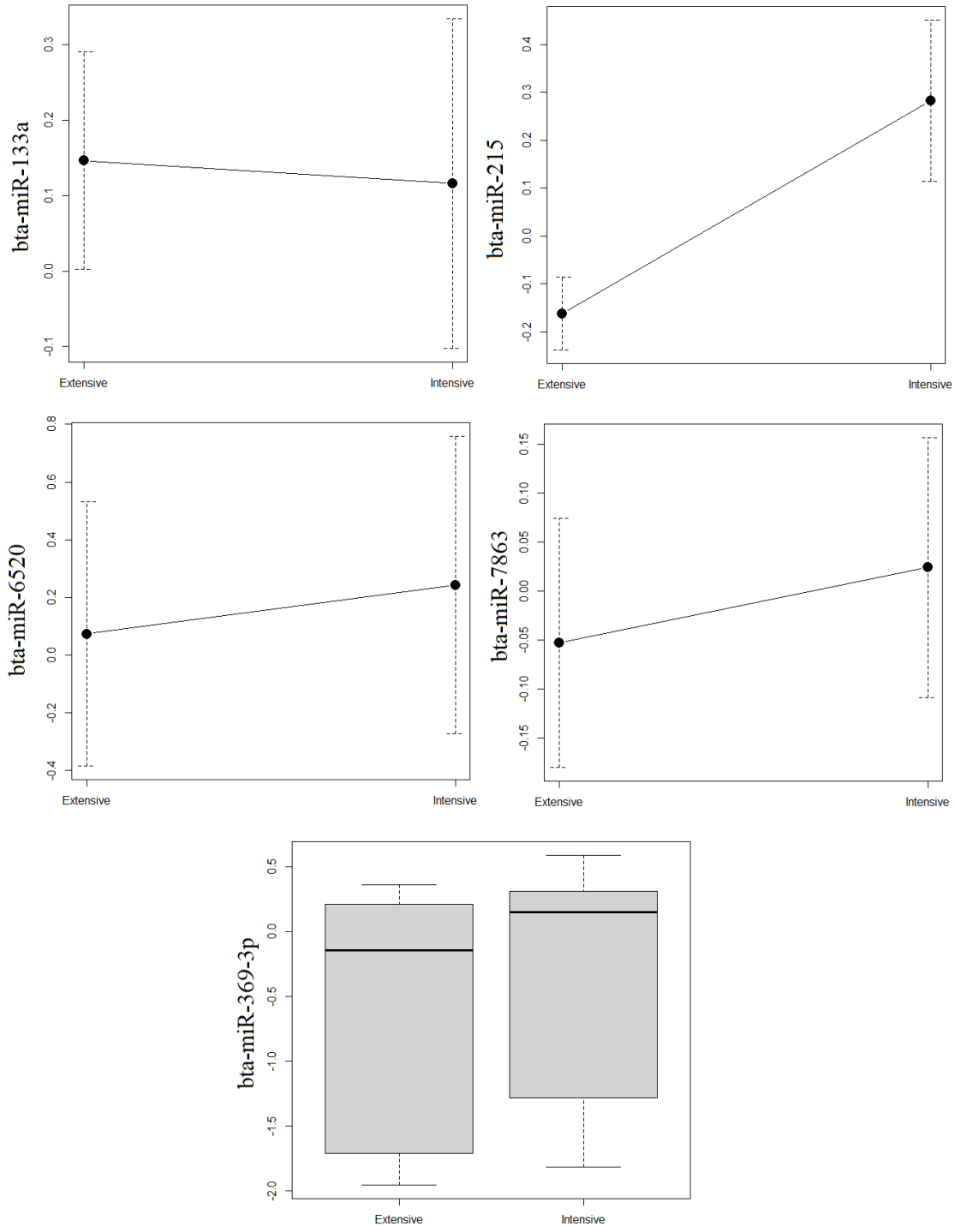

Figure 3.7. The average relative levels of miRNAs estimated by RT-qPCR in milk fat fractions from extensive and intensive farms. X-axis represents production systems, Y-axis represents average relative levels of each miRNA. The levels of *bta-miR-369-3p* did not show a normal distribution, so they are shown using a box-and-whisker plot. Error bars in the other graphs indicate the standard deviation.

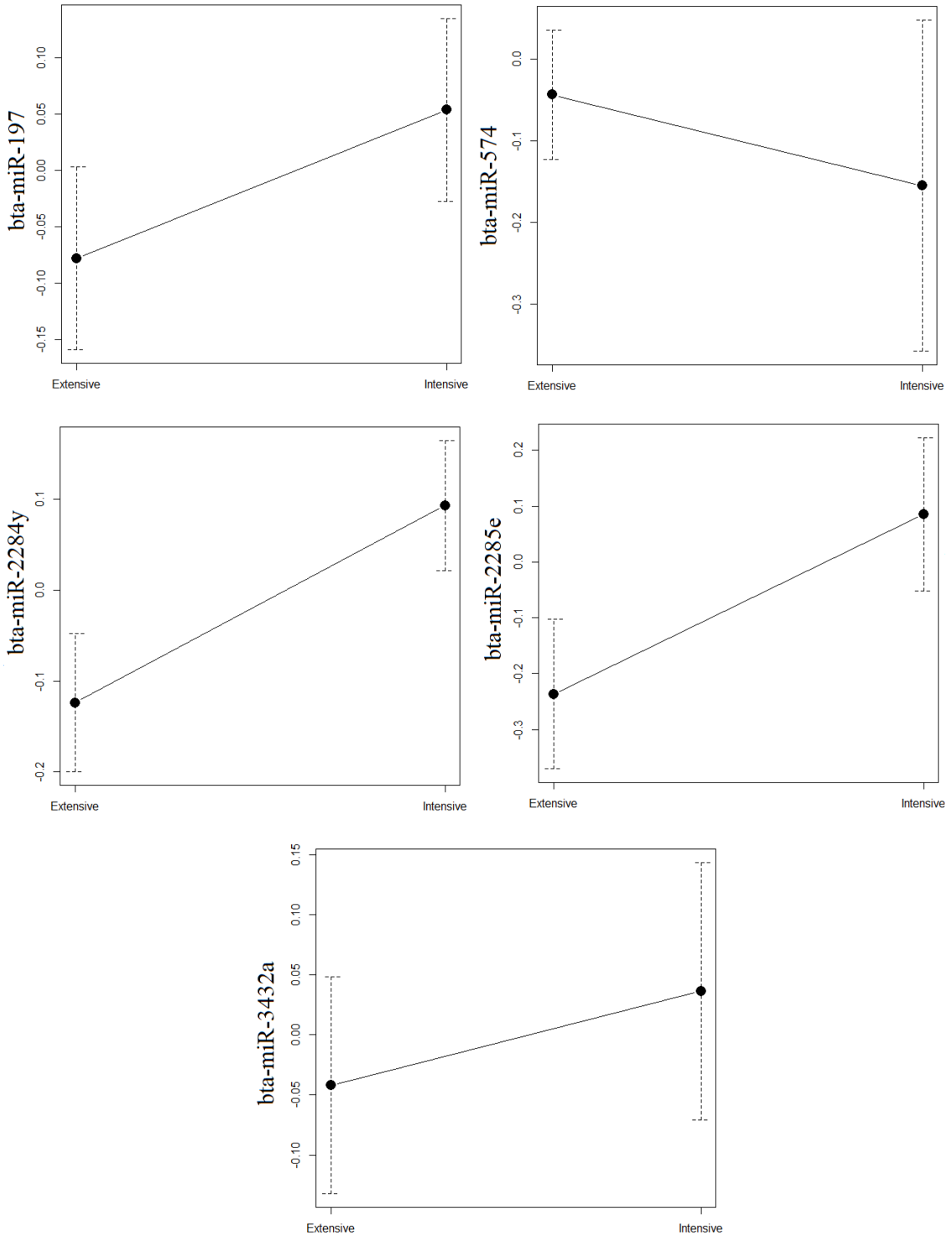

Figure 3.8. The average relative levels of miRNAs estimated by RT-qPCR in cellular fractions of milk from extensive and intensive farms. X-axis represents production systems, Y-axis represents average relative levels of each miRNA. Error bars in the other graphs indicate the standard deviation.

3.3.2. Putative target gene and pathway analyses for miRNA

Using Target Scan, 143 potential target genes were identified for *bta-miR-215*, which was abundant in intensive dairy production compared to extensive ones. Among these targets, one gene is particularly involved in lipid metabolism and energy metabolism: the fatty acid binding protein 3 (*Fabp3*).

The pathway analysis of *bta-miR-215* target genes allowed the identification of 41 associated biological pathways. Gonadotropin-releasing hormone receptor pathway and the TGF-beta 4 (Transforming Growth Factor beta 4) signaling pathway (**Figure 3.9**) were highlighted as they include the most *bta-miR-215* target genes *i.e*. *Acvr2b* (Activin Receptor Type-2B), A*cvr2a* (Activin A Receptor, Type 2A), *Mapk1*(Mitogen-Activated Protein Kinase 1), *Bmpr2* (Bone morphogenetic protein receptor type II).

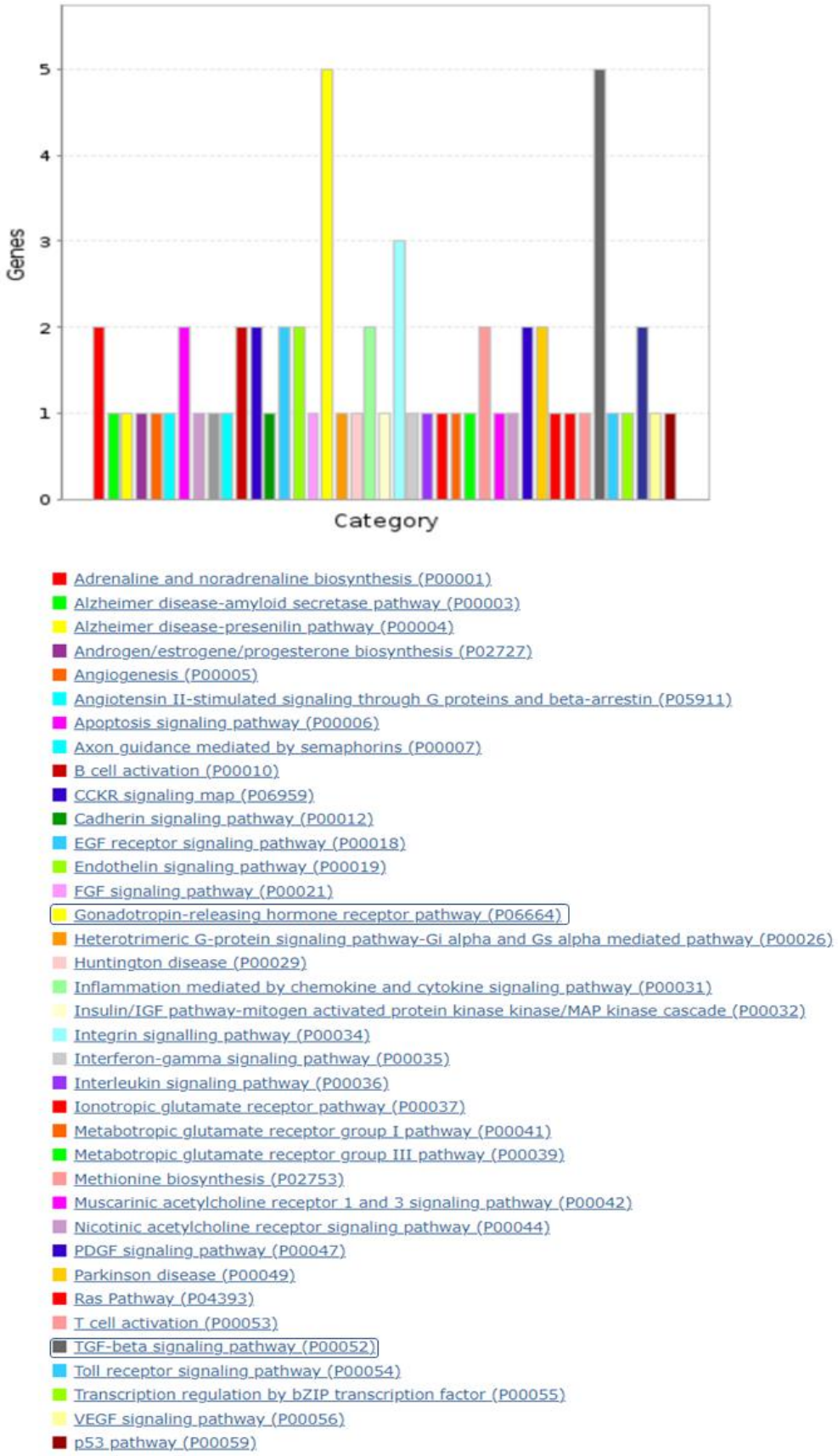

Figure 3.9. Functional Gene Ontology classification of pathway analysis and the number of targeted genes by *bta-miR-215* in each pathway.

3.4. Discussion

The objective of this study was to identify a set of miRNAs in milk whose levels differed with production system in order to determine whether miRNAs profiling can be used to authenticate milk from a given production system. To address this question, we sampled milk from dairy farms in Asturias in northern Spain that applied intensive or extensive production practices, which differ strongly in numerous characteristics that can alter milk quality, including feeding management, animal density, and access to exercise through grazing (Vicente *et al.*, 2017).

The origin of miRNAs in milk is controversial, and most miRNAs in milk are not found in blood (Alsaweed *et al.*, 2016). In addition, the miRNAs profile in milk differs across the fractions of fat, whey and cells (Li *et al.*, 2016). In the present work, we decided not to study the whey fraction because its miRNAs content is lower, its miRNAs profile is highly similar to that of milk fat (Li *et al.*, 2016), and it is not exploited in certain dairy industry practices, such as cheese production. Milk cell fraction is very heterogeneous, it is a mixture of immune and exfoliated epithelial cells that are poured into milk from the udder (Alhussien & Dang, 2018). Thus, Alsaweed *et al.*, (2016) showed that miRNAs in milk cells come mainly from epithelial cells and that lipid and cell fractions showed a higher amount and diversity than in skim milk. Another study confirmed that, in milk fat and somatic cells, total RNA concentrations were higher than in the whey fraction (Li *et al.*, 2016). Based on this the fat and cell phase were selected for this study. However, it is possible that epithelial cells do not reflect the true metabolic state of mammary gland cells because they are often dead cells (Krappmann *et al.*, 2012).

We successfully isolated total RNA from fat and cellular fractions of bovine milk. The RIN was low for RNA from milk fat, which likely reflects its abundant content of low-molecular-weight RNA different from ribosomal RNA, in a similar way as was explained in Li *et al.* (2016) in the same context.

RNA sequencing analysis confirmed different RNA profiles in the fat and cellular fractions of bovine milk. However, we did not identify significant differences in miRNAs levels between the two fractions, although we did obtain what appears to be the first evidence that miRNAs in the fat fraction differ between milk from intensive or extensive production systems. Given that milk has been proposed as a major epigenetic modulator

of gene expression of the milk recipient (Melnik *et al.*, 2019) newborn but also human consumer of bovine milk, and its modulation can be dependent on the amount of miRNAs (Del Pozo-Acebo *et al.*, 2021), our results imply that the animal production system can influence the functional properties of agri-food products of animal origin.

Sequencing results did not allow the identification of miRNAs in either the fat or cellular fraction that were specific to a given production system. Therefore, we focused on miRNAs whose levels differed between the two systems and we validated a subset of the promising miRNAs using RT-qPCR. The only miRNA that we validated to differ significantly between the two production systems was *bta-miR-215* in the fat fraction. This miRNA was upregulated in milk from intensive production. At the moment, we can only speculate why intensive production might up-regulate this miRNA. One possibility is that its up-regulation somehow compensates for poor feed conversion efficiency, as in the case of Angus cows, where up-regulation of *bta-miR-215* in cow liver was associated with less efficient animals in feed conversion (Al-Husseini *et al.*, 2016). Considering that dairy cows with medium potential showed a lower feed conversion efficiency during indoor feeding than during grazing (Knaus, 2016), it is conceivable that the variability of *bta-miR-215* levels between farm groups may be due to a response to low feed efficiency in intensive farms. Also consistent with this possibility is that heat and oxidative stress, which can easily occur in intensive production systems, upregulate *bta-miR-215* in serum of Holstein cows (Zheng *et al.*, 2014).

To understand more about the context of the miRNA *bta-miR-215*, we relied on the identification of the targets that miRNAs regulate and the pathways in which they are involved. Among the 143 genes identified by bioinformatic tools, *Fabp3* stands out for its involvement in fatty acid transport and activation in the bovine mammary gland (Zhang *et al.*, 2021). Vargas-Bello-Perez *et al.* (2020) demonstrated that when the diet of Holstein dairy cows is supplemented with hydrogenated vegetable oil, *Fabp3* is down-regulated in milk somatic cells. Likewise, when fed a high-concentrate diet, the fatty acid transporter *Fabp3* is inhibited in mammary gland (Ma *et al.*, 2022). In our study, *bta-miR-215* increased in milk in response to intensive farming conditions, which are generally associated with high-energy diets, which is consistent with its likely involvement in the downregulation of *Fabp3*. A direct relationship between *bta-miR-215* and *Fabp3* has indeed been validated in bone marrow mesenchymal stem cells (Zhou *et al.*, 2021). Apart from *Fabp3*, other two genes are of particular interest: *Mapk1* and

Bmpr2. *Mapk1* is involved in the regulation of milk protein synthesis (Lu *et al.*, 2013) and *Bmpr2* is associated with the glucose metabolism and insulin response. In fact, when *Bmpr2* is altered, it likely blunts glucose response and lipid uptake (Hemnes *et al.*, 2019). Altogether, they probably have a negative impact on protein and fat synthesis in animals bred under intensive dairy systems.

Finally, our study presents several limitations. The study was carried out in commercial farms, where managing conditions and diets are quite different even within the same group. Validation in controlled conditions at experimental farms, that reduce internal variance of the experimental groups, should help to identify miRNAs as putative biomarkers for dairy production systems.

3.5. Conclusions

We investigated differences in miRNAs profiles of raw cow tank milk from commercial farms applying intensive or extensive production systems. We affirmed that miRNAs vary between very opposite milk production systems. Then, we identified *bta-miR-215* in the fat fraction of milk with significantly higher levels in milk from intensive production systems compared to extensive production systems. Our results imply that the type of production system can influence miRNAs levels, and therefore functional properties, of bovine milk and potentially other agri-food products of animal origin.

Chapter 4

Variation of miRNAs levels in raw cow milk depending on the dairy production system

Chapter 4: Variation of miRNAs levels in raw cow milk depending on the dairy production system

4.1. Introduction

The results of the first chapter showed that some miRNAs can vary in very opposite milk production systems, and *bta-miR-215* was highlighted for its up-regulation in intensive dairy farms. The groups of farms in the first chapter were selected to maximize the differences between intensive and extensive farms and to reduce variability within each group, then, only very intensive and very extensive farms was confronted. However, the characteristics of these two groups do not represent the full variability of milk production systems.

Milk production systems are presented in different types of management that determine the degree of extensiveness/intensiveness of the farms, which can be represented by a continuous line (from 100% intensive industrial farms to 100% extensive pastoral farms). Thus, in addition to very intensive or extensive farms, many others combine certain characteristics of both extremes, where animals may be on pasture, supplemented with conserved forages and concentrates and, depending on climatic conditions, animals may be housed or on pastures (Villar-Bonet & Quintana-Ruíz, 2021; Salcedo-Díaz, 2006). The classification of farms according to the degree of extensiveness/intensiveness consists of setting thresholds or categories appropriate to different needs. Yet, besides some regional or sectoral initiatives, no national regulations clearly define the differences among this continuum of milk production systems (Ruiz *et al.*, 2017). This makes it difficult for more or less extensive farms to certify the advantages of their milk to consumers who demand socially and environmentally responsible products (Stampa *et al.*, 2020; Croissant *et al.*, 2007).

The present study explored whether levels of miRNAs in milk might serve as indicators of how extensively or intensively it was produced, thus serving as a traceability system to identify dairy production systems based on markers in the milk itself to support the certification and accurate marketing of milk from less intensive production systems. The farms have therefore been grouped according to four dairy farming systems: grazing,

zero-grazing, grass silage or corn silage (Abou el qassim, 2017). The literature review on miRNAs in cattle was updated, which allowed the inclusion of new miRNAs in our work for their relationship with feeding, management system and metabolism (Muroya *et al.*, 2016; Wang *et al.*, 2016; Li *et al.*, 2015; Muroya *et al.*, 2015); *bta-miR-29b* was selected from the work of Moruya *et al.* (2016), for its down-regulation in the serum of grazing Wagyu cattle compared to grain-fed animals. *Bta-miR-451-* was upregulated in the plasma of Japanese Shorthorn cattle after two months of grazing. This increase of *bta-miR-451* level corresponded to the same trend of this miRNA in the *biceps femoris* muscle of grazing cattle. *Bta-miR-148a* was selected from the same work for its downregulated levels after one month of grazing (Moruya *et al.*, 2015). *Bta-miR-21-5p* was affected ($p < 0.05$) by linseed and safflower oil treatments upregulated in the mammary gland (Li R. *et al.*,2015). *Bta-miR-155*, *bta-miR-181a* and *bta-miR-103* were selected from the work of Wang *et al.* (2016) testing the effects of low-quality forage use on miRNAs. *bta-miR-181a* was upregulated in the mammary gland of alfalfa-fed cows. However, *bta-miR-155* and *bta-miR-103* levels in the rumen was related to low-quality forage use. These variations of miRNAs were correlated with Nitrogen efficiency and feed efficiency in both tissues. In addition to these miRNAs, others were selected from sequencing study in the previous chapter. A total of 12 miRNAs were studied and tested in the fat phase only, as it was decided, to suspend work on milk epithelial cells, as they probably do not reflect the true metabolic state of mammary gland cells, as they are often dead cells (Krappmann *et al.*, 2012). In addition, in the previous chapter, the validation by RT- qPCR of miRNAs in the milk cell fraction did not show any significant difference between farms belonging to different production systems.

In the current study, the quantification of the levels of 12 miRNAs was performed in tank milk from four dairy farming systems: grazing, zero-grazing, grass silage and corn silage. Then, attempts were made to determine the diet or management-related factors that might be behind the variation in miRNA levels. Also, the targets of the determined miRNAs and their metabolic pathways were explored using bioinformatics tools, to understand the context of the variation of these miRNAs. By comparing the abundance levels of these miRNAs, we aim to highlight potential non-invasive biomarkers of the milk production systems, which may contribute to the authentication of socially and environmentally responsible dairy products.

4.2. Materials and Methods

4.2.1. Study farms

The sampled farms are located in different parts of Asturias (Spain) and are representative of the production systems characteristic of northern Spain. On each farm, the following data on the feeding of lactating cows were requested three days before the site visits: the composition of the diet, the amounts of the different ingredients in the ration, whether fresh grass was consumed and whether the grass was consumed as grazed or cut. Data were also collected regarding the number of lactating cows (as an indicator of herd size) and the duration of grazing per day.

4.2.2. Farm classification

Given the continuum of farm extensiveness and lack of clear regulatory definitions (Ruiz *et al.*, 2017), we defined four dairy production systems for this study based on what we considered the most relevant factors.

First, the farms were grouped according to grazing (whether the animals have access to pasture or not). The group of farms with no grazing includes a very high variability in feed management. This group was therefore also divided into subgroups: the most intensive farms, which include corn silage in their ration, and the intermediate farms, which can also be differentiated according to the consumption of grass silage or fresh grass (without grazing). These groupings are in line with the descriptions of production systems defined in Asturias (Abou el qassim, 2017). So, the farms were divided into four groups (**Table 4.1**): grazing (n = 44 farms), on all farms in this group the animals had access to pasture; furthermore, the ration included at least one of the following ingredients: fresh grass in the stable, grass silage, dried grass and, in a few cases, corn silage (grazing represents the extensive system); zero-grazing (n = 13), where all farms used fresh grass in the stable to feed the cows and, in some cases, grass silage and/ or dried grass were also included; grass silage (n = 10), where animals received ration based on grass silage with or without dried grass (zero-grazing and grass silage represent intermediate systems); and corn silage (n = 45), where animals received a ration based on corn silage, grass silage or dried silage or both (corn silage represents intensive system). In all the rations the concentrate was included. Dried grass refers to the sum of hay, alfalfa, vetch and Straw amounts in kg of fresh matter per cow per day (kg FM/cow/d).

Table 4.1 Classification of the farms based on presence or absence of certain ingredients in feed ration.

	Ration composition					
Production system	Grazing	Fresh grass in the stable	Grass silage	Dried grass	Corn silage	Concentrated feed
Grazing (n= 44)	+	-	-	-	-	+
	+	+	-	-	-	+
	+	-	+	+	-	+
	+	-	+	-	-	+
	+	-	-	+	-	+
	+	+	+	+	-	+
	+	-	+	+	+	+
	+	-	+	-	+	+
Zero-grazing (n= 13)	-	+	+	+	-	+
	-	+	-	+	-	+
	-	+	+	-	-	+
	-	+	-	-	-	+
Grass silage (n= 10)	-	-	+	+	-	+
	-	-	+	-	-	+
Corn silage (n= 45)	-	-	+	+	+	+
	-	-	+	-	+	+
	-	-	-	+	+	+

Dried grass= hay, alfalfa, vetch, straw.

4.2.3. Samples collection and processing

A total of 112 raw tank milk samples were collected, with 6 Holstein cows in the smaller farms to 250 Holstein cows in the bigger one, during autumn 2016, spring 2017, and both autumn and spring 2019 and 2021. Samples included two milking, afternoon, and morning.

Tubes containing 50 mL of each milk sample were centrifuged at 1 900 × g for 20 min. The fat layer was transferred to fresh 50 mL RNase-free tubes, then QIAzol lysis reagent was added (1 mL per milk fat gram). Tubes were vortexed until the fat was thoroughly dispersed, and samples were stored at -80 °C until RNA extraction.

4.2.4. Total RNA extraction

Total RNA was extracted from 2 mL of milk fat with QIAzol using the miRVana miRNA isolation kit following the manufacturer's instructions. RNA was eluted in 100 µL of nuclease-free water. The Nano-Drop spectrophotometer was used to assess RNA concentration and purity (A260/280 ratio).

4.2.5. RT-qPCR

The isolated total RNA was reverse transcribed using the TaqMan Advanced miRNA cDNA Synthesis Kit. Levels of miRNAs were determined by RT-qPCR using the TaqMan Advanced miRNA Assay and a StepOne thermocycler under the following conditions: 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. Template cDNA (5 µL of a 1:10 dilution) were added to 15 µL of a mix comprising 10 µL of 2× TaqMan Fast Advanced Master mix, 1 µL of 20× TaqMan Advanced miRNA Assay, and 4 µL of RNase-free water.

Specific miRNAs were quantified after being selected on the basis of previous sequencing studies. The quantified miRNAs included *bta-miR-215*, *bta-miR-369-3p*, *bta-miR-6520*, *bta-miR-7863*, and *bta-miR-532*, all of which have been identified as the most highly expressed in milk. We also quantified several miRNAs that have been associated with feeding and metabolism: *bta-miR-148a*, *bta-miR-155*, *bta-miR-451*, *bta-miR-103*, *bta-miR-181a*, *bta-miR-21-5p*, and *bta-miR-29b* (Li R. *et al.*, 2015; Muroya *et al.*, 2015, 2016; Wang *et al.*, 2016). Relative abundance of miRNAs was quantified using the △△Ct

method after normalization with *bta-miR-30a-5p* and *bta-miR-151* as described in the previous chapter. All reactions were performed in duplicate. Negative controls lacking cDNA were included in all experiments.

4.2.6. Prediction of potential functions and pathways of genes targeted by milk miRNAs

Targets of miRNAs with different levels across farms were identified using TargetScan 7.2 (Agarwal *et al.,* 2015) in cow database. Target genes were selected based on Cumulative weighted context++ score>0. Functional enrichment analysis of signaling pathways involving the miRNAs target genes was performed using DAVID Bioinformatics tools (v.6.8). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, biological process, and molecular function were analyzed (Dennis *et al.*, 2003).

The targets of the studied milk miRNAs and their associated pathways were also analyzed using DIANA miRPath 3.0 (Vlachos *et al.,* 2015). The validated miRNA targets were identified using DIANA TarBase 7.0 (http://diana.imis.athenainnovation.gr/DianaTools/). GO categories and KEGG were assessed. As bovine genes are not included in DIANA miRPath, target prediction and pathways analysis were performed based on human miRNA annotations. Statistical analyses were carried out using the integrated Fischer's exact test followed by FDR (false discovery rate) adjustment.

4.2.7. Statistical analysis

Data were analyzed using IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY: IBM Corp). One-way ANOVA was used to compare miRNA levels among the four study groups as independent biological types. When the analysis of variance gave a significant difference for the main effect, the Bonferroni post hoc test was applied (multiple comparison of means). When the assumption of equal variances was not met, Welch's ANOVA was used as an alternative to one-way ANOVA. When normality of the residues or homogeneity of variance was not verified, means were compared using the Kruskal–Wallis non-parametric test; when significant differences were attained, the Games-Howell post hoc test (multiple comparison of means) was performed. Significance was defined as $p \leq 0.05$.

To assess the individual and combined effect of the presence of the different ingredients in the ration, in addition to the quantitative factors (amounts of ingredients, duration of grazing and number of animals) analyzed as covariates, on miRNA levels, a factorial ANCOVA based on a univariate general linear model (GLM) procedure was performed.

4.3. Results

4.3.1. Description of the sampled farms and their feeding management

The average dairy herd in the sample had 40 lactating cows, with a minimum of 6 and a maximum of 250 cows per farm. The most frequent herds had less than 50 cows (**Figure 4.1**), thus reflecting a relatively small number of larger farms.

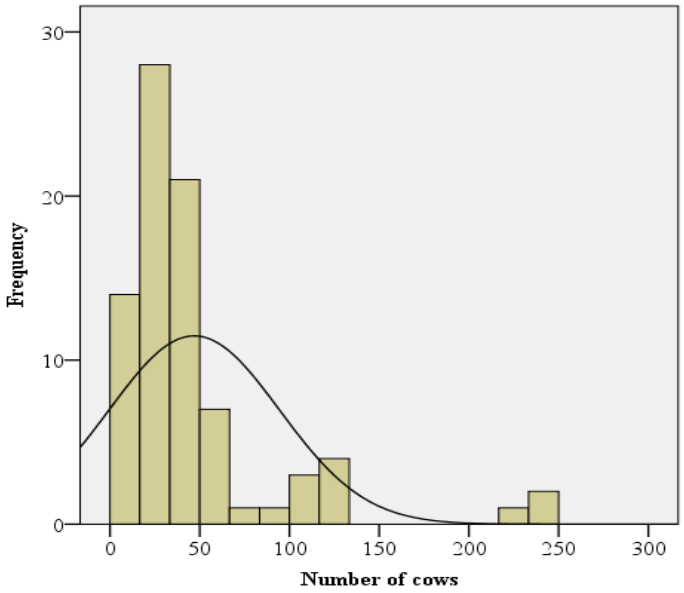

Figure 4.1. Frequency distribution of the number of cows per herd.

The 112 samples from the milk tank account for a total of 17 different rations combining the different ingredients (**Table 4.1**). According to the survey data from the sampled farms, regardless of the year and season of sampling, 51.79% of the rations included fresh grass in the diet, both in the stable and in the pasture. However, only 39.29% of the total farms adopted grazing. Corn silage was included in 50% of the rations, mainly in housed cows. In some cases (6.25% of the total) grazing cows also consume corn silage. Dried grass was consumed in 65.18% of the rations and grass silage was present in 75% of the rations (**Figure 4.2**).

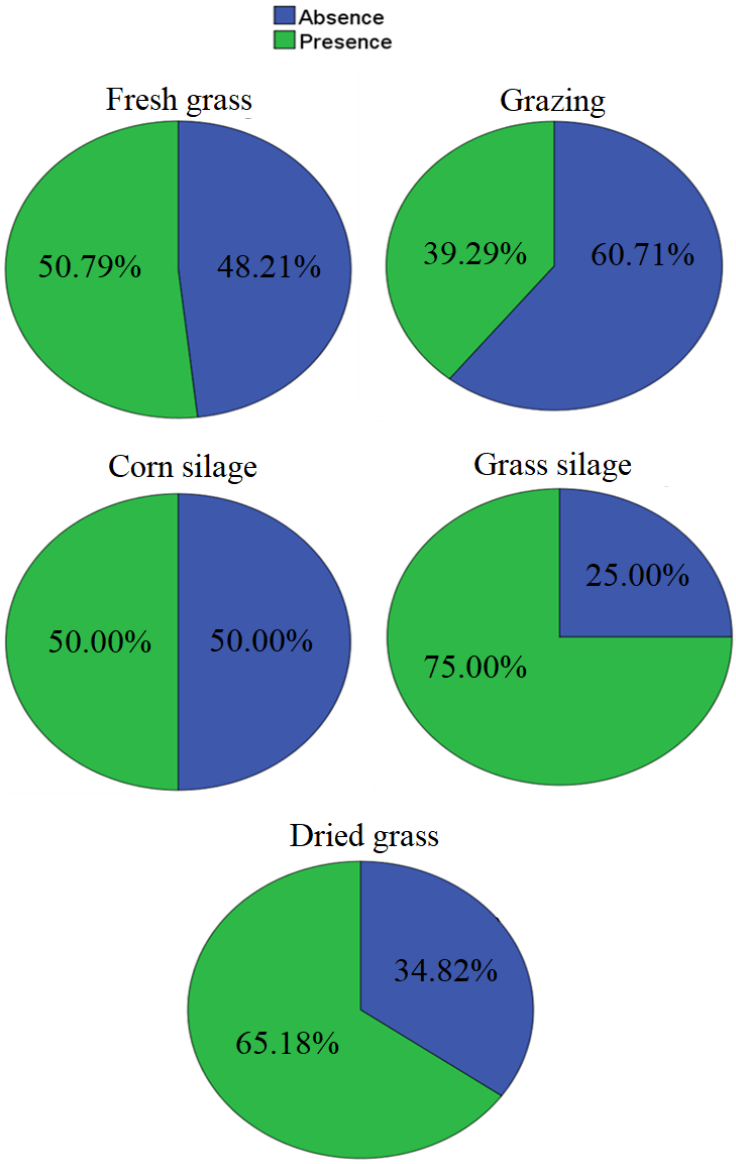

Figure 4.2. Percentages of diets including fresh grass, grazing, corn silage, grass silage and dried grass.

Considering the quantitative variables, it was observed that grazing duration ranged from 4 h per day to almost all day (except during milking hours), with an average duration of 14 h per day. The amounts of corn silage in the diets varied from 5 to 33 kg FM/cow/d, while the amounts of grass silage varied from 5 to 35 kg FM/cow/d. The inclusion of dried grass was fairly widespread, but in small quantities. Hay amounts ranged from 1 to 6 kg FM/cow/d, straw from 0.5 to 5 kg, alfalfa from 1 to 10 kg and vetch

from 1 to 13 kg. The amounts of concentrates ranged from 3 to 15 kg FM/cow/d (**Table 4.2**).

Table 4.2. Description of grazing duration and the quantities of the different ingredients in the sampled farms.

	Nº farms	Min	Max	Average	SD
Grazing duration (h/d)	45	4	24	14.36	6.00
Corn silage (kg FM/cow/d)	56	5	33	18.18	6.24
Grass silage (kg FM/cow/d)	84	5	35	15.51	7.43
Hay (kg FM/cow/d)	15	1	6	3.05	1.67
Straw (kg FM/cow/d)	39	0.50	5	1.17	0.78
Alfalfa (kg FM/cow/d)	23	1	10	3.17	2.12
Vetch (kg FM/cow/d)	16	1	13	4.63	3.44
Concentrate (kg FM/cow/d)	112	3.60	15	9.38	2.83

h/d: hour/ day, kg FM/cow/d: kg fresh matter per cow per day, SD: standard deviation, max: maximum, min: minimum

The characteristics of the parameters described above were also analyzed in the four defined groups. The Kruskal Wallis test found that the number of animals per herd was significantly higher in the corn silage group than in the other three (**Figure 4.3**). The grazing group had a minimum of 6 cows per herd and a maximum of 230, with an average of 34.80, which illustrates the great variability of this group, containing very small to large farms. The zero-grazing group had an average of 34.10 cows per herd, with a minimum of 14 and a maximum of 60. The grass silage group averaged 30 cows per herd, with a minimum of 13 and a maximum of 49. The corn silage group had higher values than the other three groups, with an average of 89.30 cows per herd, a minimum of 31 and a maximum of 250 cows per herd.

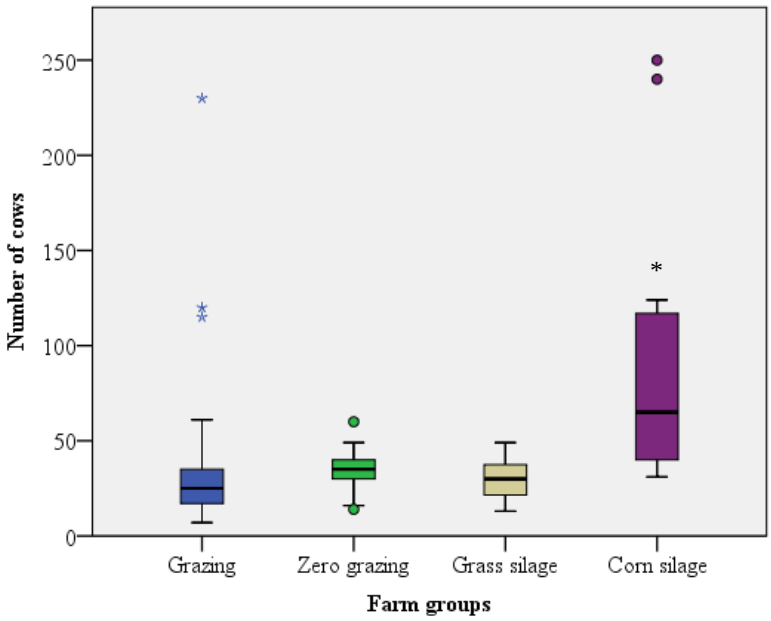

Figure 4.3. Box and whisker plot showing the distribution of the number of cows per herd according to the studied groups, in addition to their means and quartiles. *the group was significantly different

Comparing the amounts (kg of fresh matter) of the major ingredients among the four groups (**Figure 4.4**), it was observed that the amounts of corn silage in the groups where it was included (grazing and corn silage) did not show significant differences on average. In the diets of corn silage farms, the quantities of this ingredient varied considerably, with some farms providing more than 30 kg FM/cow/d, while in the grazing group, those including corn silage did not exceed 22 kg FM/cow/d.

The highest quantities of grass silage were found in the grass silage group, reaching an average of 30 kg FM/cow/d, while the other groups did not exceed 15 kg FM/cow/d on average.

The amounts of dried grass were significantly different in the corn silage group being the smallest, 1.95 kg FM/cow/d, while the three others included on average 3.94 kg FM/cow/d, 4.59 kg FM/cow/d and 5.34 kg FM/cow/d in the grazing, zero-grazing and grass silage groups respectively. Concentrate intake was significantly lower in the grazing group than in the other three groups, with an average of 7.08 kg FM/cow/d, while it was

9.73 kg FM/cow/d, 10.00 kg FM/cow/d and 11.42 kg FM/cow/d respectively in the zero-grazing, grass silage and corn silage groups.

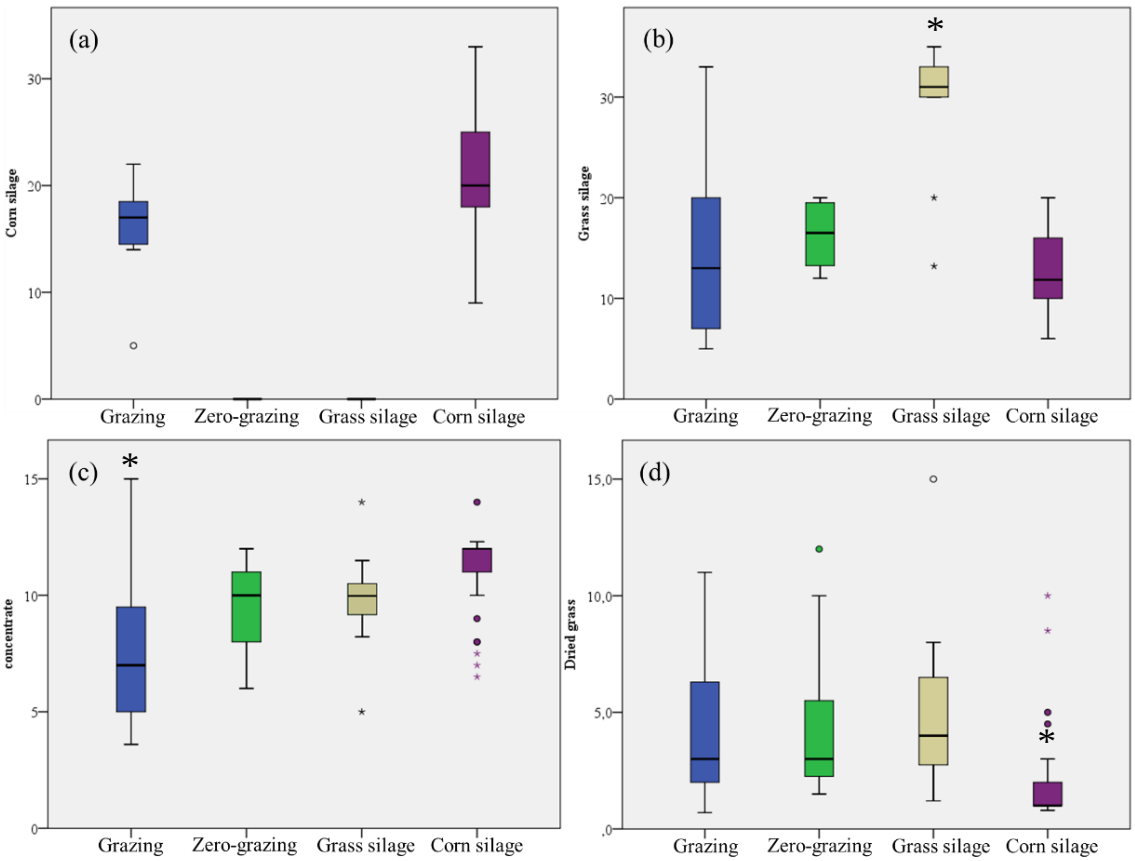

Figure 4.4. Box and whisker plot showing the distribution of the quantities of corn silage (a), grass silage (b), concentrate (c) and dried grass (c) in kg FM/cow/d, according to the studied groups, in addition to their means and quartiles. *The group is significantly different.

4.3.2. miRNAs with differential levels in cow milk according to production system

Total RNA concentrations in milk fat from the four types of milk production system varied between 84 and 144 ng/µL, and the RNA absorbance ratio was between 1.67-1.98 in all samples.

miRNA levels in milk fat across the four dairy production systems for the twelve chosen miRNAs were estimated. We found that levels of the following four miRNAs differed significantly between at least two dairy production systems: *bta-miR-155*, *bta-*

miR-103, *bta-miR-532*, and *bta-miR-7863* (**Figure 4.5**). Post hoc analysis showed that miRNAs *bta-miR-103* and *bta-miR-155* showed significant differences between grazing and corn silage groups, being more abundant in the grazing farms. *The bta-miR-532* was significantly more abundant in grazing farms than in zero-grazing, while on the contrary *bta-miR-7863* was more abundant in zero-grazing farms than in grazing. *Bta-miR-103*, *bta-miR-532* and *bta-miR-7863* showed significant differences between zero-grazing and corn silage groups with *bta-miR-103* and *bta-miR-7863* being more abundant in the zero-grazing group, but *bta-miR-532* was more abundant in the corn silage group. The miRNA *bta-miR-532* was significantly more abundant in corn silage farms than in grass silage.

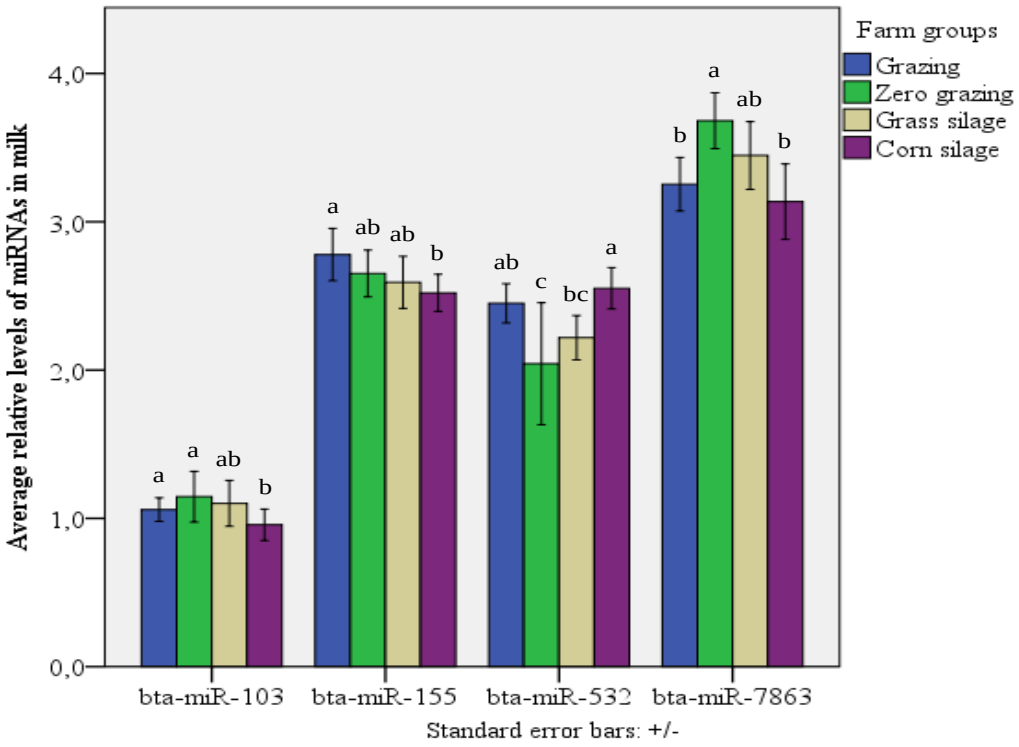

Figure 4.5. Average relative levels of the miRNAs *bta-miR-103*, *bta-miR-155*, *bta-miR-532*, and *bta-miR-7863* in raw milk from grazing (n = 44), zero-grazing (n = 13), grass silage, (n = 10), or corn silage (n = 45) milk production systems. The bar chart shows the average of miRNA levels in each farm group, and the standard error bars. Different letters show significant difference between groups.

4.3.3. Determination of intrinsic factors of farm groups that could be behind miRNA variations.

Once the miRNAs that vary between the four groups were determined, we tried to determine the effect of the presence of the different ingredients and their interaction as well as the effect of the quantitative contribution of the different ingredients, and grazing duration on the levels of the four miRNAs. The effect of the presence of fresh grass, grazing, grass silage, dried grass and corn silage in the diet and their interactions, the effect of the duration of grazing, the amount of grass silage, corn silage and concentrates in the diet on the four miRNAs levels was tested based on factorial analysis.

The critical level associated with the F-statistic ($p < 0.05$) in the corrected model row indicated that the model explained a significant part of the variation observed in the levels of *bta-miR-155*, *bta-miR-103* and *bta-miR-532*, however in the case of *bta-miR-7863* the corrected model was not significant.

The effects included in the model explaining the levels of *bta-miR-155*: grazing duration, corn silage presence and its quantities as well as the interaction of the presence of corn silage with dried grass, explained 32.70% of the miRNA level variance. The miRNA *bta-miR-155* levels seem to be affected by grazing, the presence of corn silage and its amounts in the diet, as well as the interaction between corn silage and dried grass.

Regarding *bta-miR-103*, the model also explained a significant part of the observed variation in the miRNA levels. The included factors in the model were the presence of corn silage, its quantities in the diet and the interaction between corn silage and grass silage. These factors explained 31.20% of the variance of the model describing *bta-miR-103* level. These analyses showed also that only the presence of corn silage affects *bta-miR-532* while it does not vary with the amounts of corn silage. This model explained 32.60% of the variation. However, in the case of *bta-miR-7863*, none of the studied factors seem to affect its levels. The **Table 4.3** presents the significance levels of different factors, both fixed and covariate as well as the different interactions of the factors.

Table 4.3. The effect of the inclusion of different ingredients in the diet and their amounts, the duration of grazing and the different interactions on *bta-miR-155, bta-miR-103 and bta-miR-532* levels.

	F-value Significance		
	bta-miR-155	*bta-miR-103*	*bta-miR-532*
Corrected model	0.005	0.014	0.002
Intercept	0.000	0.000	0.000
Grazing duration (h/d)	0.034	0.329	0.191
Concentrate (kg FM/cow/d)	0.485	0.227	0.711
Corn silage (kg FM/cow/d)	0.002	0.022	0.431
Grazing	0.263	0.568	0.557
Fresh grass	0.185	0.756	0.581
Corn silage	0.001	0.001	0.036
Grass silage	0.928	0.407	0.079
Dried grass	0.056	0.683	0.160
Fresh grass × Corn silage	0.051	0.077	0.211
Fresh grass × Grass silage	0.689	0.112	0.570
Fresh grass × Dried grass	0.674	0.481	0.878
Grazing × Corn silage	0.051	0.077	0.211
Grazing × Grass silage	0.653	0.838	0.459
Grazing × Dried grass	0.759	0.805	0.800
Corn silage × Grass silage	0.308	0.015	0.490
Corn silage × Dried grass	0.026	0.396	0.998
Grass silage × Dried grass	0.766	0.138	0.947
Grazing × Grass silage × Dried grass	0.790	0.065	0.905

h/d: hour/ day, kg FM/cow/d: kg fresh matter per cow per day

4.3.4. miRNA functionality and pathway analyses

To determine the possible implications of the studied miRNAs in the biological response to different production systems, we predicted the target genes of the four miRNAs as well as the functional pathways in which those target genes may participate. The 707 targets of *bta-miR-103* were associated with 71 KEGG pathways, eight biological processes, and 15 molecular functions. The 460 targets of *bta-miR-155* were associated with 106 KEGG pathways, five biological processes, and nine molecular functions. The 208 targets of bta-miR-532 were associated with 29 KEGG pathways, three biological processes, and 22 molecular functions. The 2266 targets of *bta-miR-7863* were associated with 76 KEGG pathways, 12 biological processes, and seven molecular functions.

Among these target processes, we identified 15 KEGG pathways (**Table 4.4**), eight biological processes (**Table 4.5**), and 12 molecular functions (**Table 4.6**) that were related to milk production and metabolism. In particular, the four miRNAs were all predicted to be involved in the MAPK signalling pathway (Mitogen-Activated Protein Kinases signalling pathway) and the molecular functions of transferases and serine/threonine-protein kinases. Two metabolic pathways stand out for their relationship with milk production and secretion, the oxytocin and prolactin signalling pathways.

Table 4.4. KEGG pathways that are associated with milk production and metabolism and that are predicted to be regulated by milk miRNAs with differential levels across production systems.

KEGG signaling pathway	Biomarker				No. of target genes	*p*-value
	bta-miR-103	*bta-miR-155*	*bta-miR-532*	*bta-miR-7863*		
AMPK [1]	×	×		×	38	4.6×10^{-7}
MAPK	×	×	×	×	67	3.2×10^{-6}
PI3K-Akt [2]		×	×	×	27	5.6×10^{-5}
Oxytocin	×	×		×	38	1.1×10^{-4}
Prolactin		×		×	25	1.2×10^{-4}
Insulin		×		×	35	1.4×10^{-4}
Ras [3]	×	×		×	53	1.6×10^{-4}
Growth hormone synthesis		×		×	31	1.8×10^{-4}
TGF-beta 4 [4]		×		×	25	6.2×10^{-4}
Calcium	×			×	52	7.3×10^{-4}
Glucagon				×	24	6.0×10^{-3}
Lipid and atherosclerosis	×	×			43	2.5×10^{-2}
cGMP-PKG [5]	×				32	3.3×10^{-2}
Mineral absorption				×	13	8.3×10^{-2}
Lysine degradation				×	14	9.7×10^{-2}

[1] Adenosine Monophosphate-Activated Protein Kinase (AMPK), [2] Phosphoinositide 3-kinases- Protein kinase B (PI3Ks-Akt), [3] Rat sarcoma virus (Ras), [4] Transforming Growth Factor Beta (TGF-beta 4), [5] Cyclic guanosine monophosphate- protein kinase G (cGMP-PKG)

Table 4.5. Biological processes that are associated with milk production and metabolism and that are predicted to be regulated by milk miRNAs with differential levels across production systems.

Biological process	Biomarker				No. of target genes	*p*-value
	bta-miR-103	*bta-miR-155*	*bta-miR-532*	*bta-miR-7863*		
Growth regulation				×	11	2.5×10^{-3}
Transport				×	249	1.4×10^{-2}
Ion transport				×	79	1.6×10^{-2}
Calcium transport				×	15	1.7×10^{-2}
Amino- acid transport				×	7	2.4×10^{-2}
Protein transport	×			×	67	2.7×10^{-2}
Differentiation		×			45	6.0×10^{-2}
Sodium transport	×				13	9.5×10^{-2}

Table 4.6. Molecular functions that are associated with milk production and metabolism and that are predicted to be regulated by milk miRNAs with differential levels across production systems.

Molecular function	*biomarker*				No. of target genes	*p*-value
	bta-miR-103	*bta-miR-155*	*bta-miR-532*	*bta-miR-7863*		
Transferase	×	×	×	×	279	1.4×10^{-20}
Activator	×	×		×	64	1.3×10^{-8}
Ion channel	×			×	65	3.0×10^{-8}
Serine/threonine-protein kinase	×	×	×	×	55	7.1×10^{-8}
Developmental protein	×	×		×	64	3.0×10^{-6}
Glycosyltransferase				×	31	2.5×10^{-3}
Hydrolase	×		×	×	182	2.8×10^{-3}
Growth factor				×	17	9.0×10^{-3}
Protein phosphatase			×	×	19	1.3×10^{-3}
Guanine-nucleotide releasing factor		×		×	16	1.6×10^{-2}
Calcium channel				×	8	4.0×10^{-2}
Potassium channel				×	12	4.8×10^{-2}

The miRNAs *bta-miR-103*, *bta-miR-155* and *bta-miR-532* showed high homology to human miRNAs, so we used DIANA miRPath for a second functionality analysis. Using Tarbase, we found that human target genes have been experimentally validated for *miR-103a-3p* (2156 genes), *miR-155* (1117 genes), *and miR-532* (306 genes). These targets (using the option union for KEGG and intersection for GO to merge results) were associated with 34 KEGG pathways (**Figure 4.6**) and 62 GO categories (see **the Appendix 1**). These analyses allowed us to predict pathways regulated by the three miRNAs (available in the Tarbase database). These results predicted *miR-103a-3p* and *miR-155* to be involved in fatty acid elongation and metabolism.

GO analysis revealed nine categories common to *miR-103a-3p*, *miR-155*, and *miR-532*: protein binding transcription factor activity, cellular protein modification processes, catabolic processes, biosynthetic processes, cellular nitrogen compound metabolic processes, cellular protein metabolic processes, small-molecule metabolic processes, mRNA metabolic processes, generation of precursor metabolites and energy, and regulation of glucose transport.

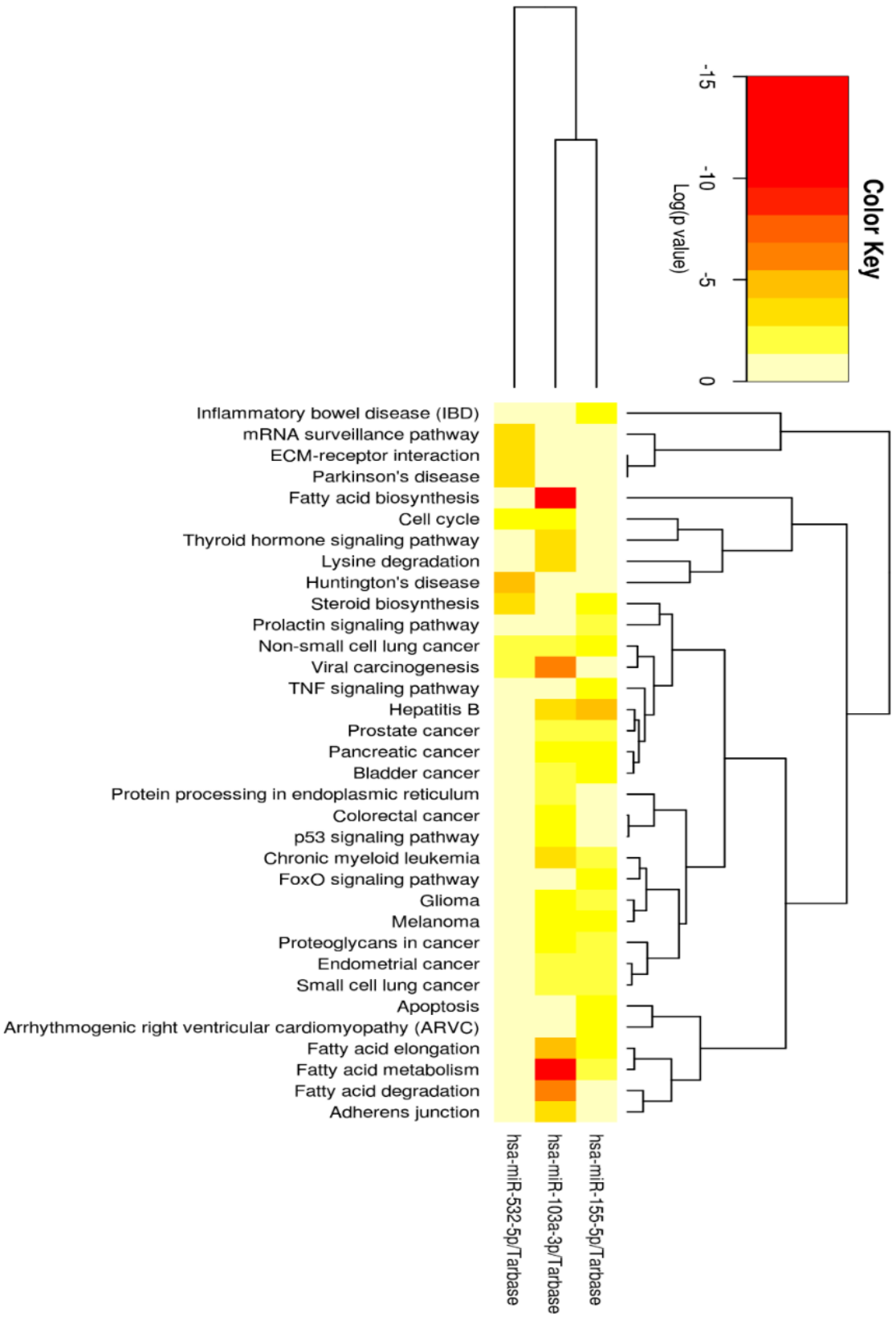

Figure 4.6. Heatmap of hierarchical clustering of *miR-103a-3p*, *miR-155* and *miR-532* based on mRNA target pathways, identified in DIANA using the Tarbase and KEGG Pathway union representation. Darker colors represent lower *p*-values.

4.4. Discussion

This work aimed to identify miRNAs that vary according to milk production system in order to differentiate milk from farms managed extensively. In the present study, among the 12 miRNAs analyzed, the levels of four miRNAs differed significantly among the types of dairy production system. These findings suggest the potential of profiling miRNAs in milk in order to certify whether it came from an extensive grazing production system.

The analysis of feeding management in the sampled farms showed the importance of the use of fresh grass in dairy cow diets in Asturias, where 50.79% of the cases reported the use of fresh grass at some time of the year to feed lactating cows, while 39.29% reported its use through grazing. Despite this importance, it seems that over the years the inclusion of fresh grass in diets and its use through grazing tends to decrease, since when comparing our results with those of a study on feeding systems in dairy farms, it was revealed that in Asturias (sample of 55 farms) (Flores-Calvete *et al.*, 2016), 74.20% of farms reported using fresh grass, while 48.30% reported using grazing. Moreover, grass silage was the predominant forage on most farms, as it was included in 75% of rations, followed by corn silage, which was used in 50% of rations, meanwhile, in the previous analysis the percentage of farms that included grass and corn silage in their rations reached 78.60% and 29.40% respectively (Flores-Calvete *et al.*, 2016). From these comparisons it is clear that the use of fresh grass tends to decrease and the use of corn silage tends to increase as a result of intensification in the region (SADEI, 2017; Jiménez-Calderón *et al.*, 2015). Theses evidence confirmed the need to preserve pastoral livestock farming in the region.

The average dairy herd in the sample had 40 cows in milking, with a dominance of farms with less than 50 head per herd. This parameter showed an increase compared to the study by Flores-Calvete *et al.*, (2016) where this average was 26.40. Due to the lack of data on farm area, it is not possible to rely on this data to evaluate the degree of intensiveness, however the number of animals per farm could indicate its size, thus indicating that small to medium sized farms predominate in the region.

On smaller farms, fresh grass was predominantly consumed in the stable or through grazing, in addition to grass silage, with a reduced use of concentrates, especially

on grazing farms. Whereas on farms with larger numbers of animals, housed cows were fed a diet dominated by corn silage, with a higher proportion of concentrates in the ration.

The previous chapter showed that the levels of the miRNA *bta-miR-215* in milk fat differed between the two extremes of extensiveness/intensiveness. The present work is not limited to extreme production systems but compared grazing livestock farms with intensive and mixed managements.

The levels of *bta-miR-103* were highest in farms including fresh grass in the diet, either grazed or harvested. Previous work showed that the expression of *bta-miR-103* in blood (Muroya *et al.*, 2015) and subcutaneous fat (Muroya *et al.*, 2020) was similar between cows fed on pasture and those fed in a free-stall barn with fresh grass harvested every morning. The fresh grass delivery mode did not affect the expression of *bta-miR-103*. In a study aiming to evaluate the effect of pasture during 3 months on stearoyl-CoA desaturase (SCD) and *miR-103a-3p* expression in milk dairy goats, the SCD was significantly higher in grazing animals compared to housed animals consuming conserved forages, whereas *miR-103a-3p* tended to be higher but not significantly (Tudisco *et al.*, 2019). Also, Lin *et al.*, (2013) showed that *miR-103a-3p* and SCD gene expression had similar trends, and that overexpression of *miR-103a-3p* in mammary gland has been linked to increased synthesis of milk fat, which is in line with the results obtained in our study linking grazing with higher milk fat content (De La Torre-Santos *et al.*, 2021), and conversely, relating higher proportion of concentrate in ration with lower milk fat content (intensive farms) (Sanh *et al.*, 2002). However, another study shows that when the grain-fed cattle were compared to the grazing cattle the *bta-miR-103* content in plasma tended to be higher in the first group ($p = 0.057$) although the difference was not significant (Muroya *et al.*, 2016). Differences between the previous studies and the present work may reflect differences in the type of tissues, and to the fact of studying the cows individually, which may introduce individual variability, such as lactation stage and number of lactations (Tudisco *et al.*, 2019). These and other factors can even mask differences in the expression of miRNAs according to production system (Avril-Sassen *et al.*, 2009).

Bta-miR-155 was abundant in milk from grazing farms compared with corn silage farms. This miRNA has been implicated in aspects of energy balance regulation: feed restriction upregulates it in mammary gland tissue of dairy cows (Billa *et al.*, 2019), while oxidative stress upregulates it in a mouse model (Vallanat *et al.*, 2010). Negative energy balance in dairy cows activates the production of reactive oxygen metabolites, which in

large quantities can create oxidative stress (Pedernera *et al.*, 2010). Grazing may improve immune function and oxidative status (Braghieri *et al.*, 2011) due to the high amounts of antioxidants in fresh grass and the exercise involved (Celi, 2011). On the contrary, other studies have reported the opposite results, with grazing favouring an increase in free radicals -without a concomitant increase in the amounts of antioxidants (Di Grigoli *et al.*, 2019). In grazing cows, the energy input may be lower than in intensive farming (Vicente Mainar *et al.*, 2012), which is probably at the origin of underlie *bta-miR-155* levels in our study.

Studies linked *miR-155* levels to a proinflammatory response in humans (Tomé-Carneiro *et al.*, 2013) and dairy cattle (Lai *et al.*, 2017). In dairy cattle, the upregulation of *bta-miR-155* was related to significantly higher risk of mastitis (Lai *et al.*, 2017). On grazing farms, it is more likely to have higher levels of somatic cells in milk compared to housed animals (De La Torre-Santos, 2021), SSC level above a certain level indicates inflammatory risks such as mastitis (Lamarche *et al.*, 2000) but below these levels may indicate resilience capacity (Bishop *et al.*, 2014). Therefore, SSC levels may be linked to the differences in *bta-miR-155* levels between grazing or housed cattle.

Furthermore, *bta-miR-7863* was more abundant in zero-grazing farms compared to grazing and corn silage animals. *Bta-miR-7863* has been studied as a mammary biomarker of mastitis caused by Staphylococcus aureus and Escherichia coli (Luoreng *et al.*, 2018). To our knowledge, no studies have investigated *bta-miR-532* expression in milk so far.

The four miRNAs identified in this study are related to the MAPK pathway, which regulates cell cycle entry and proliferation (Zhang *et al.*, 2002); and the molecular function of serine/threonine-protein kinases, which regulate cell proliferation, programmed cell death (apoptosis), cell differentiation, and embryonic development (Cross *et al.*, 2000). Thus, miRNAs *bta-miR-155*, *bta-miR-103*, *bta-miR-532* and *bta-miR-7863* may participate in cell differentiation in the mammary gland and thereby regulate milk production. Our GO enrichment analysis identified 249 target genes of the four studied miRNAs that are related to transport activity, which may indicate the great involvement of the four studied miRNAs in milk synthesis precursor transport process.

Using DIANA miRPath, experimentally validated human targets were identified, such as metabolic pathways related to fatty acid elongation and metabolism for *miR-103a-*

3p and *miR-155*. This suggests that the high levels of these two miRNAs in cow's milk are related to the increased fat synthesis due to the consumption of fresh grass, especially during grazing (De La Torre-Santos *et al.,* 2021). In parallel this leads us to think about the importance of confirming these results and to study the expression of these miRNA according to the fatty acid profile of milk.

The levels of four miRNAs showed significant differences between the four groups of farms. These differences could be due to a specific characteristic factor within the groups (e.g., grazing, presence of a specific ingredient, or its quantity), since the inclusion of a single ingredient in the diet may be behind miRNA variations in milk, as in the case of sunflower and linseed oil supplementation affecting milk miRNA expression (Li R. *et al.,* 2015; Mobuchon *et al.,* 2017).

When the effect of different ingredients on miRNAs was studied, it was revealed that the presence of corn silage in the diet may be responsible for the variation in the levels of *bta-miR-155, bta-miR-103* and *bta-miR-532,* leading to a reduction in the case of the first two and an increase in the latter miRNA. The level of *bta-miR-532* increased in the presence of corn silage in the diet, thus differentiating the "corn silage" group from the two intermediate groups, however, it did not differentiate it from the grazing group, maybe because in this group some farms included corn silage in the diet. The level of *bta-miR-103* decreased in the presence of corn silage, but also according to its amounts in the diet and the interaction between corn and grass silage in the diet. So as observed in the results the level of this miRNA was the lowest in corn silage group compared to the other three. The miRNA *bta-miR-155* levels seem to be affected by the grazing, the presence of corn silage and its amounts in the diet, as well as the interaction between corn silage and dried grass. The presence of corn silage reduced the level of *bta-miR-155* but grazing raised it, thus differentiating the extremes groups. In the case of *bta-miR-7863* none of the studied factors seemed to affect its expression, although it had a notably high level in the zero-grazing group. This could reflect the effect of a characteristic of these herds that was not contemplated in the work.

The effects of these factors explained a small part of the miRNAs variance (R^2 around 30%), which may reflect the multitude of factors that could be behind miRNAs variations. In this study, we did not consider factors, as pasture botanical composition and management, animal density, and the nutritional value of the different ingredients. All this could contribute to the variations of miRNA levels within the groups. In addition to

feeding and farm management, factors such as weather conditions on the day prior to sampling could also influence miRNA expression. (Ghorecha, *et al.*, 2013).

It was not possible to differentiate the grazing farms from the others, however the miRNAs *bta-miR-155* and *bta-miR-103* differentiated the corn silage group from the others. The inclusion of corn silage can reflect the level of intensiveness (Jiménez-Calderón *et al.*, 2015), so these miRNAs may be helpful in differentiating farms that include corn silage in the diet from those that produce milk extensively under pasture or not.

In this study, the levels of miRNAs were compared according to the diets consumed by the cows at the time of sampling, independently of the season and the year. In Asturias, lactating cows do not graze all year round, with some exceptions (Villar-Bonet & Quintana-Ruíz, 2021), so throughout the year a substitution of fresh grass for grass silage as the seasons progressed towards winter (Flores-Calvete *et al.*, 2016). This fact makes this type of milk a seasonal product, therefore, the certification does not refer to milk from grazing cows, but to milk from farms where lactating cows graze during a specific period of the year (Villar-Bonet & Quintana-Ruíz, 2021).

The sampled farms are not perfect replicates, as the samples belong to commercial farms, which increases the variability. In some groups, we were unable to obtain a large number of samples, as in the case of zero-grazing and grass silage, since few farms adopt this management system.

4.5. Conclusions

This study confirmed that the dairy production system could influence miRNA levels in milk fat. In particular, the miRNAs *bta-miR-103* and *bta-miR-155* are significantly abundant respectively in farms that base their ration on the use of fresh grass (grazed or harvested) and grazing, compared to corn silage-based farms, although they do not distinguish grazing farms from intermediate ones.

Chapter 5

Can microRNA levels in cow's milk differentiate between organic and conventional grazing dairy farms?

Chapter 5: Can microRNA levels in cow's milk differentiate between organic and conventional grazing dairy farms?

5.1. Introduction

In an exploratory study, organic and conventional pasture-based dairy farms were compared (Abou el qassim *et al.*, 2018). Although sampling was very limited, the results indicated that the level of a miRNA in milk was found to be significantly different between organic and conventional pasture-based dairy farms, with a higher level in the latter group. In this work, the aim was to test the results of the exploratory study in a larger sample, and to try to find out the factor(s) responsible for this variation.

The comparison between the different intrinsic factors of the two types of farms which may be at the origin of these differences has been determined on the basis of the description of organic farms from the Regulation (EU) 2018/848 of the European Parliament and of the Council of 30 May 2018 on organic production and labelling of organic products and repealing Council Regulation (EC) No 834/2007 (European Commission, 2018). These differences may be related to aspects linked to feeding, pasture management and pasture-animal interaction (e.g. stocking rate) and veterinary treatments. These characteristics are detailed in the **Table 5.1**.

To find out the factor(s) behind miRNA variation, several factors related to diet and management were tested. The difference in management between the two types of pasture could result in differences in the floristic composition and therefore in the diet composition of the cows (Adler *et al.*, 2013; Argamentería *et al.*, 2012; Pande, 2002). Thus, the florestic composition of organic and conventional pastures was studied.

In addition, a trial was conducted to determine the effect of the type of concentrate (organic *vs*. conventional). And finally, a principal component analysis (PCA) was used to analyze the effects of different quantitative factors related to the farm management and diet on the miRNAs levels in milk, including: the amount of concentrate (kg of fresh matter (FM)/cow/day), corn silage (kg of FM/cow/day), forage other than fresh grass (kg of FM/cow/day), grazing duration (h/day), the number of cows per farm (farm size

indicador), stocking density of animals on pasture (cow/ha) and average milk production of the animals per farm (L/cow/day)

Table 5.1. Some differences between organic and conventional pasture-based dairy farms.

	Conventional	Organic
Ingredients of the ration	Non organic	Must be organic
Concentrate	Non organic	Must be organic
Grazing	Not specified	Up to 20 % of feed may originate from the grazing or harvesting of pastures. Maximum use of grazing
% forage in the ration	Not specified	Must include a 60% minimum forage
GMO	Not specified	Prohibited use
Animal stocking density	It may be more than 2 LU/ha	Must be Maximum 2 LU/ha
Fertilization	Not specified	Shall not exceed 170 kg of nitrogen per year per ha No mineral nitrogen
Insecticides and pesticides	Could be used	Limited use
Genetic variability seeds	Not specified	Required
Chemically synthesized allopathic medicines	Could be used	Limited use
Reproductive control hormones	Could be used	Limited use

LU/ha: livestock unit per hectare; GMO: genetically modified organisms

5.2. Material and methods

5.2.1. Studied farms description

The sampled farms are located in different parts of Asturias. Visits were made to ten organic and ten non-organic grazing farms between 15 May and 15 July 2021 (**Table 5.2**). The table shows the locations and characteristics of the sampled farms as well as the sampling dates, and the **Figure 5.1** shows their geographical distribution on the map of Asturias.

Table 5.2. Locations and characteristics of sampled farms and sampling dates.

Farm code	Location	Sampling date	Type of management
Org1	Castropol	12/05/2021	Organic
Org2	Castropol	12/05/2021	Organic
Org3	Colunga	05/07/2021	Organic
Org4	Valdés	26/05/2021	Organic
Org5	Gijón	17/05/2021	Organic
Org6	Gijón	17/05/2021	Organic
Org7	Ribadesella	05/07/2021	Organic
Org8	Tineo	15/06/2021	Organic
Org9	Villaviciosa	10/06/2021	Organic
Org10	Villaviciosa	10/06/2021	Organic
Conv1	Tineo	07/06/2021	Conventional grazing
Conv2	Gozón	26/05/2021	Conventional grazing
Conv3	Villaviciosa	15/06/2021	Conventional grazing
Conv4	Siero	15/07/2021	Conventional grazing
Conv5	Tineo	07/06/2021	Conventional grazing
Conv6	Tapia de casariego	09/07/2021	Conventional grazing
Conv7	Castropol	09/07/2021	Conventional grazing
Conv8	Colunga	07/07/2021	Conventional grazing
Conv9	Colunga	07/07/2021	Conventional grazing
Conv10	Colunga	07/07/2021	Conventional grazing

Org: organic, Conv: conventional

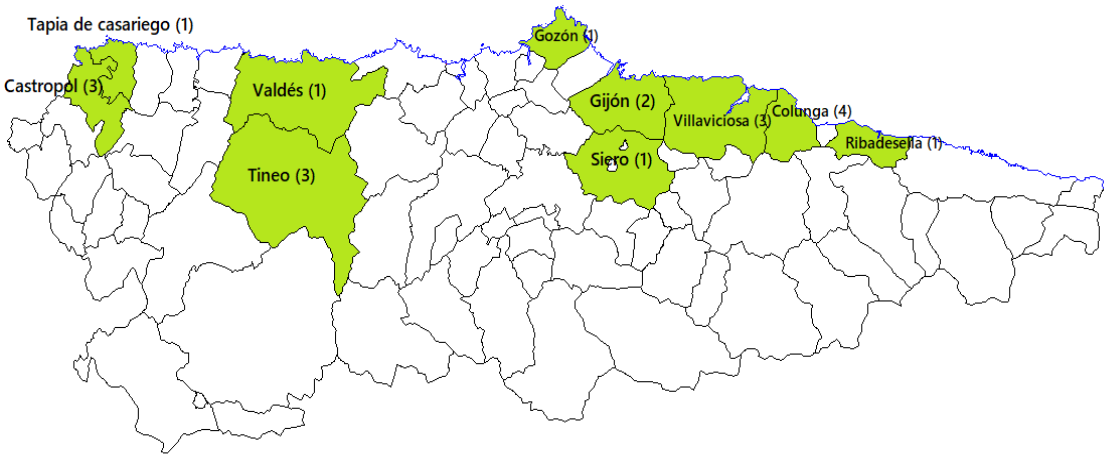

Figure 5.1. Geographical distribution of the sampled farms on the map of Asturias

For each farm, the following data for lactating cows were requested at three days before site visits: the number of cows per farm as a farm size indicator, the area devoted to grazing (ha), average milk production of the animals per farm (L/cow/day), grazing duration (h/day), and the amount of ingredients in the ration other than grazed grass expressed in kg of FM/cow/day (**Table 5.3**). From these data, the amounts of conserved forage in the diet were calculated, which constitutes the sum of the fresh matter quantities of corn silage, grass silage, straw, vetch and alfalfa in each farm, this factor does not include the amount of green forage ingested during grazing. The stocking rate on the pasture was calculated for each farm.

Table 5.3. Farm management characteristics and diet components

Farm type	Number of cows	Grazing area	Milk production	Grazing duration	Corn silage	Grass silage	Straw	Vetch	Alfalfa	Concentrate	Grazing management
Org1	26	14	17	10	0	20	0	0	0	4	Rotational
Org2	46	25	22	20	0	0	0	0	0	4	Semi-continuous
Org3	23	12	23	20	0	0	0	0	3	6	Rotational
Org4	34	18	17	10	0	20	0	0	7	5	Rationed
Org5	25	13	22	22	0	10	0	10	0	5	Semi-continuous
Org6	40	20	20	22	0	0	0	0	0	4	Rotational
Org7	26	14	24	8	0	0	0	0	0	7	Rotational
Org8	41	21	21	20	0	5	0	3	0	6	Semi-continuous
Org9	38	20	23	10	0	0	0	1	1	7	Rotational
Org10	35	18	15	9	0	0	0	0	0	4	Rotational
Conv1	61	30	25	7	0	5	0	0	0	10	Semi-continuous
Conv2	25	11	27	12	0	0	0	0	0	13	Semi-continuous
Conv3	18	13	24	18	0	22	1	0	0	5	Rotational
Conv4	25	11	25	20	0	0	0	0	0	11	Rationed
Conv5	14	10	25	6	5	5	0	0	0	9	Rationed
Conv6	45	10	29	10	22	8	0	0	0	8	Semi-continuous
Conv7	230	100	28	24	14	6	2	0	0	6	Semi-continuous
Conv8	29	32	29	20	0	0	0	2	0	6	Rotational
Conv9	24	10	20	15	0	0	0	0	0	4	Semi-continuous
Conv10	18	5	24	10	0	17	0	4	0	8	Rotational

Org: organic, Conv: conventional

5.2.2. Floristic composition study

At each visit, a study of floristic composition of the pastures where the cows were fed was carried out to check the possible effect of this factor on miRNA levels in milk. The floristic composition of the plants was determined using a square frame (0.5 m × 0.5 m). In each parcel the quadrat was placed randomly in 10 locations throughout a diagonal transect, avoiding resting areas, borders, entrances and drinking areas.

On the visited farms, three types of pasture management were adopted: rotational, rationed and semi-continuous grazing (**Table 5.3**). Rotational grazing consists of dividing a large pasture into smaller, equal plots and changing cows every day until three days. Rationed grazing consists of opening a daily plot of pasture for the animals. The area offered to the animals depends on what they need according to what they eat in the stable before going out to pasture, and also according to the available grass in the pasture. Semi-continuous grazing is when animals graze in a pasture for an extended period of time. This practice is done on farms with a large territorial base, divided into three to seven large plots (Holechek *et al.*, 1999).

In the case of rotational grazing, the floristic composition was studied in the same paddocks where the cows were grazing, except if plant recognition was difficult, in this case the study was done in neighbouring paddocks with more plant mass. In the case of semi-continuous grazing, the floristic composition was carried out in the same paddock where the cows were grazing. In the case of rationed grazing, the whole plot was studied.

Each time the square was thrown, the number and name of the different species present were recorded. The percentage of soil covered by green vegetation, the percentage covered by grasses and the percentage covered by clovers were then visually estimated. From these estimations, the following parameters were calculated:

- The percentage of the ground covered by the green vegetation which represents the mean of vegetation cover percentages among the 10 quadrats sampled at each parcel (% GVC).
- The abundance of grasses (% grasses), clover (% clover) and other species (% other species), which correspond to the mean of the area covered by grasses, clover (*Trifolium pratense* and *Trifolium repens*) and other species among the 10 quadrats sampled at each parcel.

- The frequency (%) of each species consists of the number of times a given species appears during the 10 throws of the square.
- The species richness is the estimated counting of the total number of species in each plot.
- The species density represents the average number of species within 0.25 m^2-quadrats.

5.2.3. Comparison between conventional and organic concentrate.

To test the effect of concentrate type on milk miRNA the following assay was designed.

5.2.3.1. Study site

The assay was carried out at the experimental farm of the Regional Service for Agri-Food Research and Development (SERIDA) (43◦28′20″ N, 5◦26′10″ W; 10 m above sea level) and complied with the EU Animal Welfare Directive Number 2010/63/EU.

5.2.3.2. Animals

The effect of two types of concentrate (organic *vs.* conventional) on the miRNA levels in milk fat fraction was tested in two lots of six healthy lactating Holstein cows with the characteristics described in **Table 5.4**. In the lot 1 the average day in milk (DIM) was 106.17 ± 32.08 with a mean milk production of 28.08 ± 5.21 L/day in the last week, and the lot 2 represented an average DIM of 111.50 ± 33.30 with a mean milk production of 27.86 ± 5.85 L/day in the last week.

Table 5.4. Cow characteristics descriptions for each lot.

	Lactation number	DIM	Milk production*
Lot 1	1	114	24.97
	1	167	22.68
	1	88	24.61
	2	101	27.19
	3	84	34.03
	4	83	35.05
Lot 2	1	83	24.86
	1	119	25.12
	1	165	20.15
	2	131	28.46
	3	79	36.82
	4	92	31.72

*Average daily production last 7 days (L/day), DIM: day in milk

5.2.3.3. Feeding

Each group of cows received a unifeed diet consisting of corn silage, grass silage, straw and different concentrates depending on the treatment (**Table 5.5**): conventional or organic concentrate with the nutritional values shown in the **Table 5.6**. Both concentrates were used in powder form. All cows were confined, without access to pasture. The experiment was arranged as a 2 periods × 2 lots Latin square design, each period was 5 days. To introduce a new diet to the cows, an adaptation period of 10 days was applied (**Figure 5.2**)

Table 5.5. Components and quantities of ration ingredients for each lot of cows.

kg FM/cow/day	Conventional	Organic
Corn silage	14.50	15.50
Grass silage	13.00	13.00
Straw	1.00	1.00
Conventional concentrate	8.00	---
Organic concentrate	---	8.00
Feed during milking	4.00	4.00

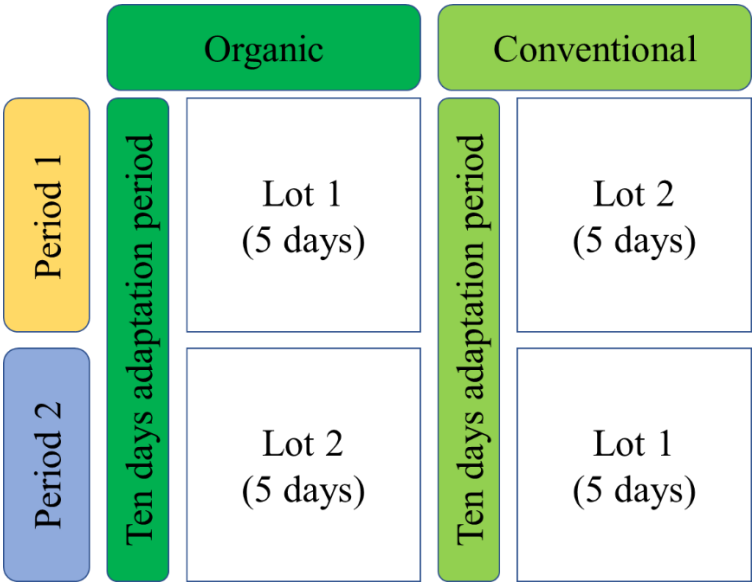

Figure 5.2. Latin square 2×2, two periods × two treatments in two cow groups

Table 5.6. Nutritional values of organic and conventional concentrates used in rations

	Unit	Conventional	Organic
Fresh matter	kg	1.00	1.00
Dry matter	%	87.40	87.40
Net Milk Energy	Mcal/kg	1.71	1.64
Crude protein	%	17.01	14.95
Crude fibre	%	4.29	4.90
Starch	%	38.41	41.93
Crude fat	%	2.90	3.01
Crude ash	%	6.08	6.26
Sodium chloride	%	0.60	0.60
Sodium	%	0.56	0.52
Calcium	%	0.58	0.79
Phosphorus	%	0.51	0.41
Magnesium	%	0.18	0.17
Copper Sulphate	mg/kg	30.00	29.90
Manganese Oxide	mg/kg	87.50	99.50
Zinc Sulphate	mg/kg	181.30	119.40
Potassium Iodide	mg/kg	2.30	2.00
Sodium selenite	mg/kg	0.60	1.20
Cobalt Acetate	mg/kg	2.00	2.00

5.2.4. Milk sampling and treatment

During visits to the 10 organic and 10 non-organic grazing farms, samples were collected from each milk tank (n=20). From the concentrate type effect assay, during each period, milk samples were collected from each lot for five days after the 10 days of adaptation to the diet, resulting in 20 milk samples.

Once in the laboratory, 50 mL milk were centrifuged at 4 °C for 20 min at 4200 × g, then milk fat fraction was isolated (Li *et al.*, 2016). The upper fat fraction was subtracted, stored in 4 mL of QIAzol at 80 °C until analysis.

5.2.5. miRNA analysis

Total RNA was isolated from 40 milk fat samples, using the mirVana miRNA Isolation Kit following the manufacturer's instructions. the TaqMan Advanced miRNA cDNA Synthesis Kit were used for the cDNA synthesis. miRNAs expression levels were quantified by RT-qPCR in a StepOne thermocycler, using the primers: *bta-miR-103, bta-miR-148a, bta-miR-155, bta-miR-181a, bta-miR-215, bta-miR-29b, bta-miR-369-3p, bta-miR-451, bta-miR-6520, bta-miR-7863* and *bta-miR-99a-3p.* Normalization was performed using internal control miRNA *bta-miR-30a-5p, bta-miR-27b* and *bta-miR-532* for the first assay and *bta-miR-30a-5p* and *bta-miR-21-5p* for the second assay determined using GeNorm algorithm as described in the chapter 1 (Vandesompele *et al.*, 2002). All reactions were performed in duplicate, and the results were expressed using the △△Ct method as $\log^2$ (Hellemans *et al.*, 2008).

5.2.6. Statistical analysis

Floristic composition parameters and miRNA levels between groups of farms were compared using t- test for data that meet the assumptions of normality and homogeneity of variances, in the opposite case, the Kruskal-Wallis test was applied.

In the experiment on the effect of concentrate type, a two-factor ANOVA was performed using feed type as the main factor and the period as the random factor, after verifying the homogeneity of variance and normality of the data. All analyses were performed with SPSS Version 22.0 software and $p < 0.05$ was accepted as statistically significant.

Redundancy analysis (RDA) was used to analyse multivariate data on floristic composition and to evaluate the significance of differences in the whole plant community between organic and conventional grazing farms. The null hypothesis was that the overall presence frequency of plant species is independent of the farm type. The data matrix included the frequency values (as proportions) of a total of 52 species (response variables) found in the 10 quadrats at each farm (e.g. 0 for absent species, 1 for species appearing in all quadrats at a specific farm), with the type of farm as the explanatory variable. The statistical significance of the model was evaluated by the F-ratio based on the trace and 499 Monte Carlo permutations. The RDA was run on CANOCO 4.5 for Windows 4.5 and plotted using CANODRAW 4.0(Ter Braak and Šmilauer, 2002)

PCA was carried out using SPSS 22.0 to assess the existing interrelationships between the factors within the two types of farms reducing the number of variables. The PCA included eight factors: amount of concentrate (kg FM/cow/day), amount of corn silage (kg of FM /cow/day), amount of forage other than fresh grass (kg of FM/cow/day), grazing duration (h/ day) number of cows per farm, stocking density of animals on pasture (cow/ ha), average milk production of the animals per farm (L/cow/day) and b*ta-miR-215* levels in milk. Before starting the PCA the data were standardized due to the disparity of units, using the formula:

$$\text{Standardized value} = \frac{initial\ value\ of\ the\ variable - mean}{standard\ deviation}$$

Next, a correlation matrix was calculated for all the factors in to identify the level of correlation between them. When factors were highly correlated ($R < -0.85$ or $R > 0.85$), only one of each pair was kept.

5.3. Results

5.3.1. Variations in miRNA levels between organic and conventional grazing farms.

The variation of eleven miRNAs was analyzed in ten organic and ten conventional grazing farms. Only the miRNA *bta-miR-215* showed a significant difference between the two groups (p = 0.041) (**Table 5.7**), with a higher level in the conventional grazing farms (**Figure 5.3**). The level of *bta-miR-215* was reduced by approximately 37% in organic farms compared to the other group.

Table 5.7. Means and standard desviations of miRNA levels between organic and conventional grazing farms (n = 10).

	Organic	Conventional	*p-value*
bta-miR-103	0.73 ± 0.02	0.65 ± 0.08	0.450
bta-miR-148a	1.52 ± 0.07	0.55 ± 0.01	0.705
bta-miR-155	2.77 ± 0.16	2.72 ± 0.98	0.450
bta-miR-181a	0.13 ± 0.01	0.21 ± 0.01	0.131
bta-miR-215	0.32 ± 0.05	0.51 ± 0.02	0.041
bta-miR-29b	0.79 ± 0.01	0.78 ± 0.09	0.364
bta-miR-369-3p	0.60 ± 0.43	0.83 ± 0.29	0.226
bta-miR-451	1.64 ± 1.35	1.84 ± 0.24	0.880
bta-miR-6520	0.90 ± 0.53	1.20 ± 0.44	0.275
bta-miR-7863	0.34 ± 0.21	0.49 ± 0.30	0.496
bta-miR-99a-3p	0.53 ± 0.05	0.64 ± 0.01	0.147

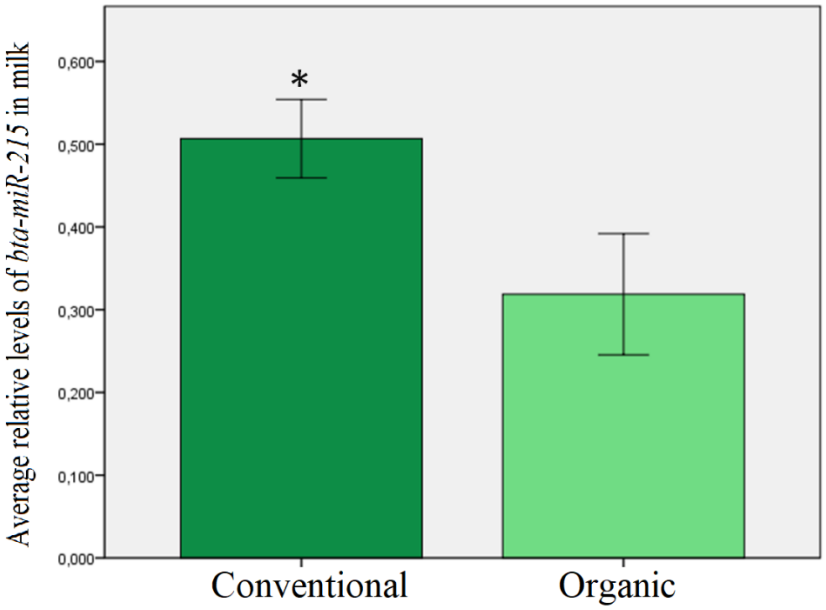

Figure 5.3. Average relative levels of the miRNA bta-miR-215 in raw milk from conventional grazing farms (n = 10) and organic farms (n = 10). The bar chart shows the average of miRNA levels in each farm group, and the standard error bars.

5.3.2. Effect of concentrate type (organic *vs.* conventional) on the levels of miRNAs in milk (controlled study)

The effect of the type of concentrate on the variation of the eleven miRNAs was studied. No significant differences were observed in the levels of miRNAs between the groups according to the type of concentrate. However, the expression of *bta-miR-181a*, *bta-miR-29b*, *bta-miR-451*, *bta-miR-7863* and *bta-miR-99a-3p* varied according to the period. It was shown that the levels of *bta-miR-181a*, *bta-miR-29b* and *bta-miR-7863* decreased from period 1 to period 2. On the contrary, the levels of the miRNAs *bta-miR-451* and *bta-miR-99a-3p* increased from period 1 to period 2. The *bta-miR-451*, and *bta-miR-7863* varied according to the interaction Period × treatment (**Table 5.8**).

Table 5.8. Average relative miRNA levels according to concentrate type and period

	Type of concentrate		Period		RSE	*p-value*		
	Org	Conv	One	two		C	P	CxP
bta-miR-103	0.36	0.38	0.35	0.39	0.51	0.816	0.594	0.291
bta-miR-148a	0.19	0.24	0.21	0.22	0.11	0.148	0.611	0.165
bta-miR-155	1.43	1.55	1.35	1.63	3.75	0.553	0.195	0.651
bta-miR-181a	0.24	0.28	0.36	0.16	0.58	0.479	0.006	0.160
bta-miR-215	0.37	0.32	0.28	0.40	0.49	0.530	0.114	0.695
bta-miR-29b	0.59	0.51	0.62	0.48	0.44	0.229	0.037	0.625
bta-miR-369-3p	0.43	0.76	0.41	0.78	6.21	0.190	0.151	0.895
bta-miR-451	3.06	2.75	2.72	3.04	5.80	0.132	0.047	0.000
bta-miR-6520	0.75	0.47	0.68	0.54	2.21	0.068	0.325	0.970
bta-miR-7863	0.17	0.25	0.26	0.16	0.33	0.134	0.039	0.008
bta-miR-99a-3p	0.62	0.46	0.43	0.65	0.95	0.073	0.026	0.914

RSE: Residual Standard Error; C: effect of concentrate type; P: effect of period; C × P: effect of interaction between concentrate type and period. Values correspond to the mean miRNA levels according to concentrate type and sampling period. Org: organic, Conv: conventional

5.3.3. Floristic composition

A total of 52 plant species were identified during the study (Appendix 1). It was observed that the percentage of vegetation cover was not significantly different between the two groups of farms, 97.83% in organic farms and 97.63% in conventional grazing farms (**Table 5.9**).

The percentage of grasses tended to be somewhat higher in the pastures of the organic farms than in the conventional ones, and conversely, the percentage of clovers (*Trifolium pratense* and *Trifolium repens*) was slightly higher in conventional grazing farms than in organic ones (**Figure 5.4**), although the differences were not statistically significant (**Table 5.9**).

There were no differences among the two pasture types in the total species richness at the studied spatial scale (25 m^2). Species richness was 18.29 ± 3.76 in the

organic farms and 17.13 ± 2.64 in conventional grazing farms (**Table 5.9**). Species density was significantly higher in organic farms with a value of 8.62 ± 1.42 *spp* per 0.25 m^2 while conventional grazing farms had a value of 6.81 ± 1.06 *spp* per 0.25 m^2 ($p < 0.05$) (**Table 5.9**).

Table 5.9. Mean and standard deviation of the percentage of vegetation cover, grasses cover, clover cover, species density and richness in organic and conventional grazing farms.

	Organic pasture	Conventional pasture	*p-value*
% of vegetation cover	97.83 ± 8.02	97.63 ± 2.30	0.714
% of grasses cover	67.83 ± 9.96	56.57 ± 16.70	0.223
% of clover cover	15.19 ± 11.28	25.68 ± 16.42	0.198
Species density	8.62 ± 1.42	6.81 ± 1.06	0.028
Species richness	18.29 ± 3.76	17.13 ± 2.64	0.321

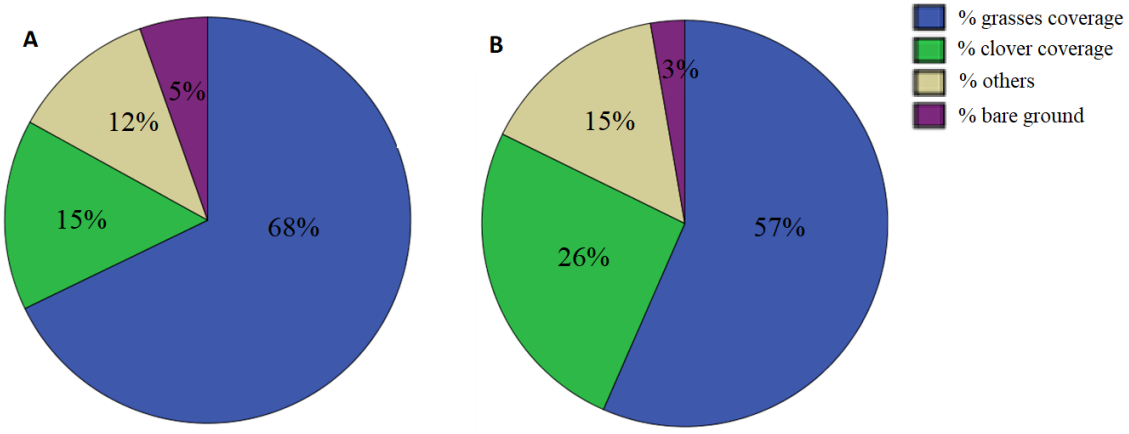

Figure 5.4. Percentage of the soil covered by grasses, clover, other species and bare ground in organic (A) and conventional (B) pastures.

To compare the frequency of the presence of each species between the two types of pasture, an RDA analysis was carried out and revealed that plant species composition did not differ between vegetation types. Also, this analysis showed that the floristic

composition did not differ significantly between organic and conventional grazing farms (F-ratio = 1.109, *p*-value = 0.3360). Thus, overall the different species found were equally present on both types of farms, although some particular species were more associated to one or another management (**Figure 5.5**). *Vicia* sp. (*Vi*) and *Trifolium pratense* (*Tp*) were the species most positively related to axis 1 mostly explained by organic farms. By contrast, *Taraxacum officinale* (*Tx*) was the species with the most negative scores along axis 1, indicating its slightly higher presence in conventional pastures.

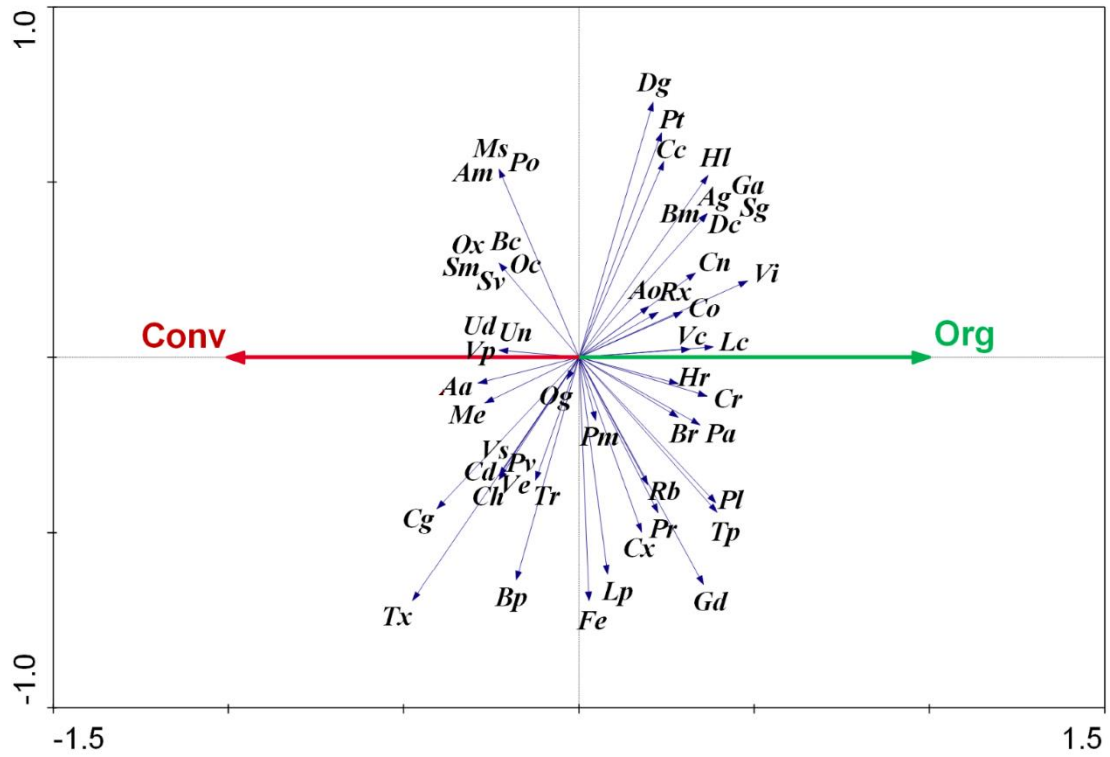

Figure 5.5. Multivariate redundancy analysis (RDA) ordination diagram of plant species in organic and conventional grazing farms. Conv (conventional pasture), Org (Organic pasture). For species abbreviations see **the Appendix 2**.

5.3.4. Relation between farm characteristics and *bta-miR-215* levels in milk

To assess the factor(s) that may be behind the variation of *bta-miR-215* between organic and conventional grazing farms, a PCA including eight factors was performed. Before this, the correlation between the different factors was studied so that only the least correlated factors could be taken into account. The correlation analysis between the eight studied factors showed that there were no strong correlations (<0.70) between the different factors (**Table 5.10**), so the eight factors have been included for the PCA.

Table 5.10. Correlation between the studied factors

	Number of Cows	Stocking. density	Milk production	Grazing duration	Corn silage	Forage	Concentrate	*Bta-miR-215*
No. Cows	1.00	0.09	0.29	0.35	0.52	0.22	-0.06	0.02
Stocking density	0.09	1.00	0.19	-0.21	0.61	0.41	0.19	0.26
Milk production	0.29	0.19	1.00	0.15	0.50	0.07	0.59	0.27
Grazing duration	0.35	-0.21	0.15	1.00	-0.02	-0.08	-0.30	-0.09
Corn silage	0.52	0.61	0.50	-0.02	1.00	0.53	0.09	0.15
Forage	0.22	0.41	0.07	-0.08	0.53	1.00	-0.16	0.08
Concentrate	-0.06	0.19	0.59	-0.30	0.09	-0.16	1.00	0.17
Bta-miR-215	0.02	0.26	0.27	-0.09	0.15	0.08	0.17	1.00

Stocking density (cow /ha), milk production (L/cow/day), grazing duration (h/day), corn silage (kg FM/cow/day), forage (kg FM/cow/day), concentrate (kg FM/cow/day), *bta-miR-215* levels in milk.

The PCA found that the first three PCs cumulate 70.6 % of the total variance. More precisely, PC1 accounts for 32.4 % of the variance, while PC2 and PC3 described 20.7 and 17.6 % of the variance, respectively. The SD, percentage variance, percentage cumulative variance, and rotated value of the selected components are shown below (**Table 5.11**). The results obtained from the PCA are plotted in **Figure 5.6** and **Figure 5.7**. The first PC describes indicators of the productivity and relative intensiveness, represented mainly by 4 factors: stocking density, milk production, corn silage and conserved forages amounts. The second PC represents farms according to grazing duration, concentrate amount and the number of cows. The PC3 is mainly explained by milk production, grazing duration and conserved forages amounts (**Table 5.11**).

Table 5.11. Principal components and percentage of variance explained by each component and their rotated values.

	Total Variance explained		
	PC1	PC2	PC3
Total	2.59	1.65	1.40
% variance	32.43	20.65	17.56
% cumulative	32.43	53.08	70.64
	Rotation of the components		
No. Cows	0.50	0.58	0.35
Stocking density	0.70	-0.13	-0.44
Milk production	0.67	-0.24	0.59
Grazing duration	-0.05	0.64	0.57
Corn silage	0.89	0.23	-0.10
Concentrate	0.36	-0.75	0.36
Bta-miR-215	0.39	-0.33	0.03
Forage	0.56	0.34	-0.52

PC1: First principal component; PC2: Second principal component; PC3: Third principal component.

Bta miR-215 correlated positively with PC1, negatively with PC2 and had very low correlation with PC3 (**Table 5.11**). In PC1, *bta-miR-215* was positively correlated with high stocking density, high milk production, high amount of corn silage, forage and concentrate in the ration. According to PC2, *bta-miR-215* was negatively correlated with the number of cows, grazing duration and forages intake, but was associated with high concentrate intake, and milk production. PC3 shows that the higher the grazing, the lower the consumption of preserved forage, so the way the forage is consumed (green *vs*. preserved) does not affect *bta-miR-215* levels.

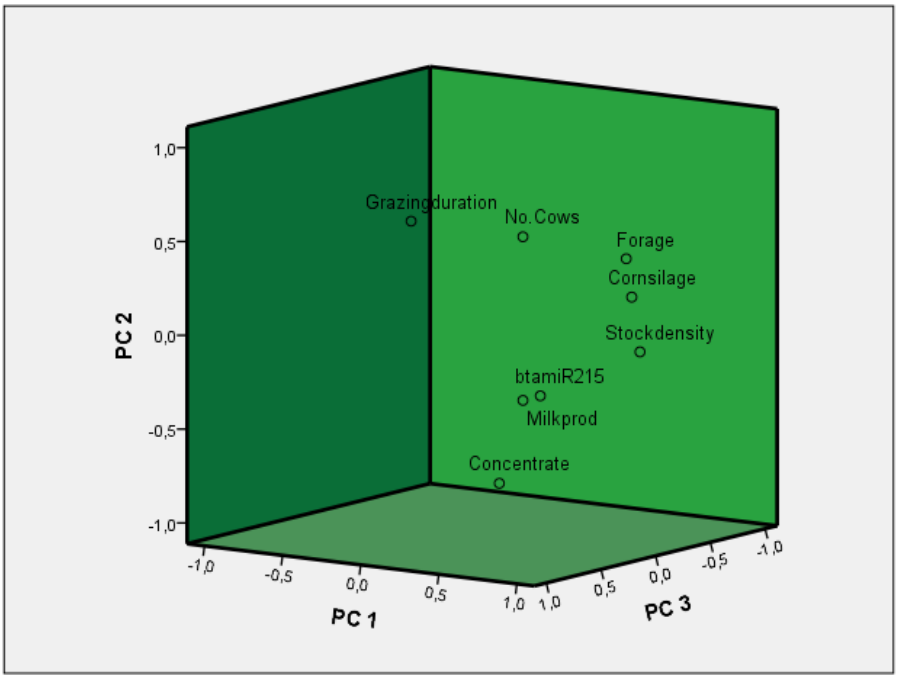

Figure 5.6. Score plot of PCA showing the Incidence of the vectors number of cows, stocking density, average milk production, grazing duration, amount of corn silage, forage and concentrate and *bta-miR-215* levels, on the three principal components.

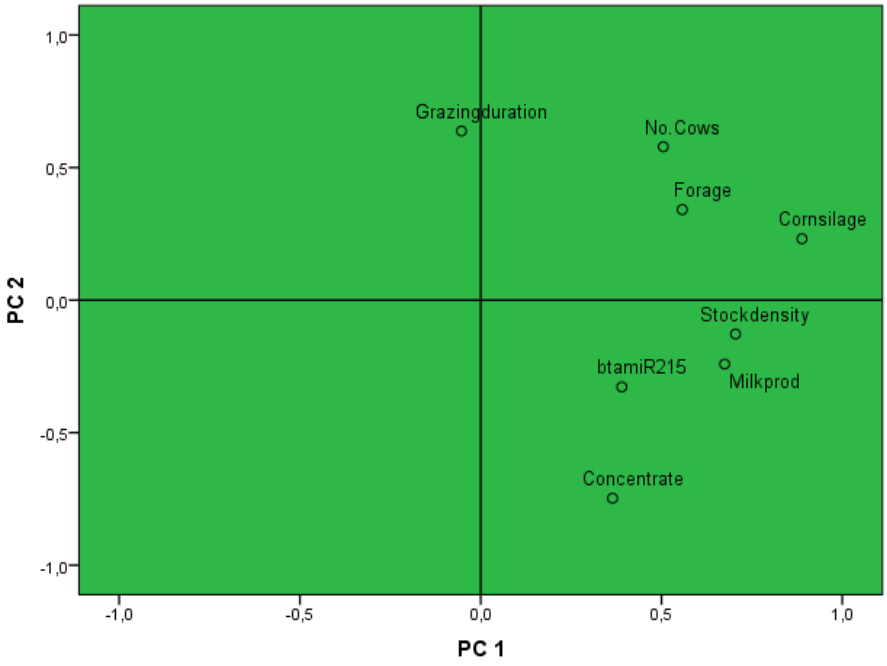

Figure 5.7. Score plot of PCA showing the incidence of the vectors number of cows, stocking density, Average milk production, grazing duration, amount of corn silage, forage and concentrate and *bta-miR-215* levels, on the two principal components.

5.4. Discussion

In a previous exploratory work, a significant difference in *bta-miR-215* levels has been observed between conventional and organic grazing farms. The level of this miRNA was significantly higher in conventional grazing farms compared to the organic ones (Abou el qassim, *et al.*, 2018). In this work, the objective was to test this difference in *bta-miR-215* levels between organic and conventional grazing farms in a larger sampling, as well as to test the levels of other miRNAs previously studied to be related to feeding, metabolism and management in dairy farms. Once the miRNAs with significant difference between the two groups were identified, we tried to identify the factor(s) of variation between these farms that may be behind the miRNA variation. Only *bta-miR-215* showed differential levels between farm groups, coinciding with the trends observed in the exploratory study (Abou el qassim, *et al.*, 2018).

Based on the Regulation 2018/848 of the European Parliament 2092/91 on organic production and labelling of organic products, an attempt has been made to draw out the factors that may be at the origin of these variations (European Commission, 2018). Factors such as the effect of the type of concentrate (organic *vs.* conventional) and the botanical composition of the pastures have been evaluated, since the latter can vary according to the management of the pastures within an organic or conventional system (Román-Trufero *et al.*, 2020; Adler *et al.*, 2013; Argamentería *et al.*, 2012; Pande, 2002).

In the trial conducted to determine the effect of concentrate type (organic *vs.* conventional), When the miRNA levels were analyzed, none of them varied according to this factor. It can therefore be concluded that whether the sub-ingredients of the concentrate are organic or not does not affect the expression of the miRNAs studied as long as their nutritional contribution is similar. However, significant differences were observed in some miRNAs depending on the sampling period, which could reflect the effect of the progress of the lactation cycle. In fact, the variation of miRNAs expression with the progress of the lactation cycle has been observed on several occasions due to their involvement in different processes that regulate mammary gland development and lactation (Xuan *et al.*, 2020; Do *et al.*, 2017; Li *et al.*, 2012; Wang *et al.*, 2012;) as they may be related to cell proliferation, differentiation and apoptosis (Li D. *et al.*, 2015). On average the lactation stage of all cows in the two groups was 109 DIM at the beginning of the experiment. As is known, a typical lactation curve starts with an accelerated

increase in milk production until it reaches a peak production around 40-50 days of lactation, followed by a slow decline until the animals dry out (Strucken *et al.*, 2015). This means that, on average, the studied cows were after the peak of lactation in the descending phase. The levels of *bta-miR-181a, bta-miR-29b* and *bta-miR-7863* decreased from period 1 to period 2. This could mean that their levels in milk decrease with the decrease of the lactation curve. For example, in a study by Do *et al.* (2017) investigated the evolution of miRNAs expression during the lactation cycle to explore their regulatory mechanisms. For this, they examined samples collected on 8 different days along the lactation curve. This study showed that miR-29b showed a significantly higher expression at 70 DIM compared with 1-7 DIM ($p < 0.05$) (Do *et al.*, 2017). On the contrary, the levels of the miRNAs *bta-miR-451* and *bta-miR-99a-3p* increased from period 1 to period 2. This means that their levels increase as lactation declines. A study in goat mammary gland showed that miR-99a-3p expression in the dry period was significantly higher than in the late lactation phase (Xuan *et al.*, 2020) showing that the increase of this miRNA in the late lactation stages may be involved in the process of remodelling and apoptosis of mammary gland tissues, as it has been shown to play a role in cell proliferation (Li J *et al.*, 2015).

Floristic composition of the pasture is one of the factors that may vary between organic and conventional grazing farms (Román-Trufero *et al.*, 2020; Adler *et al.*, 2013; Argamentería *et al.*, 2012; Pande, 2002). This variation in the floristic composition may be due to the differences in pasture management between the two types of farms (European Commission, 2018) such as grazing intensity, seeding management, herbicide use, type of fertilization, etc. (Paulisová *et al.*, 2019; Batáry *et al.*, 2016; Mrázková *et al.*, 2014; Adler *et al.*, 2013; Rodríguez & Jacobo, 2010). Different practices can lead to variations in both plant biomass in terms of yield, chemical and floristic composition resulting in a different nutritional value which could, therefore, be reflected in the composition of the milk (Dubljević *et al.*, 2020; Adler *et al.*, 2013; Argamentería *et al.*, 2012; Mackle *et al.*, 1996). The effect of fertilization is a factor that can result in a different floristic composition between the two studied management systems. Comparing two conventional grasslands fertilized with two different levels of mineral nitrogen, showed that in high N grasslands, *Lolium perenne* percentage in the pasture decreased while *Poa* spp. increased and white clover was maintained unchanged (Mackle *et al.*, 1996). Other studies have shown that, in conventional grasslands, mineral fertilization

significantly limits legume growth (Adler *et al.*, 2013; Argamentería *et al.*, 2012). Adler *et al.* (2013) studied the effect of long-term and short-term grassland management on organic and conventional grasslands. Short-term grassland management refers to those that reseed every four years or more frequently, while long-term management refers those that reseed less frequently. This study showed that pasture management affected the botanical composition of organic pastures, in such a way that short-term grassland management contained more legumes and less dandelion. While it had no effect on conventional livestock grazing (Adler *et al.*, 2013). Pande (2002) reported the same results as the previous study, showing through his work over 10 years comparing the evolution of the botanical composition of these two systems, that differences were slowly established over ten years. The study also stated that pasture renovation can be used in organic systems, as in conventional systems, to address any deficiencies in botanical composition. It was observed also that organic management positively affected the whole ecosystem (plants, non-carnivorous carabids, hunting spiders...). Whereas adopting a lower management intensity only increased the species richness of grasses and spiders (Batáry *et al.*, 2016). This probably shows the effect avoiding the use of pesticides and insecticides in organic livestock farming, increasing the overall biodiversity.

It is important to note that some characteristics of organic pasture management are not unique to it, as they can be adopted by conventional pastures and result in a similar floristic composition to organic pastures (Schwendel *et al.*, 2017). Our study did not reveal any significant difference between organic and conventional pastures in the sampled farms in Asturias. This could be due to similar practices between conventional and organic farms, as most of the selected conventional farms are managed extensively. Moreover, most organic farms have only been present in Asturias for a short time and it is possible that they have not yet reached a floristic composition that differentiates them from conventional farms, since in 2015 there were 15 organic farms in Asturias and this increased to 51 by 2020 (COPAE, 2020). Based on the results, it appears that botanical composition did not show significant differences between the studied farms and therefore its effect on *bta-miR-215* could not be proven in the present study.

PCA was used to try to find the management-related factors causing the variations in *bta-miR-215* levels in this study, regardless of the type of farm (conventional *vs.* organic grazing). The PC1 reported that, independently of grazing hours, *bta-miR-215* was related to the indicators of intensiveness. This refers to a multitude of factors related

to high consumption of concentrates, silage and conserved forages, as well as higher stocking densities that generally result in higher milk production (Vicente-Mainar *et al.*, 2013). Our results showed that farms with high stocking densities tended to use more corn silage, concentrate and conserved forages in their rations, which results in highest average milk production and highest levels of *bta-miR-215*. In addition to that, PC2 added that considering less intensiveness and productivity factors, a high consumption of forages (green or conserved) is related to reduced consumption of concentrates and reduced levels of *bta-miR-215*. Furthermore, these analyses showed through PC3 that the form of forage consumption (green or conserved) did not affect the miRNA level.

In the first chapter of this work, comparisons were made between farms with opposite management according to concentrate and corn silage intake and access to pasture (yes/no). The level of *bta-miR-215* was found to be high on farms with high concentrate intake (more than 10 kg/animal), consuming corn silage and without access to grazing. The present work confirms the previous results, adding that the miRNA levels are not directly affected by the presence or absence of grazing or its duration, but can be affected by fibre intake and the minimization of concentrates in the ration. It can be deduced that in a gradual line of intensiveness, the level of *bta-miR-215* varies positively with the latter, even within grazing farms (**Figure 5.8**).

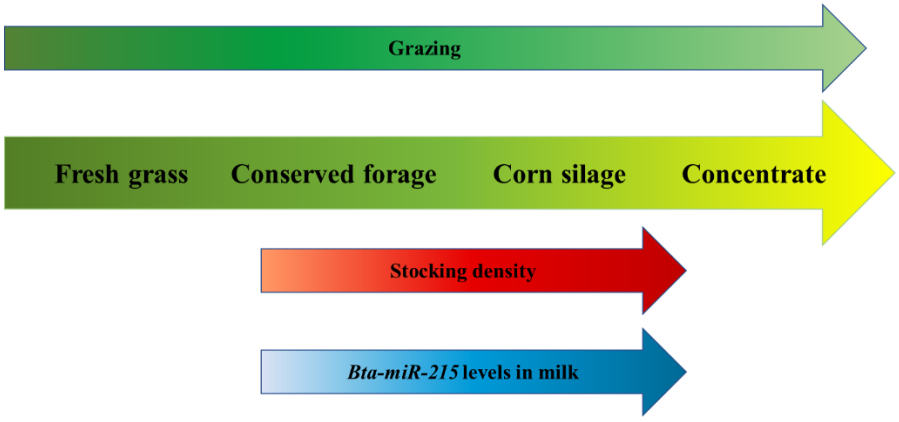

Figure 5.8. Summary of factors with possible effects on the variation of *bta-miR-215* levels in milk. (The intensity of the colour indicates the intensity of the variable).

It could be possible that all of these intensiveness factors together lead to the up-regulation of this miRNA, although its level could be related to one of these factors, especially the amount of concentrate in the diet and the animal density. While speculating

which of these factors may be implicated, it has been shown that *bta-miR-215* upregulation is probably related to low conversion efficiency in cows (Al-Husseini *et al.*, 2016), as mid-potential dairy cows showed higher conversion efficiency during pasture feeding compared to indoor feeding related to increased concentrates in the ration (Knaus, 2016). It has also been shown that oxidative stress, which can easily occur in intensive production systems, up-regulates *bta-miR-215* in serum of Holstein cows (Zheng *et al.*, 2014). Generally high densities are associated with a higher consumption of concentrates. However, in the case of our work the densities were not significantly different between the two types of farms, but the amount of concentrate was significantly different.

For all the above-mentioned reasons, it was concluded that *bta-miR-215* level is not related to a factor linked to organic farming as the type of concentrate and the botanical composition of the pasture, but it was linked to factors that could be adopted by conventional farms as well. In this regard, Schwendel *et al.* (2017) affirm that a similar diet for organic and conventional pasture-fed cows generates an obstacle for testing the authenticity of organic milk.

5.5. Conclusion

The miRNA *bta-miR-215* varied between organic and conventional grazing farms, probably not because of any intrinsic characteristics specific to organic management, but it seems that the amount of concentrate and probably stocking density were responsible for this variability. If conventional grazing farms reduce the amount of concentrates in the diet and the stocking rate on pasture, *bta-miR-215* would not be useful as a differentiator for organic farms.

Chapter 6

Cow's milk processing decreases microRNA levels in dairy products

Chapter 6: Cow's milk processing decreases microRNA levels in dairy products

6.1. Introduction

The previous chapters assessed the ability of milk miRNAs to differentiate this product according to its origin, revealing that the characteristics of milk production systems also influence the levels of miRNAs. The sensitivity of miRNA levels to aspects of animal physiology and farm conditions, coupled with their strong resistance to adverse conditions, including temperature variation, RNase, low pH (Izumi *et al.*, 2012), make them excellent candidates as biomarkers for characterizing the quality and provenance of milk (Buschmann *et al.*, 2016). One of the reasons for the high resistance of miRNAs is their packaging in vesicles such as milk exosomes and fat globule (Izumi *et al.*, 2014; Munch *et al.*, 2013; Zhou *et al.*, 2012).

From a functional point of view, it was demonstrated that after milk consumption by human adults, bovine milk exosomes can be transported to the human colon and at least some of them transferred to the bloodstream (Wolf *et al.*, 2015; Baier *et al.*, 2014), where they may affect gene expression in humans (Zempleni *et al.*, 2015). Dairy production systems influence raw milk miRNA levels, and therefore functional properties, of bovine milk and potentially other dairy miRNAs content.

The studies of miRNAs have focused on raw milk, but most milk is processed before human consumption. In our previous work, we observed that there was no significant effect of pasteurization on milk fat miRNA and cells miRNA content (Abou el qassim *et al.*, 2021) but little is known about further dairy processing on miRNAs.

Here, the objective is to study the effect of other milk technological processes such as microwave heating, yogurt fermentation and cheese ripening, to evaluate the miRNA losses. To do that we estimated the levels of seven miRNAs from farms bulk tank raw milk and after different treatments: microwave heating, fermentation (yogurt) and cheese ripening. Also, the concentration of the studied miRNAs in these products will be also estimated, to assess the implications on their potential bio-functionality.

6.2. Material and methods

6.2.1. Milk sample collection and treatments

Raw tank milk was sampled on 10 dairy farms in Asturias, during June and July 2021. The selected farms are included in different production systems (**Table 6.1**).

Table 6.1. Characteristics of the sampled farms.

Dairy farm characteristic	Number of cows	Average milk production (L/cow /d)	Grazing (h/d)	Grass silage (kg FM /cow/ d)	Straw (kg FM /cow/ d)	Vetch (kg FM /cow/ d)	Alfalfa (kg FM /cow/ d)	Corn silage (kg FM /cow/ d)	Concentrate (kg FM /cow/ d)
1	41	21.00	Yes	5.00	0.00	3.00	0.00	0.00	6.00
2	38	23.00	Yes	0.00	0.00	1.00	1.00	0.00	7.00
3	35	15.00	Yes	0.00	0.00	0.00	0.00	0.00	4.50
4	61	25.00	Yes	5.00	0.00	0.00	0.00	0.00	10.00
5	14	25.00	Yes	5.00	0.00	0.00	0.00	0.00	9.00
6	240	36.00	No	10.00	0.50	2.00	2.00	16.00	12.00
7	60	32.00	No	20.00	0.00	1.50	0.00	20.00	10.00
8	75	34.00	No	8.00	0.00	0.00	2.00	25.00	11.50
9	50	30.00	No	6.00	1.00	0.00	2.00	20.00	7.00
10	44	34.00	No	10.00	0.00	1.50	0.00	20.00	10.00

L/cow /d: liter per cow per day, h/d: hour per day, kg FM /cow/ d: kilogram of fresh matter per cow per day

The 10 samples were transported to the laboratory at 4 ºC and then processed the following day. To generate control samples (n=10), 2 mL of QIAzol lysis reagent was added to 1 g of raw milk, then samples were mixed and stored at -80 ºC. To obtain microwaved samples (n=10), 50 mL of raw milk were heated in a 700-W microwave oven for 1 min, then allowed to cool to room temperature. An aliquot of this milk (1 g) was transferred to a new RNase-free tube, 2 mL of QIAzol lysis reagent was added, and samples were mixed and stored at -80 ºC.

To obtain yogurt samples (n=10), 200 mL of raw milk were pasteurized in a thermostatic bath at 85 °C for 30 min, allowed to cool to 42 °C, then inoculated with a commercial yogurt starter, which contains *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*, at the dose recommended by the manufacturer (50 units/250 L of milk). Once inoculated, aliquots (100 mL) were transferred into two containers, which were incubated in a water bath at 42 °C until the pH reached 4.5, which occurred after approximately 4 h. Then an aliquot of yogurt (1 g) was transferred to a RNase-free tube, 2 mL of QIAzol lysis reagent was added, and samples were mixed and stored at -80 ºC. The pH and product weight were monitored at all stages.

Cheese samples (n=10) were prepared as described (Hynes *et al.*, 2000). 500 mL of raw milk was inoculated with 10 mL of the starter prepared according to the dose recommended by the manufacturer (10 units/100 L of milk). The starter culture consisted of mesophilic strains of *Lactococcus lactis subsp. lactis* and *cremoris*. Calcium chloride (1 mL, 20% w/v, final concentration= 0.02%) was added, the mixture was homogenized by stirring, then aliquots (200 mL) were transferred into two centrifuge flasks (250 mL volume) and incubated at 26-30 °C for 45 min in a water bath. Then, 65 µL of rennet (Nievi, Bizkaia, Spain; 1×10 000) was added, and the milk mixture was allowed to coagulate in a water bath at 30 ºC until reaching the appropriate consistency after approximately 90 min. The curd was cut into 5 mm cubes using a sterile stainless-steel knife, stirred for 20 min and centrifuged at 220 × g at room temperature for 10 min. The entire aqueous phase (whey) was removed, then 35 mL of saturated brine (NaCl 330g/L, pH 5.4) was added to curd and kept for 5 min. The mini- cheeses were ripened at 10-12 °C in a ripening chamber for one week. After this time, 1 g of cheese was transferred to a Falcon tube, 2 mL of QIAzol lysis reagent was added, and samples were mixed and stored at -80 ºC. As during yogurt manufacturing, pH and product weight were monitored at all stages.

6.2.2. Total RNA extraction and spike-in

Prior to RNA extraction, raw milk and dairy product samples were spiked with defined concentrations of synthetic standard miRNAs. To measure the losses of miRNAs due to different milk processing, 6 fmol of *cel-miR-238* (Norgen, Thorold, Canada) was added to the mixture of sample + QIAzol in the case of raw milk, microwaved milk, and yogurt. In the case of cheese, 54 fmol of *cel-miR-238* was added to 1 g of cheese in 2 mL

of QIAzol, based on our observation (from 10 one-week old cheeses) that 9 g of milk was necessary to obtain 1 g of cheese. In addition, to compare the amounts of miRNAs between the different products, (1 g raw milk *vs.* 1 g microwaved milk *vs.* 1 g yogurt *vs.* 1 g cheese), 6 fmol of *cel-miR-39* (Norgen, Thorold, Canada) was added to the mix (sample + QIAzol) of raw milk and cheese. The use of external synthetic reference miRNAs has been reported since the early work on circulating miRNAs (Mitchell *et al.*, 2008).

Total RNA was extracted from aliquots (2 mL) of each mix (sample + QIAzol), which amounted to 40 samples, using the mirVana miRNA isolation kit according to the manufacturer's instructions. RNA was eluted with 100 µL of RNase-free water. RNA concentration and purity (ratio of absorbance at 260 to 280 nm) were assessed using a Nano-Drop spectrophotometer.

6.2.3. RT-qPCR

Total RNA was used for cDNA synthesis using the TaqMan Advanced miRNA cDNA Synthesis Kit, and the resulting cDNA was stored at -20 °C until use. Levels of the seven miRNAs *bta-miR-148a*, *bta-miR-21-5p*, *bta-miR-215*, *bta-miR-29b*, *bta-miR-30a-5p*, *bta-miR-451*, and *bta-miR-7863*, as well as *cel-miR-238* and *cel-miR-39* were determined by RT-qPCR in a StepOne thermocycler. The final reaction solution contained 10 µL of 2× TaqMan Fast Advanced Master Mix, 1 µL of 20× TaqMan Advanced miRNA Assay, 4 µL of RNase free water, and 5 µL of 1:10 diluted cDNA. The thermocycler program was set at 95 °C for 20 s, followed by 40 cycles at 95 °C for 1 s and 60 °C for 20 s. All RT-qPCR reactions were performed in duplicate, and the results were averaged only when the duplicates differed within 0.5 threshold cycle. To assess miRNA losses due to different milk manipulations, miRNA levels were normalized to those of *cel-miR-238*, while *cel-miR-39* was used to compare the concentration of raw milk and cheese. Then miRNA levels were estimated using qbase+ 3.1 software and expressed using the △△Ct method in base $\log^2$(Hellemans *et al.*, 2008).

6.2.4. Statistical analyses

Data were expressed using mean and SD. Because sample sizes were small and some data showed a skewed distribution based on the Shapiro test, non-parametric statistical analysis was carried out. Pairwise comparisons of miRNA levels among raw

milk, heated milk, yogurt, and cheese were performed using the Wilcoxon test for paired data. Significance was defined as $p \leq 0.05$. All analyses were performed using IBM SPSS Statistics for Windows version 22.0.

6.3. Results

6.3.1. Validation of milk treatments

As expected, in yogurt fermentation, a reduction in pH, from 6.63 ± 0.19 to 4.53 ± 0.14, was observed after 4 h of the starter culture addition, (**Figure 6.1**).

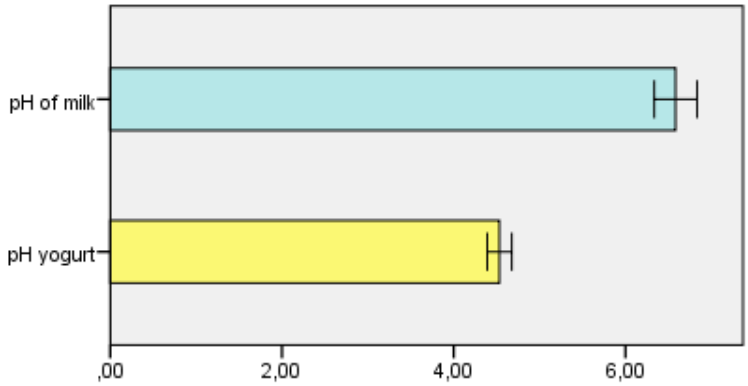

Figure 6.1. Mean pH and standard deviation for milk and yogurt.

During cheese manufacturing, pH was measured at five timepoints: raw milk, immediately after starter addition, after coagulation, as well as before and after ripening. The pH decreased significantly from the moment the ferment was inoculated. The pH decreased strongly after seven days of ripening. Overall, pH fell from 6.23 to 4.61 (**Figure 6.2**), confirming lactic fermentation.

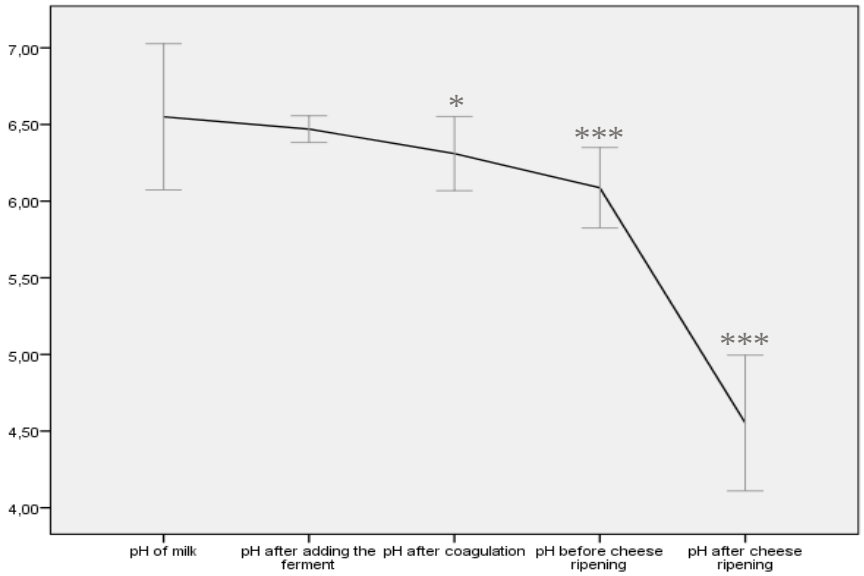

Figure 6.2. Evolution of pH in milk, during cheese manufacture and ripening. *p < 0.05, **p < 0.01, ***p < 0.001

When 206 g of raw milk was used to prepare cheese, the average weight of the fresh cheese, after removal of whey, was 60.13 ± 7.63 g, and the ripened cheese (after 7 days ripening) weighed 22.26 ± 4.07 g. This means that coagulation and whey removal reduced the weight by about 69%, and moisture decrease during ripening reduced weight by an additional 18%. Altogether, cheese yield averaged 11%.

6.3.2. miRNA losses due to milk processing

Relative levels of *bta-miR-148a*, *bta-miR-21-5p*, *bta-miR-215*, *bta-miR-29b*, *bta-miR-30a-5p*, *bta-miR-451*, and *bta-miR-7863*, normalized to the levels of spiked *cel-miR-238*, were compared across raw milk, microwaved milk, yogurt, and ripen cheese (**Table 6.2**), to assess the losses of miRNAs after the different treatments. All treatments decreased the levels of all seven miRNAs: about 31% decrease by microwave treatment and yogurt fermentation and about 43% decrease during cheese production. However not all miRNAs were affected in the same way (**Table 6.3**, **Figure 6.3**), the reductions after microwave heating varied from 17.20% for *bta-miR-30a-5p* to 39.42% for *bta-miR-451*; after yogurt fermentation, from 21.45% for *bta-miR-21-5p* to 41.62% for *bta-miR-451*; and after cheese production, from 32.73% for *bta-miR-30a-5p* to 56.32% for *bta-miR-215*. The reductions were significant for all seven miRNAs in the case of yogurt and

cheese, and only for four of seven miRNAs in the case of microwaving (*bta-miR-148a*, *bta-miR-21-5p*, *bta-miR-215* and *bta-miR-451*).

Table 6.2. Relative levels of the seven miRNAs of interest in raw milk, microwave-treated milk, yogurt, and ripen cheese. *

miRNA	Raw milk		Microwave		Yogurt		Cheese	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
bta-miR-148a	4.62	0.72	3.44	0.87	3.04	0.16	2.17	0.63
bta-miR-21-5p	4.04	0.62	2.86	1.08	3.18	0.23	2.97	0.33
bta-miR-215	3.90	0.85	2.60	1.24	2.86	0.28	1.70	0.38
bta-miR-29b	4.20	0.34	2.98	1.64	2.63	0.73	2.68	0.83
bta-miR-30a-5p	3.61	0.91	2.99	0.94	2.49	0.15	2.43	0.35
bta-miR-451	5.24	0.42	3.17	1.99	3.06	0.28	2.46	0.40
bta-miR-7863	4.28	0.39	2.33	1.75	3.21	0.18	2.39	0.36

SD, standard deviation, * Levels were normalized to those of spiked *cel-miR-238* (see Methods).

Table 6.3. Decrease in miRNA levels after treatment of raw milk. *

miRNA	Microwaved milk		Yogurt		Cheese	
	Decrease (%)	*p*-value	Decrease (%)	*p*-value	Decrease (%)	*p*-value
bta-miR-148a	25.53	0.043	34.22	0.005	53.15	0.005
bta-miR-21-5p	29.18	0.012	21.45	0.013	26.51	0.005
bta-miR-215	33.20	0.012	26.58	0.013	56.32	0.005
bta-miR-29b	29.07	0.080	37.46	0.028	36.15	0.028
bta-miR-30a-5p	17.20	0.063	31.04	0.013	32.73	0.013
bta-miR-451	39.42	0.018	41.62	0.005	53.09	0.005
bta-miR-7863	45.45	0.123	24.84	0.005	44.01	0.005
Mean	31.29		31.03		43.14	

* Decreases are expressed as the percentage in raw milk. Levels of miRNA were normalized to those of spiked *cel-miR-238*, * *p*-value *vs*. raw milk (Wilcoxon test).

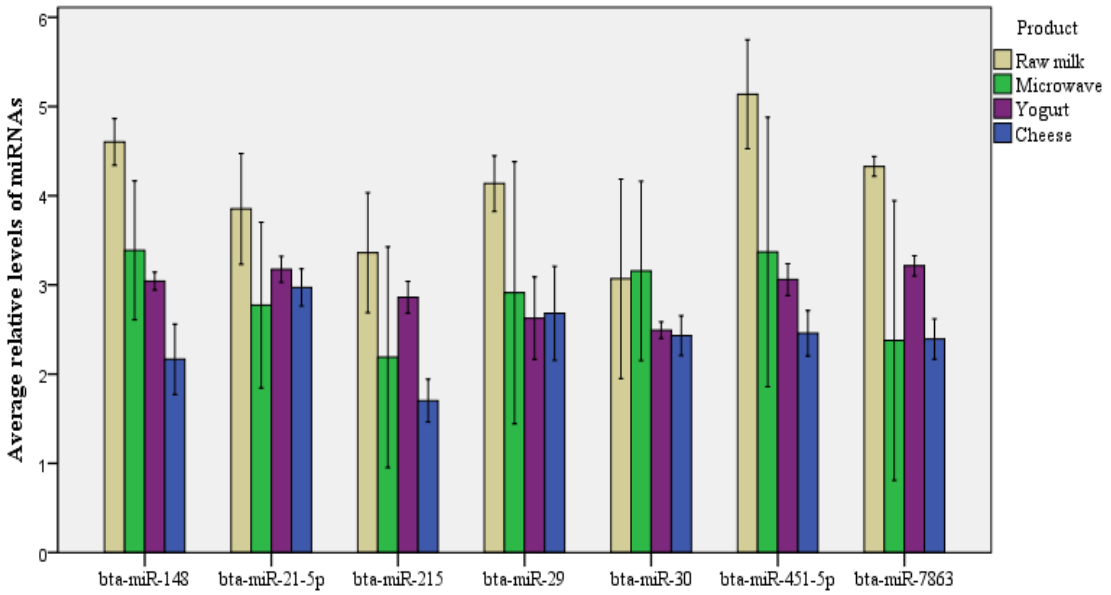

Figure 6.3. Loss of miRNA content in raw milk after the microwave heating, yogurt fermentation, and cheese manufacture. Average relative levels of *bta-miR-148a*, *bta-miR-21-5p*, *bta-miR-215*, *bta-miR-29b*, *bta-miR-30a-5p*, *bta-miR-451*, and *bta-miR-7863* in raw milk (n=10), microwave-heated milk (n=10), yogurt (n=10), and cheese (n=10). Levels were normalized to those of spiked cel-miR-238. The bar chart shows the average of miRNA levels for each product, and the standard error bars.

6.3.3. miRNA concentrations in milk and cheese.

Relative levels of *bta-miR-148a*, *bta-miR-21-5p*, *bta-miR-215*, *bta-miR-29b*, *bta-miR-30a-5p*, *bta-miR-451*, and *bta-miR-7863*, normalized to the levels of spiked *cel-miR-39*, were compared across raw milk, and ripen cheese, to assess the concentration of miRNAs in raw milk, and ripen cheese. The differences in levels between the same amount of raw milk and cheese were not significant for the studied miRNAs, which might indicate that their concentrations were similar between raw milk and cheese (**Table 6.4**).

Table 6.4. Relative abundance levels of seven miRNAs in raw milk and cheese. *

miRNA	Raw milk		Cheese		*p*-value
	Mean	SD	Mean	SD	(Wilcoxon test)
bta-miR-148a	2.32	1.35	2.10	0.30	0.76
bta-miR-21-5p	2.30	2.01	2.97	1.64	0.33
bta-miR-215	1.68	2.06	1.60	0.17	> 0.99
bta-miR-29b	1.72	2.26	3.16	1.63	0.11
bta-miR-30a-5p	1.77	1.95	2.89	0.33	0.13
bta-miR-451	2.24	2.58	1.50	0.11	0.31
bta-miR-7863	1.99	1.46	2.43	1.21	0.40

* Levels were normalized to those of spiked *cel-miR-39*.

6.4. Discussion

Here we evaluated the effects of different cow milk treatments on miRNA content, in order to guide efforts to define biomarkers for assessing the quality or provenance of dairy products destined for human consumption, and also, to evaluate the contribution of different dairy products in miRNAs, considering them bioactive compounds (Rani *et al.*, 2017). Our results confirmed the presence of miRNAs in the studied dairy products and suggest that thermal treatment of raw milk by the microwave as well as yogurt or cheese production can substantially reduce miRNA levels, indicating that the levels of potential biomarker miRNAs in raw milk cannot be necessarily extrapolated to dairy products derived from that milk.

Microwave heating has been shown to affect certain physical and chemical characteristics of milk (Musto *et al.*, 2014) and to damage DNA (Dehghan *et al.*, 2012), which led us to hypothesize that this treatment could affect miRNAs. Indeed, we found that microwave treatment of raw milk significantly decreased the amounts of *bta-miR-148a*, *bta-miR-215*, *bta-miR-21-5p*, and *bta-miR-451*. A previous study also described a significant decrease in *bta-miR-21-5p*, but not *bta-miR-29b* (Zhao *et al.*, 2018). We did not observe a significant decrease in *bta-miR-29b*, yet other work reported a 40% loss in *miR-29b* (Howard *et al.*, 2015). This discrepancy may be due to the storage of raw milk prior to treatment. Howard *et al.* (2015) studied the stability of miRNAs in milk after 15 days of cold storage, being after that heated by microwave (n=3). No significant difference was seen in *miR-29b* after 15 days of cold storage, but the significant difference appeared after microwave heating (Howard *et al.*, 2015).

The fact that milk treatment significantly affects some miRNAs and not others may depend on the different fractions of milk where the miRNAs are found (Li *et al.*, 2016). Intracellular miRNAs are less stable than extracellular miRNAs within exosomes, microvesicles, apoptotic bodies, high-density lipoproteins, or protein complexes (Sohel, 2016). In the case of milk fat, the fat globule membrane appears to be more resistant to gastrointestinal enzymes (Le *et al.*, 2012) and microwave heating (Rodríguez-Alcalá *et al.*, 2014) than other milk components.

In contrast to microwave heating, milk pasteurization has been reported not to significantly affect miRNA content of fat or milk cells (Abou el qassim *et al.*, 2021) or EVs in milk (Hansen *et al.*, 2022).

As cheese and yogurt can be presented in different forms on the market, a simple model of these treatments was elaborated, representing the general characteristics of each product. In contrast to the effect of microwaving, fermentation of previously pasteurized milk to make yogurt significantly reduced levels of many miRNAs (Yu *et al.*, 2017; Howard *et al.*, 2015). The reduction in miRNAs during milk processing is not surprising given the range of changes that occur during milk fermentation to make yogurt and cheese.

In yogurt production, the starter culture can lower the pH below 4.6, leading to aggregation of caseins (Settachaimongkon *et al.*, 2014). Lactose is converted into lactic acid and several amino acids and FAs (especially stearic and oleic) are released into yogurt. During bacterial fermentation, vitamin B content increases and minerals are converted into an ionic form (Sfakianakis & Tzia. 2014; Vedamuthu. 2006; Walstra *et al.*, 2005). This study revealed that fermentation of previously pasteurized milk to make yogurt significantly reduced the levels of many miRNAs, with a loss of up to 41.62% in some cases.

We suspect that much of the miRNA loss in our study can be attributed to degradation of exosomes, as others have proposed (Howard *et al.*, 2015). In one study, fermentation was found to reduce by 90% the protein content of milk exosomes assuming that, under the effect of bacterial proteases, exosomes can be altered, consequently the miRNAs contained in these exosomes can be easily degraded (Yu *et al.*, 2017). As pasteurization does not significantly reduce the miRNA content (Abou el qassim *et al.*, 2021), most of the reduction in the miRNA content could be attributed to exosome degradation described before.

In our study, we prepared cheese following the modified protocol of Hynes *et al.* (2000) obtaining similar yields that those reported in the original work. We observed a more acidic pH on day 7 (4.61) than in that work (5.21), perhaps because we used raw milk for cheese making, so the natural bacteria in the milk could also metabolize lactose. Pasteurization, in contrast, destroys most bacteria, limiting the acidification (Bánkuti *et*

al., 2017). The pH can also vary depending on the starter culture used (Ressutt *et al.*, 2020).

As it was expected the greatest losses of miRNAs occurred during cheese manufacturing, given the losses due to fermentation (as in yogurt) but also because of the removal of whey, which is known to contain a wide variety of miRNAs (Li *et al.*, 2016; Alsaweed *et al.*, 2015).

However, we found that concentrations in the final product of the seven miRNAs (1 g of milk *vs.* 1 g of cheese, spiked by *cel-miR-39*) did not differ significantly between raw milk and cheese (**Table 6.4**). Similarly, two studies reported even higher miRNA concentrations in two types of cheese (camembert and Fresco queso dip) than in raw milk (Oh *et al.*, 2017; Howard *et al.*, 2015). We suspect that fermentation and whey drainage may reduce absolute miRNA levels, but that the subsequent water loss during ripening increases their concentrations.

6.5. Conclusions

We confirmed that bovine milk contains several miRNAs even after microwaving, pasteurization followed by fermentation to make yogurt, and cheese manufacturing. We showed that these treatments decreased all miRNA levels.

Our results clearly argue for caution in efforts to identify miRNAs that may be useful biomarkers: they may need to be assessed in the final products for human consumption, and not merely extrapolated from assays of the raw milk from which they are produced.

Finally, considering miRNAs as bioactive component in milk and dairy products, raw milk and cheese supposedly may provide similar concentrations of miRNAs, higher than those of yogurt and microwaved milk. Although the miRNA profile may differ between these two products. Additional studies are needed to explore the complete profiles and availability of miRNAs in dairy products and, subsequently, their putative functionality in human cells.

Chapter 7

Characterization of cow's milk exosomes and study of in silico functionality of microRNAs that vary in these structures according to the milk production system.

Chapter 7: Characterization of cow's milk exosomes and study of in silico functionality of microRNAs that vary in these structures according to the milk production system

7.1. Introduction

Most miRNAs are found in the cellular environment, but there are others found in body fluids, known as extracellular or circulating miRNAs (Sohel, 2016). These miRNAs are characterized by the fact that they are quite stable and resistant to adverse conditions (Izumi *et al.*, 2012); as they are enveloped by membrane vesicles such as exosomes, microvesicles, apoptotic bodies (Sohel, 2016), thus enabling cell-to-cell communications (Hwang, 2013). Therefore, beyond the functions that miRNAs perform in the cells that produce them, they can also be transferred to other cells and thus act on them. (Chen *et al.*, 2012). Milk has been shown to be a rich source of miRNAs (Carrillo-Lozano *et al.*, 2020) where they are found packaged in vesicles such as milk exosomes and the fat globules (Izumi *et al.*, 2014; Munch *et al.*, 2013; Zhou *et al.*, 2012).

In view of this, some studies have challenged the dogma that miRNAs are endogenous regulators, suggesting that dietary miRNAs can be absorbed and bioavailable in the recipient organism. Furthermore, these miRNAs can regulate gene expression in host cells of different organisms. Several studies confirmed that milk miRNAs contained in exosomes can be transferred into human cells after milk consumption and that these miRNAs are able to regulate gene expression in the recipient cell (Benmoussa *et al.*, 2020; Reif *et al.*, 2019; Samuel *et al.*, 2017), thus conferring a functional character to milk (Zempleni *et al.*, 2015).

miRNAs are involved in diverse regulatory pathways (Chang *et al.*, 2004), they recognize and target multiple mRNAs by base-pairing with their target transcripts using the RISC silencing complex, consequently, they lead to the downregulation or repression of target genes (Bartel, 2004). The target recognition is first determined by the seed-sequence (Lewis *et al.*, 2003). A complete understanding of the regulatory function of miRNAs relies on the identification of the targets that miRNAs regulate and the pathways

in which they are involved (Pian *et al.*, 2020), based on several computational and experimental approaches (Stenvang *et al.*, 2012).

Experimental approaches consist of overexpressing or silencing specific miRNAs (gain- and loss-of-function strategies) to test their functions either *in-vitro* or *in-vivo* (Vidigal & Ventura 2015; Stenvang *et al.*, 2012).

Several computational approaches have also been implemented for miRNA target gene prediction. These approaches are based on predicting the degree of sequence complementarity between a miRNA and its target (Lim *et al.*, 2003). When the search is limited to 7-nucleotide patterns the accuracy of the prediction increases further (Lewis *et al.*, 2005). Several tools and databases have been proposed so far: DIANA-miRPath (Vlachos *et al.*, 2015), TargetScan (Agarwal *et al.*, 2015), there are even databases only devoted to ruminants, such as RumimiR (Bourdon *et al.*, 2019). Some of them include experimentally supported targets in different species, such as TarBase, where the information is collected manually from the literature (Sethupathy *et al.*, 2006). However, perfect seed pairing is not necessarily a reliable indicator of miRNA interactions, which could explain the non-functionality of some predicted target sites. Hence the importance of experimental verification of computationally identified targets (Didiano & Hobert 2006). Despite the importance of exosomes, their isolation was challenging due to the heterogeneous composition of EVs which could overlap with exosomes during their isolation (Oliveira-Rodriguez *et al.*, 2016) and the presence of contaminating molecules such as casein (Vaswani *et al.*, 2017). Therefore, to ensure the success of this extraction, enriched exosomes must be validated and characterized.

For this purpose, in this work exosomes were characterized by particle concentration and size through nanoparticle tracking analysis (NTA), size and distribution by Dynamic light scattering (DLS), protein concentration by bicinchoninic acid assay (BCA) and the presence of exosomal markers by immunoblotting, for further investigations in cell cultures on the putative functionality of bovine milk miRNAs in human cells. Also, the studied miRNAs contained in these structures were also evaluated by measuring their levels according to the production system. In parallel, we identified the human target genes of the miRNAs contained in these exosomes with differential levels between the two production systems, using bioinformatics tools, to examine how certain miRNAs can reveal functional properties of agri-food products.

7.2. Material and methods

7.2.1. Milk sampling

Tank milk samples were collected from 8 extensive and 9 intensive farms (Asturias), during June and August 2021 (**Table 7.1**). The samples were stored at 4 ºC and immediately transported to the laboratory for processing.

Table 7.1. Characteristics of the sampled farms.

	Number of cows	Average milk production (L/cow /d)	Grazing	Corn silage (kg FM /cow/ d)	Grass silage (kg FM /cow/ d)	Straw (kg FM /cow/ d)	Vetch (kg FM /cow/ d)	Alfalfa (kg FM /cow/ d)	Concentrate (kg FM /cow/ d)
Extensive farms	41	21	Yes	0	5	0	3.00	0	6.00
	38	23	Yes	0	0	0	1.00	1.00	7.00
	35	15	Yes	0	0	0	0	0	4.50
	61	25	Yes	0	5	0	0	0	10.00
	14	25	Yes	0	5	0	0	0	9.00
	30	20	Yes	0	0	0	0	4.00	7.00
	35	26	Yes	0	5	0	2.00	2.50	9.00
	24	23	Yes	0	0	0	0	2.00	8.00
Intensive farms	240	36	No	16	10	0.50	2.00	2.00	12.00
	60	32	No	20	20	0	1.50	0	10.00
	75	34	No	25	8	0	0	2.00	11.50
	50	30	No	20	6	1.00	0	2.00	7.00
	44	34	No	20	10	0	1.50	0	10.00
	66	35	No	25	10	0	0	0	12.00
	120	36	No	30	5	1.00	2.00	1.50	12.00
	85	34	No	22	15	1.00	1.50	2.00	11.00
	66	40	No	25	10	0	0	2.00	12.00

L/cow /d: liter per day, kg FM /cow/ d: kilogram of fresh matter per cow per day

7.2.2. Exosome Isolation

Milk was well mixed, 100 mL of each sample were centrifuged at 3000 *rcf* for 15 min at 4 °C, to remove milk fat globules, somatic cells and cellular debris, then whey was transferred to a new RNase-free tube. 300 µL of acetic acid was added to each 40 mL whey, the mix was vortexed and centrifuge during 10 min at 3000 *rcf* to suspend casein. After the separation, the 17 skim samples were stored at -80 ºC. EVs were isolated from milk skim by differential centrifugation as described by Vaswani *et al.* (2017).

Each sample was collected in three OptiSeal ultracentrifuge tubes (14 mL, 95mm), (Beckman Coulter, Gladesville, Australia) and subjected to successive ultracentrifugation steps: 12 000 *rcf* for 60 min, 35 000 *rcf* for 60 min, and then at 70 000 *rcf* for 60 min at 4 °C (Beckman, SW 40 Ti Swinging-Bucket ultracentrifuge rotor). The supernatant was filtered through 0.45 and 0.22 µm syringe filters and centrifuged at 135 000 *rcf* for 90 min at 4 °C, to sediment EVs. The EVs were resuspended in 300 µL PBS. The pellet was kept in the refrigerator at 4°C for 12 h, then mixed with a pipette and stored at -80°C for later use.

7.2.3. Exosomes characterization

7.2.3.1. Dynamic light scattering (DLS)

The extracted exosomes were diluted 10-folds in PBS and then transferred into the cuvette (ZEN 0040 disposable cuvette), which was filled up to 100 µL of this dilution. Size distribution of EVs were carried out using a Zetasizer (Malvern Instruments, Malvern, UK) equipped with a solid-state He-Ne laser (λ=633) with a detection angle of 173° backscatter and a temperature set at 20 ºC. The EVs-containing cuvette was loaded into the DLS device to start measuring. Zetasizer software version 7.11 was used for data analysis and exosome size data refers to the scattering distribution by number (%).

7.2.3.2. BCA protein quantification

Protein concentration was determined based on bicinchoninic acid (BCA) Thermo Scientific™ Kit protein assay BCA Pierce™ (Thermo Scientific, Massachusetts, USA). Before starting, the samples were prepared as follows: 50 µL of the sample, 50 µL RIPA 2× (Abcam, Cambridge, MA, USA) and 1 µL of protease inhibitor were added into a tube. The mixture was vigorously vortexed and transferred to ice for 10 min. Part of the

mixture was used for the determination of protein concentration and the other part was stored at - 80 °C for later use.

On an Elisa plate, 5 µL of exosomes in duplicate and 200 µL buffer (Thermo Scientific, Massachusetts, USA) were added and compared in duplicate with serially diluted bovine serum albumin (BSA) as standard according to the manufacturer's instructions. To perform the calibration curve, 0, 1, 2.5, 5, 7.5, 10, 20 µL BSA and 200 µL Buffer were added in duplicate. The plate was placed in an incubator at 37°C 1/2 h, the response was measured at λ=562 nm using a spectrophotometer (Tecan, Crailsheim, Germany) and a calibration curve representing a third order polynomial equation was established.

7.2.3.3. Immunoblotting

Once the amount of proteins in the exosome samples was quantified, the presence of the characteristic membrane proteins of the exosomes was identified by immunodetection with specific antibodies against CD 81 and CD 9. The purity of the exosomes and the absence of casein micelles were also verified with specific antibodies against casein. For this, 30 µL of 4× LSD buffer (Abcam, Cambridge, MA, USA) was added to 90 µL of the previously prepared RIPA sample, then the exosomal protein mixtures were incubated for 10 min at 70 °C.

To estimate the size of the different proteins, the molecular weight ladder (Abcam, Cambridge, MA, USA) was used, 8 µL of the Ladder marker protein was added to 40 µL of 1× LDS, then 20 µL of this mixture was loaded on each side of the gel. To carry out electrophoresis, two gels were prepared and placed on top of each other. To prepare the migration/protein separation gel, the following components were added:
5 mL Deuterium-depleted water (DDW), 2.5 mL lower 4× buffer consisting of 1.5 M Tris-HCl (pH=8.8), 0.4% SDS and DDW, which, due to its pH, negatively charges the proteins, favouring their migration through this gel, 2.5 mL Acrylamide / Bisacrylamide (Biological Industries, Beit HaEmek, Israel) at a final concentration of 10% and 60 µL Ammonium Persulphate (APS) (Bio-Rad Laboratories, inc Japan) at 10% and 7.5 µL TEMED (TCI EUROPE N.V., Zwijndrecht, Belgium) to promote acrylamide gelation. For the protein pre-concentration gel the following reactives were included:
500 µL Acrylamide / Bisacrylamide to a final concentration of 5%, upper 4× buffer consisting of 0.5 M Tris-HCl pH=6.8, 0.4% SDS and DDW, which, thanks to its pH,

keeps the proteins neutrally charged and maintains their migration speed low. To promote acrylamide gelation, 30 µL APS 10% and 7.5 µL TEMED were added.

Once the gel is prepared in the electrophoresis cassette, 30 µL of the exosome lysate and 20 µL of the molecular weight marker were added. The electrophoresis started at 120V in the presence of run electrophoresis buffer (100 mL) (Bio-Rad, Laboratories, Inc USA), and DDW (900 mL), for 1 h. After this time, proteins were transferred from SDS-PAGE to a polyvinyl difluoride (PVDF) (Bio-Rad Laboratories, USA) membrane, in the presence of transfer electrophoresis buffer (100 mL) (Bio-Rad, Laboratories, Inc USA), Methanol 20% (200 mL) and DDW (700 mL) for 1 h at 300V.

To prevent non-specific binding of antibodies to the membrane, this was incubated for 1 h with blocking buffer composed of tris-buffered salline (TBST) (50 mL) DDW (500 mL) and Polysorbate 80% (Tween 20) (125 µL). Membranes were then probed with appropriate primary antibodies: CD81 (1:1000; Cosmo BioTokyo, Japan), CD9 (1:1000; Cosmo BioTokyo, Japan) and anti-α-casein diluted 1:2000 (Cosmo Bio, Tokyo, Japan) at 4 °C overnight. This was followed by secondary antibody horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit (1:3000; Cell Signaling Technology) and NIH image was detected using chemiluminescence detection.

7.2.3.4. Nanoparticle tracking analysis (NTA)

After confirming the presence of the characteristic proteins of the exosomes, we used Malvern NanoSight™ NS500 instrument (NanoSight™, NTA 3.0 Nanoparticle Tracking and Analysis Release Version Build 0064; Amesbury, United Kingdom) to display the dimensions, the shape of these particles as well as their concentration. To ensure that the exosomes are intact and unbroken. Samples from the two groups of farms were pooled, and for each group, three serial concentrations were prepared:1/100, 1/1000 and 1/10000. Measurements were made from the most diluted sample to the least diluted. The concentration (particles/mL) was determined to the 1/100, in five replicates.

7.2.4. Study of miRNAs in exosomes.

7.2.4.1. miRNAs extraction

To study the exosome content of miRNAs, total RNAs were extracted from the 17 samples using the Direct-zol RNA MicroPrep RNA extraction kit (Zymo Research,

CA, USA). Following the manufacturer's instructions, the cells were lysed using TRIzol reagent and then centrifuged at 12 000 × g for 1 min at 4 °C. The supernatant was then incubated with DNase I at room temperature for 15 min. This mixture was transferred into Zymo-Spin™ IC columns and centrifuged at 12 000 × g for 1 min at 4 °C. Finally, the RNA was washed with RNA wash buffer and eluted with 40 μL of RNase-free water. The purity of the extracted RNA was quantified and evaluated with the Nano-Drop 2000 (Thermo Scientific™, Wilmington, Delaware USA). cDNA was synthesized from the extracted RNA using the high capacity RNA-cDNA kit (Applied Biosystems, Foster City, CA, USA) using 1 mg of total RNA isolated from exosomes.

7.2.4.2. RT-qPCR

The expression of the miRNAs *bta-miR-148a, bta-miR-215, bta-miR-30a-5p*, and *bta-miR-451* was studied in relation to the expression of the normalizer RNU6, using RT-qPCR with fast SYYBR Green master mix (Applied Biosystems, Foster City, CA, USA) using the StepOne Plus Real-Time PCR System. PCR reactions were 1 cycle at 95 ºC for 5 min, 40 cycles at 95 for 5 s, and 60 ºC for 30 s. The relative amounts of miRNAs were determined using ΔΔCt method.

7.2.4.3. Bioinformatics analysis

In order to identify potential human target mRNAs of cow's milk miRNAs, identified to have levels that vary between intensive and extensive farms in exosomes, it is important to verify the similarity of their sequences to human miRNAs. To this end, the miRBase platform (version 22.1, 2022). was used to identify the sequence of cow's milk miRNAs and their human homologues. Putative targets of the differentiating miRNAs were then predicted with a high degree of accuracy based on TarBase v8-DIANA tools. miRNA pathways analysis was performed using diana miRPath v2.0 (Vlachos *et al.*, 2015).

7.2.5. Statistics

As the sample size of this assay is less than 10, the Kruskal-Wallis test was used to compare the data based on SPSS software Version 22.0 and $p < 0.05$ was accepted as statistically significant.

7.3. Results

7.3.1. DLS

To confirm the exosome isolation, size distributions of raw milk derived EVs (n=17) were measured using DLS. Peaks representing the size of the largest particles population, Z-mean values measuring the average size of the set of particles measured and the distribution polydispersity index (dPI), a representation of the population size distribution within the samples, were detected and the results are recorded in the **Table 7.2.** When measuring the size of milk exosomes in the samples, the most numerous particle population had a diameter size ranging from 31.69 nm to 92.01 nm and on average 76.34 ± 34.76 nm. The Z-average of the samples ranged from 141.10 nm to 184.70 nm with a mean of 160.15 nm, while the dPI ranged from 0.22 to 0.40 with a mean of 0.28.

Table 7.2. Average particles size and distribution values measurements by DLS.

Samples	Peak size (d.nm)	SD	PdI (d.nm)	Z-average (d.nm)
1	30.16	15.30	0.40	156.00
2	59.68	32.16	0.29	173.30
3	78.21	37.24	0.28	163.80
4	90.00	30.00	0.25	164.37
5	81.01	36.27	0.26	155.95
6	84.71	38.51	0.33	165.50
7	88.48	41.11	0.27	158.97
8	82.43	34.91	0.29	156.50
9	79.58	37.62	0.24	155.40
10	31.69	17.60	0.35	169.40
11	68.22	35.30	0.29	178.00
12	88.64	51.94	0.37	184.70
13	84.79	32.82	0.22	141.10
14	92.01	38.58	0.22	151.10
15	88.06	38.43	0.22	148.80
16	85.69	36.09	0.23	148.60
17	76.14	36.04	0.27	146.20

d.nm diameter in nanometres

7.3.2. Protein quantification

After performing the protein concentration assay, the corresponding calibration curve was generated (**Figure 7.1**). This curve showed a linear correlation coefficient (R^2) higher than 0.90 and is therefore considered statistically significant and valid for the interpolation of the absorbance of the samples. The final protein concentration obtained in the simples (n= 17) varied between 1.35 and 3.70 µg/µL. Protein concentration values for each sample were represented in the **Table 7.3**.

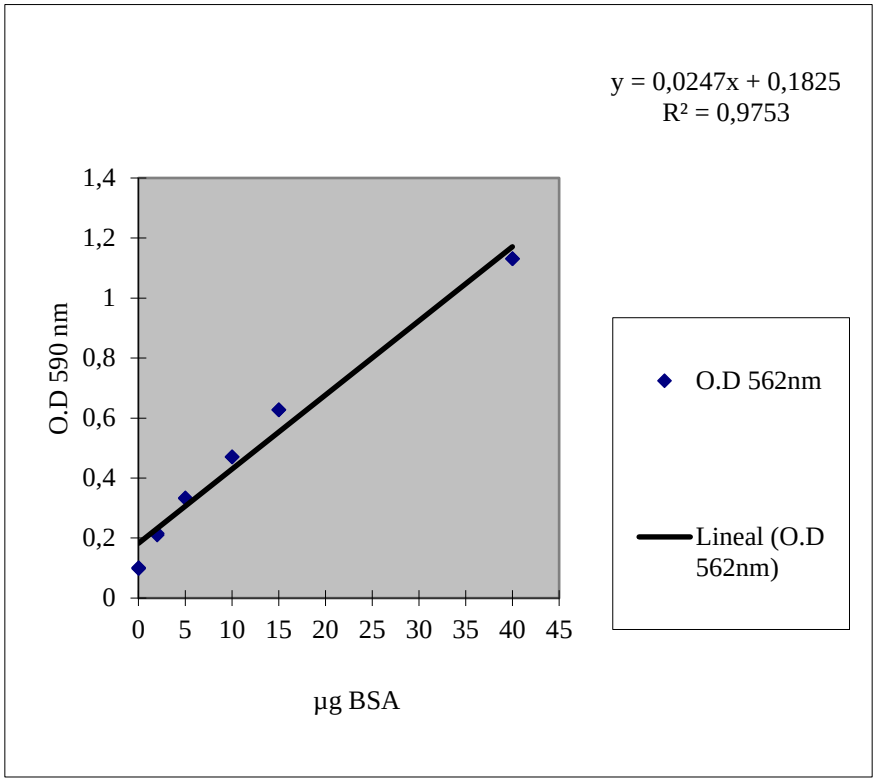

Figure 7.1. Calibration curve of BSA at 562 nm for the Pierce tm BCA protein assay kit.

7.3.3. Immunoblotting

Once the amount of proteins in the exosome sample was quantified, the presence of the characteristic proteins of the exosomes was verified by immunodetection with specific antibodies against the proteins CD81 and CD9, which are characteristic of exosomes. In addition, the purity of the exosomes and the absence of casein micelles were verified by casein-specific antibodies. After developing the photographic film, after electrophoresis and sample transfer, the presence of a band between 17-20 kDa

corresponding to CD 81 and CD 9 proteins was observed (**Figure 7.2**) and the absence of the band between 25-35 kDa corresponding to casein (**Figure 7.2**), which confirmed the presence of exosomes in the sample and their purity considering the absence of casein micelles.

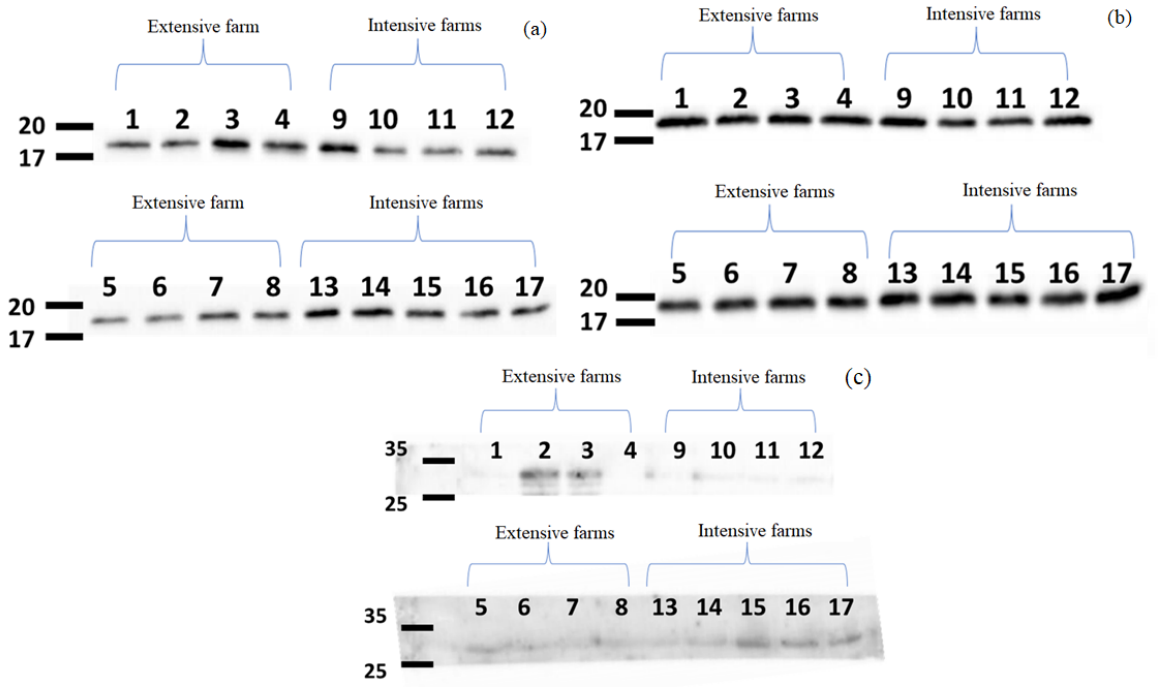

Figure 7.2. Identification of the presence of exosomes by immunoblotting against CD 9 (a) and C 81 (b) proteins and verification of exosomes purity against anti-α-casein (c).

7.3.4. Nanoparticle tracking analisis

NTA was used to determine the exosomes concentration (particles/ mL). The yield of exosomes obtained in extensive farms milk was around $1.4 \times 10^9 \pm 2.0 \times 10^8$ particles/mL. Whereas in intensive farming the concentration was much lower, around $6.8 \times 10^8 \pm 1.1 \times 10^8$particles/mL. NTA indicated an average particle size of 166 and 178 nm in the samples from intensive and extensive farms, respectively (**Figure 7.3**).

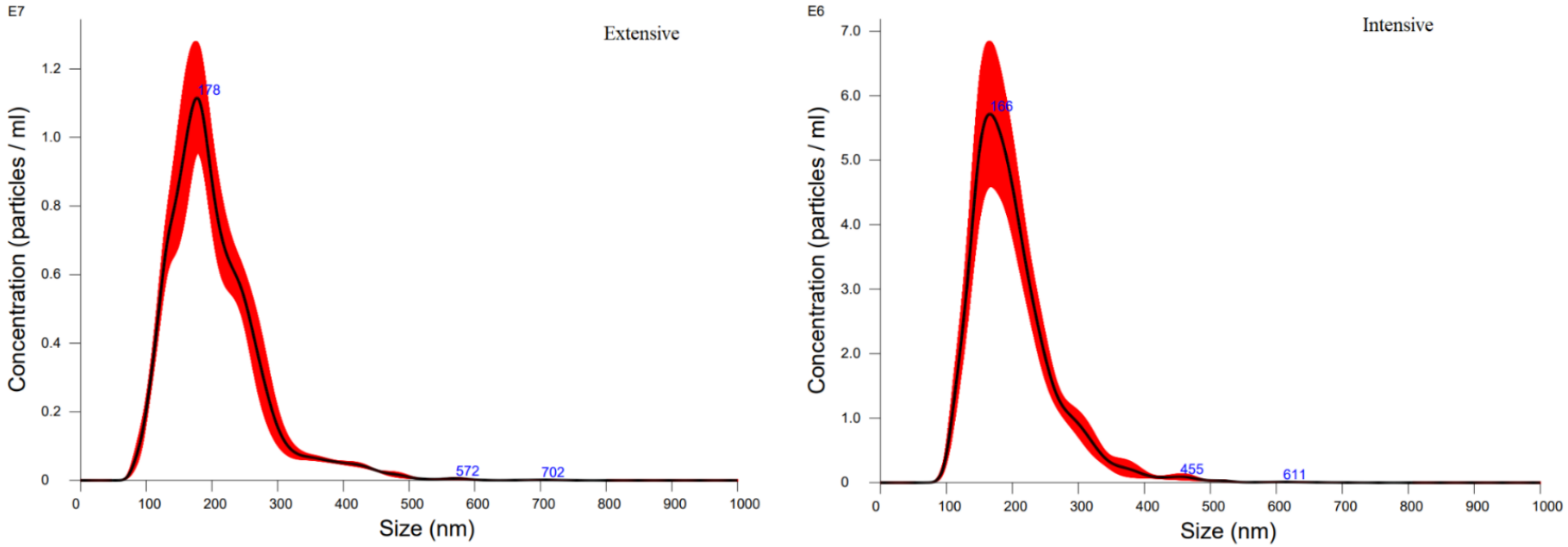

Figure 7.3. Average particle size distribution of particle concentration in the sample from extensive and intensive farms.

7.3.5. RT-qPCR

To assess the quality of the samples used for RT-qPCR, RNA concentration and purity (A260/280 ratio) was assessed by the Nano-Drop spectrophotometer. The total mean RNA concentration was 4.85 ± 2.35 ng/µL in milk exosomes, the purity value was about 1.34 ± 0.26. Detailed values for all samples are presented in the **Table 7.3**.

Table 7.3. RNA concentration (ng/ µL), A260/280 ratio of the extracted total RNA and protein concentration (ng/ µL).

Samples	RNA concentration (ng/ µL)	A260/280 ratio	Protein concentration (ng/ µL)
1	3.60	1.28	1.35
2	2.70	2.21	1.86
3	4.80	1.37	1.99
4	3.70	1.28	1.64
5	11.60	1.43	2.75
6	3.50	1.13	2.28
7	7.50	1.25	1.75
8	6.70	1.41	2.37
9	3.70	1.14	2.46
10	2.40	1.05	3.7
11	7.50	1.51	2.26
12	5.40	1.19	2.28
13	3.30	1.24	2.22
14	4.50	1.22	1.93
15	2.50	1.36	2.01
16	4.10	1.2	1.95
17	5.00	1.53	2.18

The only miRNA in exosomes that differed significantly between intensive and extensive production systems was *bta-miR-451*. This miRNA was significantly more abundant in exosomes of milk from extensive farms (*p = 0.021)* (**Figure 7.4**, **Table 7.4**).

The miRNA *bta-miR-148a* showed a trend towards lower levels in milk from extensive production, but the differences did not reach statistical significance (*p = 0.114).*

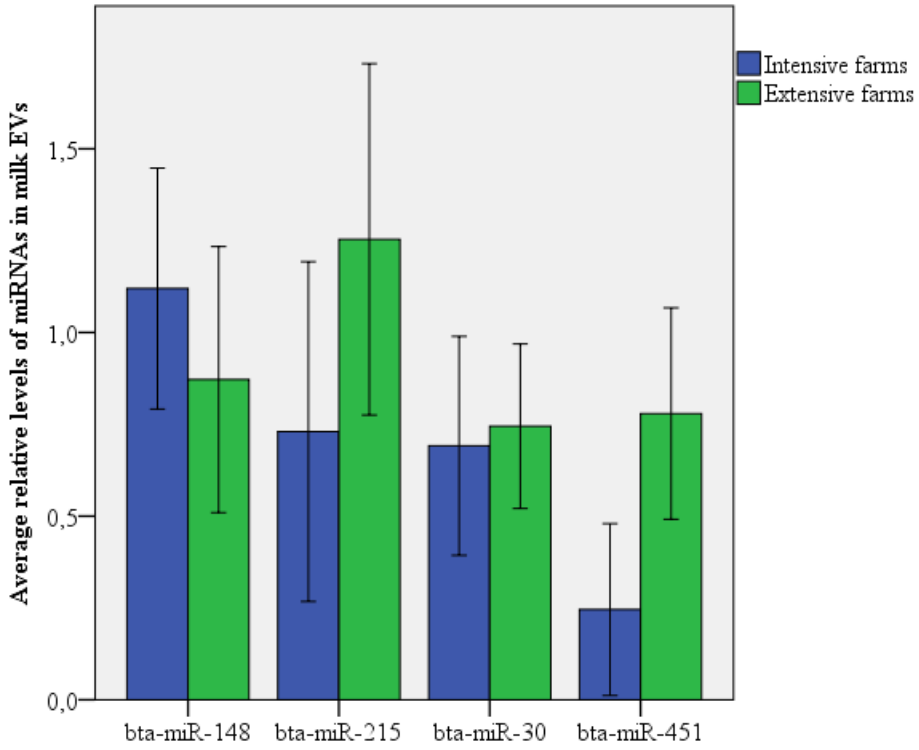

Figure 7.4. Average relative level of miRNAs in exosomes between intensive and extensive farms.

Table 7.4. Average relative level of miRNAs in milk exosomes in intensive and extensive farms and the significance test of the difference between the two groups.

	Average relative level of miRNAs in milk exosomes		Sign
	Intensive farms	Extensive farms	
bta-miR-148a	1.120	0.872	0.114
bta-miR-215	0.730	1.254	0.167
bta-miR-30a-5p	0.691	0.745	0.888
bta-miR-451	0.246	0.779	0.021

7.3.6. miRNA functionality and pathway analyses

The miRNA *bta-miR-451* showed high homology to human miRNAs and their sequences are:

bta-miR-451 : aaaccguuaccauuacugaguuu

hsa-miR-451a: aaaccguuaccauuacugaguu

This showed that it is possible that *bta-miR-451* recognize the same targets as its homologue *hsa-miR-451*, and therefore base pairing with its target may lead to the same regulation as that of the human miRNA.

To determine the possible implications of the upregulation of *bta-miR-451* in grazing cow milk exosomes on the human consumer, the target genes as well as the functional pathways in which these target genes may be involved were determined using bioinformatics tools. Using Tarbase, we found that 27 human target genes were experimentally validated for *miR-451*. These targets were associated with 4 KEGG pathways (**Table 7.5.**). These analyses allowed us to predict pathways regulated by the miRNA.

Table 7.5. KEGG pathways that are associated with *miR-451* using Tarbase.

KEGG pathway	*p*-value	Genes
Parkinson's disease	<0.01	2
mTOR signaling pathway	<0.01	5
Thyroid cancer	<0.01	2
Colorectal cancer	0.03	3

7.4. Discussion

The pellets of EVs obtained after ultracentrifugation may contain exosomes as well as other vesicles, macromolecules and protein aggregates (Vaswani *et al.*, 2017). To ensure the nature of the isolated molecules, a series of analyses have been carried out, since it is difficult to establish a clear classification of EVs due to their considerable variability and, above all, their overlapping sizes. The term "extracellular vesicles" was established to refer to all vesicles secreted into biological fluids, whereas the use of the term "exosomes" implies that certain conditions must be fulfilled (Lötvall *et al.*, 2014). Many definitions for EVs exist in the literature. Microvesicles are EVs that are secreted into the extracellular space, and on average tend to be larger in size (i.e. 100–1000 nm) compared to exosomes (30–120 nm) (Sohel, 2016).

DLS analysis confirms that the most numerous particle population had a diameter size (d. nm) ranging from 31.69 nm to 92.01 nm, which fit within the recognized sizes of the exosomes. However, the average size of the set of particles varied between 141.10 nm and 184.70 nm, demonstrating that other EVs were also isolated. Vesicles of 80 to 300 nm in diameter correspond to the microsomal fraction, including exosomal particles (Jeppesen *et al.*, 2014). Reflecting the complexity of separating the two groups using the existing techniques (Vaswani *et al.*, 2017).

After confirming the presence of exosome-sized molecules, the next step was to confirm that the isolated vesicles are exosomes is to ensure that they carry specific markers, such as CD9 and CD81 using immunoblotting. Caseins are not intrinsic components of exosomes but could be a co-isolated impurities (Sedykh *et al.*, 2020), so immunoblotting was used to confirm purity and the absence of casein. Exosomal markers were clearly observed by immunoblotting as they were enriched in the exosomal fractions. Aggregated proteins were eluted in most samples.

The exosomes concentration in the milk from grazing cows was around $1.4 \times 10^9 \pm 2.0 \times 10^8$ particles/mL, whereas in intensive farms the concentration was much lower, about $6.8 \times 10^8 \pm 1.1 \times 10^8$particles/mL. These concentrations are included in a range determined by other studies: 4.8×10^8 particles/mL (Adriano *et al.*, 2021) to 7.2×10^{11} particles/mL (Vaswani *et al.*, 2019). The concentration of milk exosomes can vary depending on the method of extraction of these molecules (Adriano *et al.*, 2021), but also depending on various physiological states (Zhang *et al.*, 2016). Mitchell, *et al.* (2016)

showed that the concentration of exosomes in the plasma was 50% greater in fertile cows compared to subfertile ones. The diet also seems to influence these concentrations in milk, thus, alfalfa hay and corn silage substitution by whole cottonseed and soybean hulls in the diet of dairy cows led to an increase in exosome concentration in cows consuming cottonseed and soybean hulls (Quan *et al.*, 2020). Suggesting that the variation in exosome concentration in this study was probably due to a change in the rumen fermentation pattern, increased feed conversion ratio and the high concentrations of minerals contained in these by-products, especially phosphorus, which is involved in the synthesis of sphingolipids, a key component of EVs biogenesis (Verderio *et al.*, 2018). Therefore, the concentration of exosomes in milk could vary according to the feeding management of the dairy cows. However, according to our sequencing results the concentration of miRNAs was higher in intensively produced milk.

We also assessed some miRNAs levels from exosomes between intensive and extensive farms. The first observation was the very low concentration of total RNA, which averaged around 4.85 ng/µL. Previous studies reported that the RNA concentration in bovine milk whey-derived exosomes were about 4.80 - 33 ng /µL (Bozack *et al.*, 2021) and 0.50 - 1 ng/µL (Quan *et al.*, 2020).

When RT-qPCR was performed, a significantly higher level of *bta-miR-451* was observed in milk exosomes in grazing cattle, which is consistent with the study by Muroya *et al.*, (2015) where the elevation of *bta-miR-451* level in plasma exosomes and biceps femoris muscle of grazing Japanese Shorthorn cattle has been demonstrated, suggesting the relation between secretion of *bta-miR-451* from skeletal muscle cells and circulation during grazing. As some miRNAs in milk may originate from blood (Alsaweed *et al.*, 2016), and given that *bta-miR-451* represented high levels in the plasma of cows with access to pasture, it could be assumed that the level of this miRNA in milk also varies with exercise during grazing.

Functional analysis revealed four validated metabolic pathways where *miR-451a* can be involved in human cells. A significantly lower expression of *hsa-miR-451* was observed in parkinson patients compared with healthy patients suggesting that low levels of this miRNA can facilitate post-transcriptional repression of their mRNA targets related to several neurodegenerative signalling pathway as Parkinson's disease (Chen *et al.*, 2016). Another study demonstrated the ability of *miR-451a* as a potential therapeutic

agent by targeting cancer cells. It was shown that this miRNA targets the tuberous sclerosis 1 (TSC1) gene which activates PI3K/Akt/mTOR signalling in cancer cells, and furthermore this miRNA enhances the synergistic effect of anti-myeloma drugs by increasing cell apoptosis, decreasing clonogenicity, and decreasing MDR1 mRNA expression (Du *et al.*, 2015). *miR-451a* acts as a tumor suppressor in papillary thyroid carcinoma (Minna *et al.*, 2016) and colorrectal cancer by targeting macrophage migration inhibitory factor (MIF) (Ma *et al.*, 2020). This miRNA is recognized for being able to represses cell proliferation and invasion (Zeng *et al.*, 2014).

This suggests a bioactive role of grazing milk with high levels of *miR-451a* and its possible ability to act on human cells, by testing the bioinformatic results in an *in-vitro* or *in-vivo* assay.

7.5. Conclusions

It seems that the concentration of exosomes in milk from extensive dairy farms was higher than milk from intensive farms.

The miRNA *bta-miR-451* in milk whey exosomes represented high levels in grazing farms compared to intensive farms.

Chapter 8

General discussion and conclusions.

Chapter 8: General discussion and conclusions.

En la Cornisa Cantábrica coexisten varios modelos de producción de leche que, si bien son diferentes, comparten determinados rasgos comunes como son, el carácter familiar de las explotaciones y la importancia de la base forrajera propia (Flores-Calvete *et al.*, 2016). Las explotaciones más pequeñas, que constituyen el 60% de las granjas, gestionan el 37% de la superficie agraria útil (SAU) y son responsables del 20% de la producción de leche de la zona norte. En estas explotaciones, el 80% de la SAU está destinada a hierba de prados y praderas, siendo el resto cultivos anuales. De ellas más del 85% de las explotaciones utilizan la hierba fresca en la alimentación de las vacas en lactación, fundamentalmente como pastoreo, y algo menos del 75% dependían del ensilado de hierba como principal forraje conservado, con un consumo de concentrado por debajo del 30% de la materia seca de la ración (Flores-Calvete *et al.*, 2016). Sin embargo, un aspecto preocupante ha sido la constatación de que estas explotaciones muestran una elevada intención de abandono, y que menos del 20% de las granjas que pretendían abandonar o cuyo titular tenía más de 55 años, tenían la sucesión asegurada. Para ayudar a impedir el abandono de estas tierras y de mantener estos ecosistemas plurianuales, surge la motivación general de esta memoria de tesis: la conservación y valorización de un sistema de producción de leche, basado en el uso de los recursos existentes en zonas con condiciones edafo-climáticas favorables para el crecimiento de hierba y forrajes, como es el caso de la Cornisa Cantábrica en general y de Asturias en particular.

Para ello, se pretende definir un sistema de certificación basado en moléculas que están presentes en la leche y que varían dependiendo de factores externos que caracterizan el sistema de producción, como la dieta, el ejercicio durante el pastoreo y el bienestar

animal. Para certificar el origen de la leche se han propuesto varias moléculas propias de la leche como marcadores, como los ácidos grasos, los antioxidantes liposolubles, y también los microARN (De la Torre, 2021; Abou el qassim 2017). La apuesta en este trabajo ha sido el estudio de las variaciones de los miARN en muestras de leche cruda de tanque de granjas comerciales ubicadas en Asturias.

Al comienzo de este trabajo, el conocimiento al respecto de miARN en leche bovina no era lo suficientemente amplio como para poder seleccionar una lista de miARN candidatos a partir de la bibliografía con el propósito de diferenciar la leche producida en base a pastos. Lo que si estaba claro en la bibliografía es que los perfiles de miARN no eran los mismos según la matriz o fracción de la leche estudiada (Li *et al.*, 2016), y se decidió que las fracciones a estudiar fueran la grasa y la de células. Se descartó estudiar el suero, por dos motivos: primero porque su contenido era similar al de la grasa (Li *et al.*, 2016) y segundo porque el suero de la leche es una fracción que se descarta durante la fabricación de algunos productos lácteos como por ejemplo el queso. Por lo tanto, en un primer intento, se exploró el miARNoma en las células y la grasa de la leche mediante la secuenciación en dos sistemas de producción de leche muy opuestos, para maximizar las posibilidades de identificar los miARN diferenciadores. Los resultados de la secuenciación no permitieron identificar miARN en la fracción grasa o celular que fueran específicos de un determinado sistema de producción. Sin embargo, si se detectó que, en la grasa de la leche de los sistemas extensivos, la cantidad total de miARN es mayor que en sistemas intensivos, lo que consideramos la primera evidencia de que el sistema de producción puede modificar la funcionalidad de los alimentos de origen animal. A partir de ese momento el trabajo se centró en los miARN cuyos niveles diferían entre los dos sistemas. A pesar de que se seleccionaron solo 3 ganaderías intensivas *vs.* 3 ganaderías extensiva para la secuenciación, y que las ganaderías del mismo grupo no forman replicas reales (son ganaderías comerciales), se pudieron identificar miARN candidatos diferenciadores entre los dos sistemas, de los cuales se seleccionaron 5 miARN en la grasa y 5 miARN en las células para su validación. Cuando validamos este subconjunto de miARN mediante RT-qPCR en una muestra de ganaderías más grande (10 *vs.* 10) de manejo opuesto, con características iguales a las ganaderías de secuenciación, se encontraron diferencias significativas entre en el *bta-miR-215* en la fracción de la grasa, con niveles más altos en intensivo. En la fracción de células en cambio, no se ha podido

validar ningún miARN. lo que atribuimos a que los miARN en esta fracción de la leche no representan el estado fisiológico real de la glándula mamaria (Krappmann *et al.*, 2012). A partir de aquí, se ha decidido seguir investigando solo el uso de los miARN en la grasa de la leche como biomarcadores del sistema de producción.

El siguiente paso fue actualizar la revisión bibliográfica sobre miARN en ganado vacuno por su relación con la alimentación, el sistema de manejo y el metabolismo (Li R. *et al.*, 2015; Muroya *et al.*, 2015; Muroya *et al.*, 2016; Wang *et al.*, 2016), lo que permitió incluir nuevos miARN en nuestro trabajo.

Ahora bien, hemos visto que los miARN varían, según algún factor intrínseco o la interacción de varios, entre dos sistemas de producción opuestos, pero lo que nos interesa es toda la variabilidad de sistemas de producción de leche partiendo teóricamente de ganaderías 100% intensivas a otras 100% extensivas, pasando por las intermedias que representan características de los dos extremos (Ruiz *et al.,* 2017; Salcedo-Díaz, 2006; Villar-Bonet & Quintana-Ruíz, 2021). Hay una multitud de factores que pueden ser útiles para la caracterización de los sistemas de producción (Marín, 1996), como por ejemplo el manejo alimentario, el aporte de hierba fresca tanto a través del pastoreo o en el pesebre, el uso de forrajes conservados y la cantidad de concentrados en la ración (Ruiz *et al.*, 2017; Vicente-Mainar *et al.,* 2013). Sin embargo, no existe una definición clara de los diferentes sistemas, incluso de los más extremos. Las ganaderías puramente extensivas serían las que se basan en el pastoreo exclusivamente, con un mínimo uso de concentrados suficiente para el funcionamiento correcto del rumen. Avanzar hacia lo intensivo significaría la introducción de forrajes conservados, más cantidades de concentrado, menos horas o ausencia de pastoreo sustituida por el consumo de hierba fresca cortada en el pesebre y combinada con otros forrajes (henos o/y silos). También implica el remplazo gradual de la hierba fresca por los silos, que puede llegar hasta un uso exclusivo de forrajes conservados, especialmente el silo de maíz (Jiménez-Calderón *et al.,* 2015) y altas cantidades de concentrados (Díaz de Otálora *et al.,* 2022). Además del manejo alimentario, factores como la densidad animal, se relaciona con ganaderías intensivas cuando es alta (Vicente Mainar *et al.,* 2013). Sin embargo, el solapamiento de los diferentes factores hace que la caracterización sea más compleja (Ruiz *et al.,* 2017), como el caso de las ganaderías de pastoreo con un manejo intensivo; densidades animales altas, consumo de silo de maíz y altas cantidades concentrados.

Es importante señalar que, en Asturias, las vacas en lactación no pastan todo el año, salvo algunas excepciones (Villar-Bonet & Quintana-Ruíz, 2021), por lo que a lo largo del año se produce una sustitución de hierba fresca por ensilado de hierba a medida que avanza la estación hacia el invierno (Flores-Calvete *et al.,* 2016). Este hecho hace que la certificación de leche de pastos no se refiera a la leche producida exclusivamente por vacas en pastoreo, que implicaría un tipo de leche de producción estacional, sino a leche de explotaciones en las que las vacas en lactación pastan durante un periodo concreto del año (Villar-Bonet & Quintana-Ruíz, 2021). Los muestreos realizados en este trabajo, independientemente del tipo de ganadería y manejo del rebaño, se llevaron siempre a cabo en primavera u otoño, periodos en los que se puede realizar el pastoreo, para evitar diferencias en el contenido de miARN debidas a la época de muestreo.

Frente a esta complejidad y la ausencia de una normativa nacional que defina claramente las diferencias entre esta línea continua que representan los diferentes sistemas de producción de leche (Ruiz *et al.,* 2017), se ha muestreado la leche en ganaderías con diferentes dietas representando esta variabilidad. Así que en capítulo 4 se ha intentado abordar la siguiente cuestión: ¿cómo varían los miARN a lo largo de la línea de intensividad/extensividad y cómo pueden los miARN diferenciar las ganaderías de pastoreo del resto? Para ello se han considerado 4 grupos (Abou el qassim, 2017): pastoreo (extensiva), pesebre, silo de hierba (intermedias), silo de maíz (intensivas). Se compraron las ganaderías de pastoreo sin considerar la dieta que hay detrás, ni las horas de pasto al día, con ganaderías que se basan en el uso de la hierba verde en pesebre, silo de hierba y silo de maíz, y se encontraron 4 miARN que mostraban diferencias significativas entre los grupos:

El *bta-miR-155* diferencia las ganaderías de pastoreo de las de silo de maíz, sin ser capaz de diferenciar las del pastoreo de las intermedias. El *bta-miR-103* diferencia las ganaderías que consumen hierba fresca, ya sea pastada o en pesebre, de las del silo de maíz, sin poder diferenciarlas de las de silo de hierba. El *bta-miR-532* diferencia las del maíz de las intermedias (pesebre y silo de hierba), y las del pasto de las de pesebre. El *bta-miR-7863* diferencia las ganaderías del pesebre de las de pastoreo y las del silo de maíz sin que se puedan diferenciar de las de silo de hierba. Los dos últimos miARN diferencian las intermedias de las de pastoreo y las de silo de maíz, pero no diferencian las de pastoreo de las de maíz. Estas diferencias pueden deberse al conjunto de factores que caracteriza cada grupo, pero también puede deberse a un factor en concreto (presencia

/ ausencia de un factor, por ejemplo, el pastoreo a través del consumo de la hierba fresca o el ejercicio, el silo maíz, y las cantidades incluidas de los concentrados, *etc.* (Li R. *et al.*, 2015; Mobuchon *et al.*, 2017; Muroya *et al.*, 2015; Muroya *et al.*, 2016).

El análisis factorial muestra que el silo de maíz y el pastoreo son los principales factores en las diferenciales que aparecen. Además, otros factores pueden entrar en juego, ya que los modelos que incluyen los diferentes ingredientes como factores logran explicar tan solo el 30% de la varianza. De esta manera se confirma la complejidad que puede existir detrás de la expresión de los miARN (Bianchi *et al.*, 2017; Friedman *et al.*, 2009), lo que constituye una de las limitaciones de su uso como biomarcadores comparando los sistemas de producción de leche que a su turno se caracterizan con una multitud de factores.

El análisis funcional de los 4 miARN identificados ha mostrado su implicación en la vía MAPK, que regula la entrada en el ciclo celular y la proliferación (Zhang *et al.*, 2002); y la función molecular de las proteínas quinasas de serina/treonina, que regulan la proliferación celular, la muerte celular programada (apoptosis), la diferenciación celular y el desarrollo embrionario (Cross *et al.*, 2000). Estos resultados sugieren que estos miARN pueden participar en la diferenciación celular en la glándula mamaria y, por tanto, regular la producción de leche. El análisis de enriquecimiento GO identificó 249 genes diana de los cuatro miARN relacionados con la actividad de transporte, lo que puede indicar su gran implicación de en el proceso de transporte de precursores de la síntesis de leche.

En este capítulo se han determinado dos miARN capaces de diferenciar las ganaderías de pastoreo de las de silo de maíz y por otra parte las ganaderías que se basan sobre el consumo de hierba fresca pastada o cortada de las ganaderías de silo de maíz.

Según una definición más concreta de las ganaderías en base a pastos, definiendo las horas de pastoreo al día, los periodos de pastoreo al año, el aporte mínimo de forrajes, silos y concentrados y la densidad animal, se podrían definir miARN capaces de segregar según los criterios definidos.

Las ganaderías de pastoreo representan una enorme variabilidad interna de dietas y manejos por ejemplo la composición botánica de los pastos, el estado fisiológico de la hierba pastada, horas de pastoreo al día, la dieta aparte de la hierba fresca pastada, la

densidad animal y el tipo de manejo ecológico o convencional. En un intento exploratorio para ver cómo pueden variar los miARN entre las ganaderías de pastoreo ecológicas y convencionales se ha mostrado que el *bta-miR-215* varía según si la ganadería es ecológica o de pastoreo convencional, siendo más abundante en las últimas.

El capítulo 6 confirmó estos resultados mostrando que las diferencias que aparecen el en *bta-miR-215* son probablemente debidas a cuantidad de pienso y el nivel de intensidad incluso cuando todas las ganaderías adoptan el pastoreo. Sugiriendo así la importancia de comprobar mediante un ensayo controlado el efecto de concentrado sobre *bta-miR-215*.

El nivel de *bta-miR-215* no se ha visto relacionado con un factor vinculado a la ganadería ecológica como es el tipo de concentrado y la composición botánica de los pastos, sino que se relacionó con factores que también podrían adoptar las explotaciones convencionales, afirmando asi que una dieta similar para las vacas alimentadas con pastos ecológicos y convencionales genera un obstáculo para comprobar la autenticidad de la leche ecológica (Schwendel *et al.*, 2017). Así pues, sugerimos que, si las explotaciones de pastoreo convencionales reducen la cantidad de concentrados en la dieta y la carga ganadera en los pastos, el *bta-miR-215* no sería útil como diferenciador para las explotaciones ecológicas.

El *bta-miR-215* aparece en el capítulo 3 como diferenciador entre dos ganaderías de manejos opuestos (18 horas en promedio de pastoreo *vs.* cero pastoreo; cero silo maíz *vs.* silo de maíz; 4-6 kg de concentrado *vs.* más de 10 kg de concentrado). Junto a los resultados del capítulo 6, esto sugiere que este miARN representa niveles diferentes en ganaderías más extremas usando el concentrado. Sin embargo, en el capítulo cuatro este miARN, no aparece como diferenciador entre el grupo del pastoreo *vs.* el grupo de silo de maíz, lo que probablemente podría ser debido a la implicación de más factores que interfieren en esa diferencia, o bien que estos dos grupos no son lo suficientemente opuestas con relación a este criterio. De ahí de importancia de definir bien el grupo que se necesita diferenciar y determinar los miARN que son capaces de diferenciarlo.

Los miARN siempre se han descrito como resistentes a las condiciones adversas como el bajo pH, las RNAasas y las variaciónes de temperatura (Izumi *et al.*, 2012), gracias a la protección proporcionada por las vesículas extracelulares (VEs) (Munch *et*

al., 2013; Zhou *et al.*, 2012; Izumi *et al.*, 2014). No obstante, algunos procesos tecnológicos afectan a los miARN probablemente porque esos procesos rompen las VEs. Excepto la pasteurización de la leche, todos los procesos estudiados reducen los niveles de miARN considerando la misma cuantidad de leche inicial utilizada para fabricar el producto. Sin embargo, durante el proceso de fabricación del queso, una gran proporción de miARN se pierde, pero en la misma cantidad de queso y leche aparecen niveles similares de miARN, sugiriendo que el proceso de fabricación de queso concentra el contenido de miARN.

El perfil de miARN en la leche difiere entre las fracciones de grasa, suero y células (Li *et al.*, 2016). En los capítulos anteriores se han explorado los perfiles y niveles de los miARN en las células y la grasa, pero no en el suero porque su contenido en miARN es menor, sus perfiles de miARN son muy similar al de la grasa de la leche (Li *et al.*, 2016), además que no se aprovecha en ciertas prácticas de la industria láctea, como la producción de queso. Sin embargo, otro estudio mostró que la mayoría de los miARN presentes en el suero de leche cruda bovina se expresaron a niveles significativamente más altos en los exosomas que en otras fracciones de la leche (Izumi *et al.*, 2015). Además, el contenido de los exosomas en miARN traduce estados fisiológicos correlativos a los del organismo, lo que hace de ellos buenos biomarcadores (Li *et al.*, 2022; Mori *et al.*, 2019)

En el capítulo 7 se ha abordado el tema de exosomas de la leche de vaca por su importancia tanto funcional para los humanos (Benmoussa *et al.*, 2020; Reif *et al.*, 2019; Samuel *et al.*, 2017). o de posible uso como biomarcador (Mori *et al.*, 2019).

Mediante la metodología de Vaswani *et al.* (2017) ha sido posible aislar los exosomas que se ha confirmado mediante su caracterizacion. Igualmente, se ha observado que la concentración de los exosomas en la leche de las ganaderías extensivas es superior que la leche de las ganaderías intensivas, sugiriendo mayor aportación de miARN por la leche en base a pastos. Se ha estudiado también el contenido en algunos miARN en estos exosomas, donde se revelo miARN *bta-miR-451* en los exosomas de la leche con altos niveles en las ganaderías de pastoreo comparados con las intensivas. El análisis bio-informático del homólogo de este miARN en humano ha mostrado su implicación en 4 rutas metabólicas enfermedad de Parkinson, vía de señalización mTOR, cáncer de tiroides y cáncer colorrectal, donde se ha observado su papel de facilitar la represión post-transcripcional de sus objetivos de ARNm relacionados con varias enfermedades

neurodegenerativo (Chen *et al.*, 2016), y diferentes tipos de cáncer (Du *et al.*, 2015; Minna *et al.*, 2016; Ma *et al.*, 2020), por su capacidad de reprimir la proliferación y la invasión celular reconocida (Zeng *et al.*, 2014). Lo que evoca la importancia de realizar un estudio de funcionalidad de estos exosomas en vitro o en vivo para comprobar la bioactividad de la leche del pastoreo. Hoy en día, existen dos hipótesis enfrentadas: la hipótesis funcional, según la cual los miARN de la leche se transfieren a otros organismos y ejercen funciones reguladoras fisiológicas, y la hipótesis nutricional, que propone se limitan a proporcionar nutrición sin conferir señales reguladoras activas al organismo receptor (Mar-Aguilar *et al.*, 2020; Melnik *et al.*, 2016; Zempleni *et al.*, 2015).

Muchos trabajos proponen el uso de los miARN contenidos en los exosomas como biomarcadores por varias ventajas citadas (Izumi *et al.*, 2015; Li *et al.*, 2022; Mori *et al.*, 2019). Según los resultados expuestos se podría confirmar que los niveles de los miARN difieren según la fracción de donde provienen, ya *bta-miR-451* varía entre los dos grupos de ganaderías solo en el suero lo que podría proponer su origen sanguíneo considerando también el resultado del estudio Muroya *et al.*, (2015). Al analizar el nivel de este miARN en la grasa no se han vistos diferencias significativas. En nuestro estudio el uso de los miARN como herramienta para la trazabilidad de la leche en base a pastos requiere ser una rápida ya que se aplicaría a muchas ganaderías periódicamente. El uso de los miARN en los exosomas requiere varias etapas previas para caracterizar estas vesículas, aumentando así el tiempo y los costes de la herramienta.

Según el análisis de las dietas realizado en el capítulo 4 en comparación con un estudio anterior, se puede confirmar que la tendencia en las ganaderías de leche en Asturias va hacia más intensividad. confirmando la necesidad de valorar las ganaderías de pastoreo en la zona creando una marca de calidad permitiendo diferenciar ese producto, destacar sus ventajas y ayudar a mantener su existencia (Villar-Bonet & Quintana-Ruíz, 2021).

La siguiente etapa para realizar eso, sería la determinación formal y regulada de los factores característicos de las ganaderías de pastoreo que cumplen los requisitos de sostenibilidad. Se podría determinar miARN en la grasa de leche que varían según estos factores en la fase de grasa y que sean capaces de segregar las ganaderías de interés del resto. En la fase de validación de esta herramienta estaría bien relacionar los niveles de miARN a otros indicadores ya estudiados y fáciles de medir cuyos niveles indican

fácilmente el nivel fisiológico de lo que se requiere medir mediante los miARN. Por ejemplo, se sabe que el consumo de hierba fresca se ha relacionado con perfiles determinados de grasa en la leche, entonces en la fase de validación se podría tomar estos niveles en cuenta (Alothman *et al.*, 2019; De la Torre-Santos *et al.*, 2020)

Una vez determinados los miARN relacionados con los diferentes factores de caracterización de los sistemas de producción de leche basándose sobre la cuantificación de miARN mediante sondas de nanopartículas de oro (Yang *et al.*, 2008) Es una herramienta que permite una determinación sensible, específica y cuantificación absoluta de miARN que además no requiere normalización. Esta técnica consiste en la hibridación del miARN diana con la sonda de captura y la sonda de nanopartículas de oro y así quedan inmovilizados en la superficie de una microplaca, lo que implica la amplifica de la señal mediante una fluorescencia que es proporcional a los niveles de un miARN dado (Yang *et al.*, 2008).

La herramienta descrita presenta las siguientes limitaciones:

- ✓ La complejidad de los factores que podrían ser implicados en la expresión de los miARN frente a la multitud de factores implicados en un sistema de producción de leche
- ✓ No se sabe cuánto tiempo se requiere para el cambio de los niveles de miARN durante la transición de un sistema de gestión a otro.

Conclusiones

A partir de este trabajo concluimos que:

1. El sistema de producción de leche modifica el contenido total de miARN en la grasa de la leche cruda de vaca, siendo mayor en sistemas extensivos.
2. Se han identificado tres miARN cuyos niveles varían según el tipo de sistema de explotación de las vacas en lactación:
    a. *bta-mir-215*: cuyo nivel aumentan a mayor intensificación del sistema.
    b. *bta-miR-103*: cuyo nivel es mayor en las ganaderías que incluyen hierba fresca, tanto pastada como en pesebre.

    c. *bta-miR-155*: cuyo nivel es mayor en ganaderías donde los animales pastan.
3. La concentración de los exosomas en la leche cruda de las ganaderías de pastoreo es superior que en la leche de las ganaderías intensivas.
4. El contenido de *bta-miR-451* en los exosomas de la leche cruda es mayor en las ganaderías de pastoreo comparadas con las intensivas.
5. El sistema de producción influye en los niveles de exosomas y miARN, y por tanto potencialmente en las propiedades funcionales, de la leche bovina.

Conclusions (english)

From this work we conclude that:

1. The milk production system modifies the total miRNA content in raw cow's milk fat, being higher in extensive systems.
2. Three miRNAs have been identified whose levels vary according to the type of farming system of the lactating cows:
    a. *bta-mir-215*: whose level increases with greater intensification of the system.
    b. *bta-miR-103*: whose level is higher in farms that include fresh grass, both grazed or harvested and consumed indoors.
    c. *bta-miR-155*: whose level is higher in farms where animals graze.
3. The concentration of exosomes in raw milk from grazing farms is higher than in milk from intensive farms.
4. The content of *bta-miR-451* in raw milk exosomes is higher in grazing farms compared to intensive ones.
5. The production system influences the exosome and miRNA levels, and thus potentially the functional properties, of bovine milk.

Chapter 9

Bibliography

Chapter 9: Bibliography

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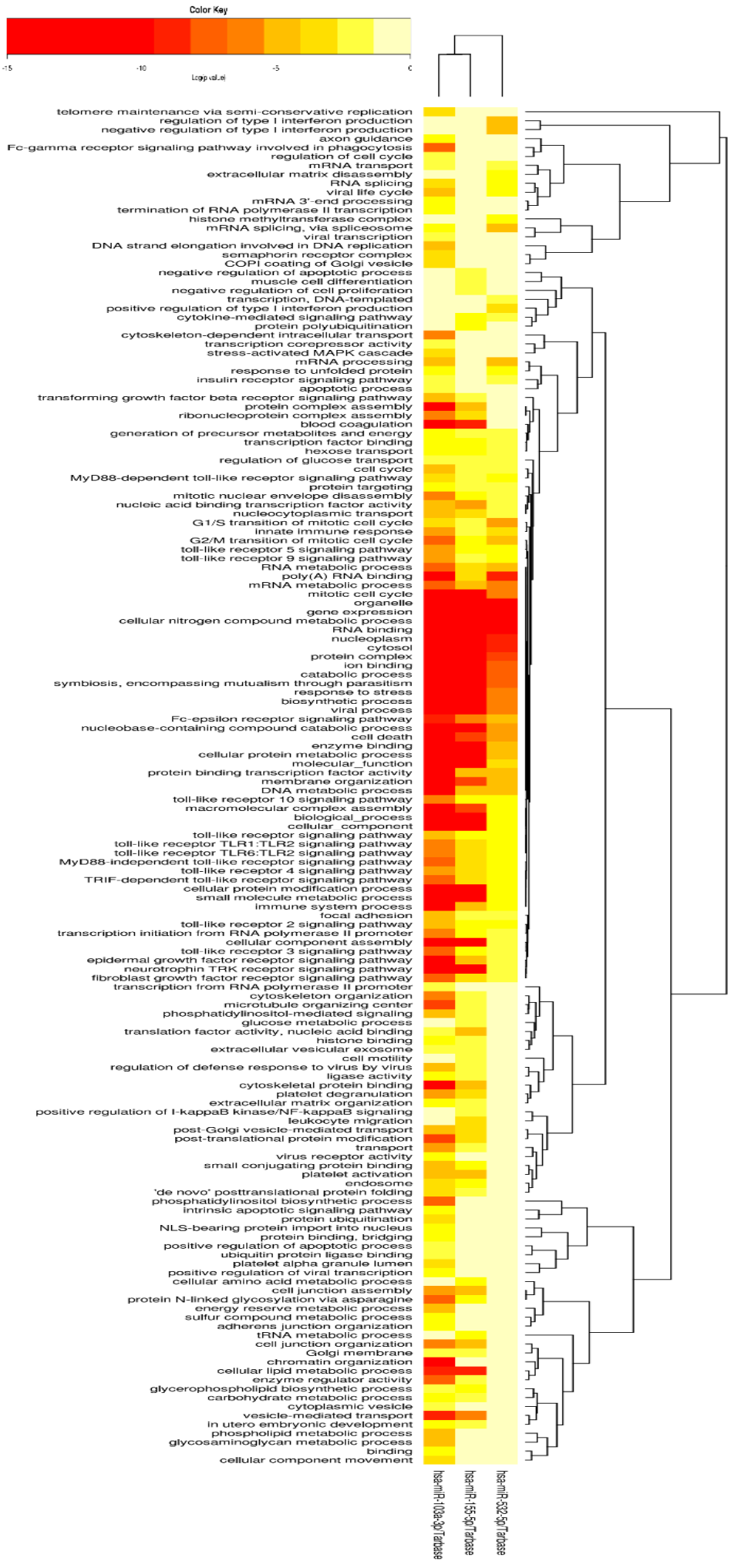
Chapter 10

Annexes

Chapter 10: Annexes

Appendix 1. Heatmap of hierarchical clustering of *miR-103, miR-155* and *miR-532* based on mRNA target pathways, identified in DIANA using the Tarbase and GO intersection representation. Darker colors represent lower p-values.


Color Key
-15
-10
-5
0
Log p value
telomere maintenance via semi-conservative replication
regulation of type I interferon production
negative regulation of type I interferon production
axon guidance
Fc-gamma receptor signaling pathway involved in phagocytosis
regulation of cell cycle
mRNA transport
extracellular matrix disassembly
RNA splicing
viral life cycle
mRNA 3'-end processing
termination of RNA polymerase II transcription
histone methyltransferase complex
mRNA splicing, via spliceosome
viral transcription
DNA strand elongation involved in DNA replication
semaphorin receptor complex
COPI coating of Golgi vesicle
negative regulation of apoptotic process
muscle cell differentiation
negative regulation of cell proliferation
transcription, DNA-templated
positive regulation of type I interferon production
cytokine-mediated signaling pathway
protein polyubiquitination
cytoskeleton-dependent intracellular transport
transcription corepressor activity
stress-activated MAPK cascade
mRNA processing
response to unfolded protein
insulin receptor signaling pathway
apoptotic process
transforming growth factor beta receptor signaling pathway
protein complex assembly
ribonucleoprotein complex assembly
blood coagulation
generation of precursor metabolites and energy
transcription factor binding
hexose transport
regulation of glucose transport
cell cycle
MyD88-dependent toll-like receptor signaling pathway
protein targeting
mitotic nuclear envelope disassembly
nucleic acid binding transcription factor activity
nucleocytoplasmic transport
G1/S transition of mitotic cell cycle
innate immune response
G2/M transition of mitotic cell cycle
toll-like receptor 5 signaling pathway
toll-like receptor 9 signaling pathway
RNA metabolic process
poly(A) RNA binding
mRNA metabolic process
mitotic cell cycle
organelle
gene expression
cellular nitrogen compound metabolic process
RNA binding
nucleoplasm
cytosol
protein complex
ion binding
catabolic process
symbiosis, encompassing mutualism through parasitism
response to stress
biosynthetic process
viral process
Fc-epsilon receptor signaling pathway
nucleobase-containing compound catabolic process
cell death
enzyme binding
cellular protein metabolic process
molecular_function
protein binding transcription factor activity
membrane organization
DNA metabolic process
toll-like receptor 10 signaling pathway
macromolecular complex assembly
biological_process
cellular_component
toll-like receptor signaling pathway
toll-like receptor TLR1:TLR2 signaling pathway
toll-like receptor TLR6:TLR2 signaling pathway
MyD88-independent toll-like receptor signaling pathway
toll-like receptor 4 signaling pathway
TRIF-dependent toll-like receptor signaling pathway
cellular protein modification process
small molecule metabolic process
immune system process
focal adhesion
toll-like receptor 2 signaling pathway
transcription initiation from RNA polymerase II promoter
cellular component assembly
toll-like receptor 3 signaling pathway
epidermal growth factor receptor signaling pathway
neurotrophin TRK receptor signaling pathway
fibroblast growth factor receptor signaling pathway
transcription from RNA polymerase II promoter
cytoskeleton organization
microtubule organizing center
phosphatidylinositol-mediated signaling
glucose metabolic process
translation factor activity, nucleic acid binding
histone binding
extracellular vesicular exosome
cell motility
regulation of defense response to virus by virus
ligase activity
cytoskeletal protein binding
platelet degranulation
extracellular matrix organization
positive regulation of I-kappaB kinase/NF-kappaB signaling
leukocyte migration
post-Golgi vesicle-mediated transport
post-translational protein modification
transport
virus receptor activity
small conjugating protein binding
platelet activation
endosome
'de novo' posttranslational protein folding
phosphatidylinositol biosynthetic process
intrinsic apoptotic signaling pathway
protein ubiquitination
NLS-bearing protein import into nucleus
protein binding, bridging
positive regulation of apoptotic process
ubiquitin protein ligase binding
platelet alpha granule lumen
positive regulation of viral transcription
cellular amino acid metabolic process
cell junction assembly
protein N-linked glycosylation via asparagine
energy reserve metabolic process
sulfur compound metabolic process
adherens junction organization
tRNA metabolic process
cell junction organization
Golgi membrane
chromatin organization
cellular lipid metabolic process
enzyme regulator activity
glycerophospholipid biosynthetic process
carbohydrate metabolic process
cytoplasmic vesicle
vesicle-mediated transport
in utero embryonic development
phospholipid metabolic process
glycosaminoglycan metabolic process
binding
cellular component movement
hsa-miR-103a-3p Tarbase
hsa-miR-155-5p Tarbase
hsa-miR-532-5p Tarbase

Appendix 2. Plant species identified in the studied pastures

Plant species	**Abbreviation**
Achillea millefolium	Am
Agrostis sp.	Ag
Anagallis arvensis	Aa
Anthoxanthum odoratum	Ao
Bellis perennis	Bp
Briza media	Bm
Bromus commutatus	Bc
Bromus sp.	Br
Carex divulsa	Cx
Centaurea nigra	Cn
Cerastium glomeratum	Cg
Chamaemelum nobile	Ch
Convolvulus arvensis	Co
Crepis sp.	Cr
Cynodon dactylon	Cd
Cynosurus cristatus	Cc
Dactylis glomerata	Dg
Daucus carota	Dc
Festuca sp.	Fe
Galium sp.	Ga
Geranium dissectum	Gd
Holcus lanatus	Hl
Hypochaeris radicata	Hr
Lolium multiflorum	Lm
Lolium perenne	Lp
Lotus corniculatus	Lc
Medicago sativa	Ms
Mentha suaveolens	Me
Oenanthe crocata	Oc
Oxalis sp.	Ox

Pilosella officinarum	Po
Plantago lanceolata	Pl
Plantago major	Pm
Poa annua	Pa
Poa trivialis	Pt
Potentilla reptans	Pr
Prunella vulgaris	Pv
Ranunculus bulbosus	Rb
Rumex sp.	Rx
Senecio vulgaris	Sv
Stellaria graminea	Sg
Stellaria media	Sm
Taraxacum officinale	Tx
Trifolium pratense	Tp
Trifolium repens	Tr
Urtica dioica	Ud
Veronica chamaedrys	Vc
Veronica persica	Vp
Veronica serpyllifolia	Vs
Veronica sp.	Ve
Vicia sp.	Vi

Appendix 3. Communication On the effect of pasteurisation of milk on its miRNAs, presented at the 72nd EAAP congress in Davos.

Book of Abstracts of the 72nd Annual Meeting of the European Federation of Animal Science

Book of abstracts No. 27 (2021)
Davos, Switzerland
30 August – 3 September 2021

Poster Session 74

The effect of pasteurization in the expression of bovine milk microRNA

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The edaphoclimatic conditions in Asturias (northwest Spain) enable the presence of milk production systems oscillating between farms based on grazing and low consumption of conserved feed and forages as well as a low density of animals per surface, and intensive farms with confined animals, with a higher density, and higher consumption of conserved feed and forages. Identifying milk according to its origin is a useful way to highlight the positive effects of grazing farms on the environment, milk quality, animal welfare, and social aspects. MicroRNAs (miRNAs) are 21-25-nucleotide small RNAs that perform various functions within cells, including gene expression regulation. They can be profiled through microarray, quantitative real-time Polymerase Chain Reaction (RT-qPCR), or sequencing. Their expressions vary according to the genetic context and external factors to the animal (feeding, handling, etc.). Besides, these molecules are resistant to adverse physicochemical conditions, which make them potential biomarkers. In previous studies, a set of miRNAs from raw milk with differential expression according to some ingredients of the diet were determined. However, it is important to evaluate the effect of technological treatments, in milk factories, (pasteurization, fermentation, sterilization, etc.) on these miRNAs. Furthermore, taking into account the potential bioactivity of milk according to its miRNAs content, it is important to assess miRNAs milk content intended for human consumption. In the present study, we analysed the effect of pasteurization on the miRNAs determined as biomarkers. For that purpose, the expression of nine miRNAs was analysed in the same volume of twenty tank milk samples before and after pasteurization. The expression analysis of the different biomarkers in pasteurized and non-pasteurized milk showed that the expression of most miRNAs was lower after pasteurization, although without significant differences ($P<0.05$). Moreover, pasteurization of raw milk affects differently fat and cell fraction. These results suggest that miRNAs are quite resistant to pasteurization.

The effect of pasteurization in the expression of bovine milk microRNA.

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Introduction

The edaphoclimatic conditions in Asturias (northwest Spain) enable the presence of different milk production systems, oscillating between intensive and extensive farms. Both have different intrinsic characteristics: the breeds, the feeding management as well as animal density, etc. Mixed systems share characteristics with extensive systems, for adopting grazing at a period of the year, and with the intensive, for housing the livestock the rest of the year. Likewise, each of these systems requires different productive factors (land, capital, labor), which results in differences in the quantity and quality of the products (Marín, 1996). Collective awareness of the consumption of local products with less negative effect on the environment, human health and animal welfare, implies that grazing is being promoted over cattle housed indoors, so mechanisms of authentication are required to ensure consumers the quality of the milk they purchase. The use of miRNAs as biomarkers was attempted in different areas, the determination of diseases (Lin *et al.*, 2017), the detection of early pregnancy in cattle (Schanzenbach *et al.*, 2017), the characterization of miRNA related to heat stress in dairy cattle (Li *et al.*, 2018) or related to mastitis Holstein cattle (Li *et al.*, 2014).

MiRNAs (miRNAs) are small non-coding RNAs of 21–25-nucleotide, they have a central role in gene regulation in eukaryotes. Its expression varies according to its genetic context and external factors to the animal (feeding, handling) (He & Hannon, 2004). Actually, miRNA fits with most criteria of a good biomarker for being conserved among different species, stable in various body fluids, its expression level can be easily assessed by various methods (Etheridge *et al.*, 2011). The regulatory role of miRNAs on gene expression results in the repression of protein synthesis, due to its complementarity with messenger RNA. Beyond the functions they exert in the cells that produce them, miRNAs can also be transferred to other cells, or other species, in protein complexes or through extracellular vesicles. (Zhang et *al.*, 2015; Zhou et *al.*, 2017). Zhang et al., (2012) show

that humans and mice can absorb miR-168a miRNA from rice and that this miRNA alters the expression of a protein when consumed in the diet. There is also persuasive evidence that human use miRNAs from cow's milk in genetic regulation (Zempleni et *al.* 2015) highlighting the bioactive characteristic of milk.

In previous studies, a set of miRNAs from raw milk with differential expression according to some ingredients of the diet were determined (Abou el qassim, 2017; Abou el qassim et *al.,* 2018). Taking into account that the majority of milk is processed (pasteurization, fermentation, sterilization) before the consumption, it is important to assess the effect of processing on miRNA milk cargo, for two reasons: 1) in order to avoid the possible bias on miRNAs profile by the effect of technological treatments. 2) to characterize miRNAs in treated milk, and assess its content intended for human consumption as a bioactive food. So that, this work focuses on determining the effect of pasteurization on milk miRNAs.

Material and methods

Ten samples were collected from commercial tank milk before and after pasteurization. Once in the lab, 50ml of milk were centrifuged at 4 ° C for 20 minutes at 4200xg, then milk cells and fat phase were isolated to be analyzed separately, because both fractions contain different cargos (Li et *al.,* 2016). Total RNA was isolated from 20 milk fat and 20 cell samples, using the mirVana miRNA Isolation Kit following the manufacturer's instructions (LifeTechnologies). Using the TaqMan Advanced miRNA cDNA Synthesis Kit (LifeTechnolgies) the cDNA was synthesized. miRNAs expression levels were quantified by quantitative real-time Polymerase Chain Reaction (RT-qPCR) (TaqMan Advanced miRNA Assays; ThermoFisher Scientific) in a StepOne thermocycler (LifeTechnologies) using the following primers: bta-mir-30, bta-mir-21, bta-mir-148, bta-mir-29 bta-mir-451, bta-mir-151, bta-mir-215, bta-mir-155 and bta-mir-7863 (Abou el qassim et *al.,* 2017). The quantification with (RT-qPCR) is highly sensitive and specific, however, during the experimental steps, technical variables that hide the true biological response can be introduced (Faraldi et *al.,* 2019), so, different normalization strategies are commonly used. In this study exogenous normalization was setting up, by spike-in 3 f mol of the Cel-mir-54 (from the organism *C. elegans)*. This was added during RNA extraction to assess for variations from this step. Differential expression of miRNAs was analyzed using the software Qbase+ (Biogazelle) and significant differences between the groups was established using T-test statistical for paired data at $p < 0,05$.

Results and discussion

The expression analysis of the different biomarkers in pasteurized and non-pasteurized milk, showed that the expression of most miRNAs is lower after pasteurization, although without significant differences (p <0.05) (Table 1). Izumi et *al.*, (2012) showed that miRNAs are stable under degradative conditions such as low pH or treatment with RNase for miRNA encapsulation in extracellular vesicles. However, a study of Howard et *al.* (2015) about the effect of pasteurization on whole raw milk shows a loss of about 60% in processed milk(n=3) for two miRNAs (bta-mir200c and bta-mir29). When we analyses our results using t-test for independent sample (used in Howard study) we get significant differences in some miRNA in the cellular fraction. T-test for paired data help to reduce aleatory effects.

Table1: Comparison of means between raw and pasteurized milk in cells and fat, by t-test for paired data at p <0.05.

	Bta-miR-148	*Bta-miR-21*	*Bta-miR-29*	*Bta-miR-30*	*Bta-miR-155*	*Bta-miR-215*	*Bta-miR-451*	*Bta-miR-7863*	*Bta-miR-151*
Cells	0,148	0,070	0,092	0,342	0,103	0,198	0,192	0,192	0,453
Fat	0,447	0,483	0,465	0,468	0,378	0,276	0,371	0,366	0,357

Pasteurization of raw milk affect differently fat and cells fraction (Figure 1), thus the percentage of miRNA lost after pasteurization does not exceed 11% in the fat, however the percentage of miRNA lost in cells is much higher. It has been shown that a large proportion of miRNA in milk are contained in extracellular vesicles, providing protection against degradation (Zhou et *al.*, 2012). Also, it is known that milk fat globules are secreted by mammary epithelial cells through a mechanism of exocytosis (Huston y Patton, 1990). That constitutes a second layer of protection (Lago-Novais et *al.*,2016).

As a conclusion we can say that pasteurization does not critically affect miRNA cargo, and that miRNA linked to milk fat seems to be more protected from degradation than those from cellular fraction, although the putative functionality of these miRNAs needs to be tested.

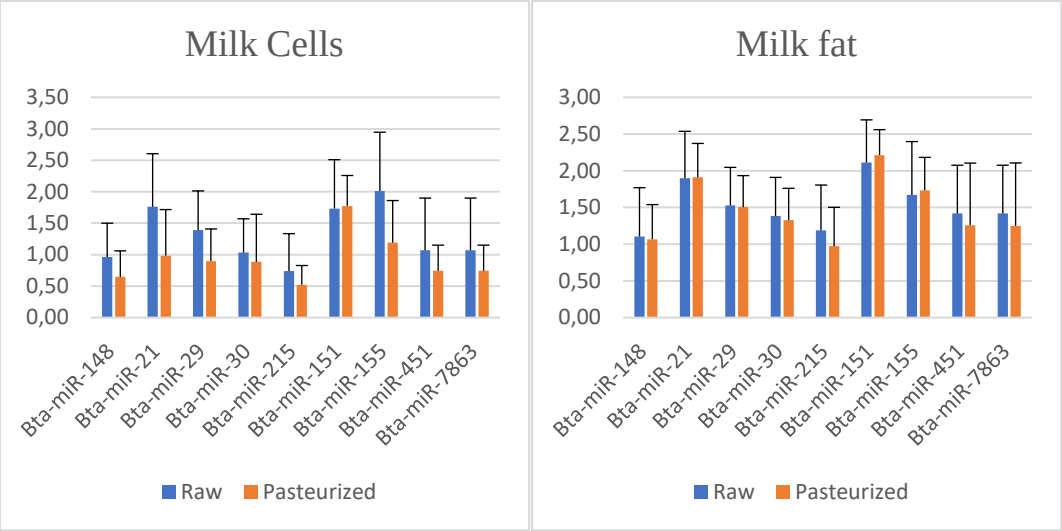

Figure 1: Means and standard deviations of miRNA loss after milk pasteurization in fat and cells milk fractions (n=10 replicates)

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Chapter 11

Scientific communications

Chapter 11: Scientific communications

Publications in peer-reviewed impact journals:

Veterinary Sciences (2022), 9 (12), https://doi.org/10.3390/vetsci9120661

Differences in the microRNAs levels of raw milk from dairy cattle raised under extensive or intensive production systems

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Abstract: Studying microRNA (miRNAs) in certain agri-food products is attractive because (1) they have potential as biomarkers that may allow traceability and authentication of such products; and (2) they may reveal insights into the products' functional potential. The present study evaluated differences in miRNAs levels in fat and cellular fractions of tank milk collected from commercial farms which employ extensive or intensive dairy production systems. We first sequenced miRNAs in three milk samples from each production system, and then validated miRNAs whose levels in the cellular and fat fraction differed significantly between the two production systems. To accomplish this, we used quantitative PCR with both fractions of tank milk samples from another 20 commercial farms. Differences in miRNAs were identified in fat fractions: overall levels of miRNAs, and, specifically, the levels of bta-mir-215, were higher in intensive systems

than in extensive systems. Bovine mRNA targets for bta-miR-215 and their pathway analysis were performed. While the causes of these miRNAs differences remain to be elucidated, our results suggest that the type of production system could affect miRNAs levels and potential functionality of agri-food products of animal origin.

Keywords: milk; miRNA; dairy production systems; biomarker

International Journal of Molecular Science (2022), 23 (19) https://doi.org/10.3390/ijms231911681

Variation of miRNA content in cow raw milk depending on the dairy production system

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Abstract: Pasture-based milk presents several advantages over milk from intensive industrial farming in terms of human health, the environment, animal welfare, and social aspects. This highlights the need for reliable methods to differentiate milk according to its origin on the market. Here we explored whether miRNA profiles could serve as a marker of milk production systems. We compared levels of previously described miRNAs in milk from four production systems (altogether 112 milk samples): grazing, zero-grazing, grass silage or corn silage. Total RNA was extracted from the fat phase, and miRNAs levels were quantified by real-time quantitative PCR. The levels of the miRNAs bta-miR-155 and bta-miR-103 were higher in the grazing system than in corn silage farms. The levels of bta-miR-532, bta-miR-103 and bta-miR-7863 showed differences between different farm managements. The miRNAs bta-miR-155 and bta-miR-103 were predicted to participate in common functions related to fat metabolism and fatty acid elongation. All four differentially expressed miRNAs were predicted to participate in transport, cell differentiation, and metabolism. These results suggest that the dairy production system influences the levels of some miRNAs in milk fat, and that bta-miR-155 and bta-miR-103 may be potential biomarkers to identify milk from pas-ture-managed systems.

Keywords: milk; microRNA; biomarker; dairy production systems.

Communications to conferences

Abou el qassim, L., Alonso, J., & Royo, L. J. (2022). **Grazing farms differentiation through the expression of microARNs and AI algorithm.** *Grassland Science in Europe*. 29th General meeting of european grassland federation (26-30 Junio Caen 2022). Oral presentation

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Abou el qassim, L., & Royo, L. J. (2020). La leche puede contar la vida de la vaca. *La Nueva España*.

Master's thesis/ bachelor's degree

Co-supervisor of a Master's thesis with Luis J. Royo, in the studies of the Master's Degree in Food Biotechnology at the University of Oviedo in the academic year 2021-2022, with the title: **Diferencias en el contenido de microARN en leche de vaca entre el ordeño de mañana y el de tarde**.

Co-supervisor of a degree project with Luis J. Royo, in the studies of Biology for the 2022-2023 academic year, with the title: **Variaciones en el contenido de miRNA en leches comerciales**

Scientific diffusion conferences

Semana de la Ciencia en el SERIDA, noviembre 2021, charla "**La PCR que ye eso**"