Protein Content of Milk Extracellular Vesicles

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Proteins represent one of the main components of milk, with high heterogeneity in terms of the number of protein species that can result from alternative splicing, single point mutations, and different post-translational modifications (PTMs).

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1. Introduction

The human and animal milk proteomes have been analyzed in order to better understand the nutritional value of this important food across the species. In particular, the analysis of different milk fractions evidenced specific protein profiles with various abundant proteins and some other proteins less represented but with very important functions [1]. Indeed, in addition to their nutritional value, milk proteins can have enzymatic and hormonal activity, as well as immunomodulatory effects [2]. Moreover, the interest in farm animal milk is also due to its use in human diet and to the economic value of the dairy product industry. For these reasons and to define the molecular processes related to lactation and the possible changes due to mammary infection, the proteome of the different milk fractions including MFG, skim milk, and milk whey of humans and farm animals has been extensively characterized [1][3][4][5]. The complexity of milk proteome, in part due to the plethora of PTMs (phosphorylation, acetylation, glycosylation, and lipid conjugation) that differently affect the structural and biological properties of proteins, has been related to its digestibility and allergenicity [6]. In contrast, few studies have analyzed the protein cargo of milk exosomes or, in general, of milk EVs, even if the presence of membrane-delimitated particles distinct from MFG were evidenced in skim milk in the late 1970s and later confirmed [7][8][9].

Like other milk fractions, human and bovine milk EVs have been extensively characterized, while data from other animal species are still limited. In addition, different proteomic approaches and different quantitative mass spectrometry (MS) methods have been used, and a large part of information has been obtained by comparing EV proteomic results with specific database such as Vesiclepedia, EVpedia, and ExoCarta or with protein milk databases.

The paucity of studies about the proteomic analysis of milk EVs is surprising as proteins can be part of their bioactive components and can contribute to the various properties of EVs cited above. Indeed, the immunomodulatory function of milk EVs in the development of the infant's immune system has been proposed in 2007 by Admyre and co-workers [10], who analyzed for the first time the proteome of the exosomes isolated from

human breast milk and colostrum. In these vesicles, high levels of mucin-1, MHC di class II, and tetraspanins CD63 and CD81 were found, whereas some typical protein markers of exosomes including MHC class I, CD54, CD40, CD80, and CD86 were scarcely represented or not detectable. MS analysis evidenced also CD36, polymeric-Ig receptor precursor, immunoglobulins, as well as cytosolic proteins and enzymes. Interestingly, the presence of proteins involved in vesicle budding and endocytosis was also evidenced such as ADP-ribosylation factor and testilin [10]. Moreover, the presence of typical milk proteins including lactadherin, butyrophilin, and xanthine oxidase, that had never been detected in EVs derived from other biofluids or secreted by other cell types, was first assessed in both human [10] and bovine milk exosomes [11]. These data were confirmed by other studies using different methods [12][13]. However, Reinhard and co-workers found that the abundance of these proteins in bovine milk exosomes was lower than in MFG (15–30%-fold less) [11]. On the contrary, milk bovine exosome resulted to be enriched in low abundant milk proteins that are under-represented and/or missing in MFG. This first proteomic analysis of the milk bovine exosomes also evidenced a high enrichment in both Rab proteins and annexins that play key roles in vesicle fusion and trafficking events. Moreover, KEGG pathway analysis includes the proteins specifically identified in bovine exosome in endocytosis, regulation of actin cytoskeleton, and tight junction, highlighting their involvement in milk secretion and exosome formation [11].

Notably, Reinhardt and co-workers identified more than 2000 protein species in bovine exosome [11]. Further studies, using different approaches, confirmed that EVs are the milk fractions with the highest protein contents [13] [14][15]. In particular, a total of 1963 proteins were identified in human milk EVs, of which 198 seem to be specific of milk EVs, because they resulted to be not present in the EV database Vesiclepedia [14]. Moreover, by comparing the proteins detected in milk EVs with proteome of other milk fractions (e.g., whole milk, skim milk, whey milk, MFG, and casein fraction), reported in previously published studies, 1330 proteins resulted to be common [14]. Proteins identified as exclusive of milk EVs resulted to be linked to cell signaling, as well as cell growth and cell maintenance [14]. In another study, the differentially abundant proteins in milk exosome compared to other milk fractions were also associated to ribosome and regulation of actin cytoskeleton [16]. Moreover, GO annotation confirmed the different cell sources of milk EVs as their proteins were associated to breast, mammary gland, and mammary epithelium, as well as to dendritic cell, CD4 T cell, platelet, monocyte, and B cell [14]. The contribution of bovine mammary epithelial cells (BMECs) as a source of milk EVs was confirmed by Zang and co-workers, who compared the proteome of these cells with the protein database of milk exosomes [17]. Briefly, 77 proteins found in both milk EVs and BMECs resulted to be mainly involved in signaling pathways associated with milk biosynthesis and cell proliferation, according to KEGG pathway annotation.

The overlapping between proteome of exosome and other milk fractions was analyzed in silico [13]. By aggregating the proteins reported in 20 proteomic studies, a protein atlas of milk proteins was generated including more than 4500 protein species. Among them, 3139 proteins resulted to be specifically present only in the exosomes and 95 proteins were common in all milk fractions [13]. Milk EVs are usually isolated from skim milk; therefore, as expected, some proteins must be shared among these two fractions. In contrast, MGF contamination in milk EVs could be limited or excluded as they are obtained from the hydrophobic and hydrophilic fractions of whole milk, respectively. These two phases are usually easily separated in the first step of every protocol used for milk fraction preparations.

The protein cargo differs also between EV subtypes, isolated from commercial skimmed, filtered, and pasteurized cow's milk, by differential centrifugation. In particular, the proteome of fraction 35K (pelleted at 35,000× *g*) and fraction 100 K (pelleted at 100,000× *g*) was compared [18]. The 20 proteins specifically associated with the 35K fraction resulted to be involved in the regulation of translation, proliferation, and cell survival, whereas 40 proteins identified only in the 100 K fraction were related to metabolism, extracellular matrix turnover, and immunity. In particular, five proteins were highly represented in the 35 K fraction (epidermal growth factor receptor substrate 15, phosphoglycerate dehydrogenase, dynactin subunit 2, protein kinase camp-dependent type regulatory subunit beta, and glutaredoxin-3) and five proteins were specifically identified in the 100 K pellet (complement c8 beta chain, c1galt1-specific chaperone 1, cartilage-associated protein, alpha-mannosidase 2×, and procollagen-lysine 2-oxoglutarate 5-dioxygenase 3). Considering that these proteins discriminating EV subtype have different cellular localization, the authors speculated that the EVs present in the two fractions could be derived from different cellular biogenesis [18]. Furthermore, the glycoproteome of bovine milk exosome has also been characterized [19]. A total of 86 glycoproteins were found to be differentially glycosylated in bovine milk exosome and milk whey. In particular, the fucosylated and sialylated proteins were the most abundant in milk exosomes. Bioinformatic analysis evidenced that milk exosome glycoproteins were mainly involved in immune-related pathways, as well as in signal transduction and cell adhesion [19]. Glycoproteins can be important for the cell crosstalk and drug delivery applications, as it has been reported that the different glycoproteome of EV surface can either assist or inhibit their internalization [20].

Among the less-investigated animal species, horse milk exosomes were analyzed by using a 2D electrophoresis-based approach [21]. Few protein spots were evidenced that corresponded to different serum protein species, i.e., albumin, lactoferrin, lactadherin, beta-lactoglobulin, xanthine dehydrogenase, and kappa-, beta-, and alpha-S1 caseins. This is probably due to the specific proteomic approach used which evidenced only the most representative proteins, in turn masking the less abundant. The proteomic analysis of porcine milk EVs identified 639 proteins. These were mainly annotated as cytoplasmatic and membrane proteins and resulted to be involved in carbohydrate metabolism, immunity, and disease-related pathways [22].

2. Changes of Proteome Milk EVs according to Lactation Stages

The proteome of milk EVs changes during different lactation stages, like that of other milk fractions [10][23][24][25]. Colostrum exosomes are significantly enriched in proteins involved in the immune response such as acute phase proteins, antimicrobial peptides, and complement activation proteins [10][16][23]. Moreover, differences between the proteome of bovine milk and colostrum exosomes are more pronounced than those observed comparing the exosome proteome of human milk and colostrum [16]. Interestingly, by comparing the milk exosome proteins from human and bovine colostrum, 22 milk exosome proteins differentially expressed were annotated in the immune system processes. In particular, lactoferrin-like protein and plastin-2 were the most abundant proteins in bovine and human exosomes, respectively. Lactoferrin protects newborns from the development of necrotizing enterocolitis and regulates cell survival, while plastin-2 is involved in leukocyte function and in the defense against bacteria

invasion [16]. Exosomes from both colostrum and mature milk showed high abundance of proteins associated with ribosome and regulation of actin cytoskeleton [16], as well as of different integrins, other typical milk proteins, and proteins regulating cell growth and proliferation [23]. Moreover, the proteome of bovine colostrum EVs progressively changes within 72 h after partum, becoming more similar to those from mature milk [23]. Proteomics analysis evidenced also different protein cargo of exosomes from porcine colostrum and mature milk [25]. Among the identified proteins (637), 166 were found to be differentially abundant. The colostrum EV proteins related to the regulation of hemostasis and cellular lipid intake that can be indicative of the process occurring in the adaptation for extrauterine life were highly represented, whereas the most abundant proteins in mature porcine milk EVs were linked to endothelial barrier, endothelial cell development, and establishment or maintenance of apical/basal cells, probably related to the cellular development linked to the transition from colostrum to milk [25]. Furthermore, the changes of the protein cargo of milk EVs occurred also in the late stage of cow lactation. The comparison between proteome of bovine EVs isolated from late-stage lactating cows and the bovine milk EVs proteomic database evidenced a high abundance of proteins involved in the modulation of immune response, gut functions of infants, lipid intake, as well as structural and functional changes occurring in the mammary gland in late lactations [24].

3. Milk Protein EVs as Putative Biomarkers

Changes in milk EVs proteome have made milk EVs a potential source of putative biomarkers assessing the mammary gland pathological or physiological status, since the abundance of several proteins (e.g., casein) in the other milk fractions can limit the detection of less represented proteins, even if these can be differentially modulated in pathological conditions [26][27][28]. The comparison between MFG, whey, and EVs isolated from healthy and Staphylococcus aureus infected cows evidenced higher, larger numbers of protein changes in whey and MFG, compared to EVs [26]. On the contrary, the proteome of milk EVs resulted to be significantly modified by bovine leukemia virus infection [27]. Twenty-six proteins were found to be differentially expressed in milk EVs from infected cattle as compared to uninfected cattle. Bioinformatic analysis annotated these proteins in metabolic processes, binding, catalytic activities, cancer-related pathways, and focal adhesion. Thus, the viral oncogenic disease can alter the proteins encapsulated in bovine milk EVs, and these proteins could be putative markers of clinical stages of the viral disease in cattle [27]. Furthermore, milk exosomes seem to be also promising for the early detection of pregnancy. Notably, in whey EVs from pregnant cows, a significant abundance of proteins was found, such as polymeric immunoglobulin receptor, sulfhydryl oxidase, mucin-1, and lymphocyte antigen 96, already described to be up-regulated in other biofluids or tissues of pregnant cows [28].

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