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Bioactive components in the marsupial pouch and milk

Manujaya W. Jayamanna Mohottige[1,2], Chloe E. Gardner[3], Mitchell G. Nye-Wood[1], Katherine A. Farquharson[2,3], Angéla Juhász[1,2], Katherine Belov[2,3], Carolyn J. Hogg[2,3], Emma Peel[2,3] and Michelle L. Colgrave[1,2,4]

[1]School of Science, Edith Cowan University, Joondalup, WA, Australia; [2]Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science, Australia; [3]Faculty of Science, School of Life and Environmental Sciences, The University of Sydney, Sydney, Australia and [4]Commonwealth Scientific and Industrial Research Organization, Agriculture and Food, Brisbane, QLD, Australia

Review Article

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Corresponding author:
Michelle L. Colgrave;
Email: m.colgrave@ecu.edu.au

Abstract

Marsupials give birth to immunologically naïve young after a relatively short gestation period compared with eutherians. Consequently, the joey relies significantly on maternal protection, which is the focus of the present review. The milk and the pouch environment are essential contributors to maternal protection for the healthy development of joeys. In this review, we discuss bioactive components found in the marsupial pouch and milk that form cornerstones of maternal protection. These bioactive components include immune cells, immunoglobulins, the S100 family of calcium-binding proteins, lysozymes, whey proteins, antimicrobial peptides and other immune proteins. Furthermore, we investigated the possibility of the presence of plurifunctional components in milk and pouches that are potentially bioactive. These compounds include caseins, vitamins and minerals, oligosaccharides, lipids and microRNAs. Where applicable, this review addresses variability in bioactive components during different phases of lactation, designed to fulfil the immunological needs of the growing pouch young. Yet, there are numerous additional research opportunities to pursue, including uncovering novel bioactive components and investigating their modes of action, dynamics, stability and ability to penetrate the gut epithelium to facilitate systemic effects.

Introduction

Milk is a complete food source for mammalian young. It is particularly important for marsupials, given their unique reproductive and developmental biology. Marsupials have a very short gestation period of up to 30 d, after which they give birth to altricial young that undergo the majority of development *ex utero* within the pouch[1]. The development of pouch young is supported by a complex and extended lactation period, with examples such as koalas (*Phascolarctos cinereus*)[2] which can lactate for up to 12 months. Conversely, eutherian mammals, such as humans, have an extended *in utero* development and comparatively consistent milk composition throughout lactation[3].

Lactation in marsupials studied to date is divided into three phases that differ in nutrients (protein, carbohydrate and fat) and bioactive compounds. For a thorough review of the nutritional components of marsupial milk, see Refs.[4,5]. The timing and definition of these phases differ between species. In this review, we will use the phases defined for tammar wallaby (*Notamacropus eugenii*) and brushtail possum (*Trichosurus vulpecula*) as their milk is the most well-studied amongst marsupials. Phase 1 represents pregnancy, phase 2A (early lactation) is when the joey is permanently attached to the teat, phase 2B (mid-lactation) is when the joey suckles intermittently, and phase 3 (late lactation) is when the joey starts to exit the pouch and is eventually weaned at approximately 9 months[6,7].

Marsupial young are immunologically naïve at birth and lack mature immune tissues and cells for up to 120 d postpartum[8]. Given that the pouch is not sterile and contains a diverse range of microorganisms[9,10], the young require immunological support during development. Multiple mechanisms ensure the protection of joeys during this time, including passive immunity via the milk and bioactive compounds in the pouch skin. Numerous immune cells and proteins, such as immunoglobulins, have been identified in marsupial milk, which change in composition and abundance during lactation[11,12]. Bioactive peptides within the milk and pouch, such as antimicrobial peptides (cathelicidins and defensins), lysozyme and marsupial-specific growth factors, also provide immune and nutritional support[11,12].

Marsupial joeys can be orphaned due to threats such as habitat loss and fragmentation, vehicle strike and fire[13]. Orphan joeys in care are hand-raised using commercial colostrum and milk substitutes such as Wombaroo (Woombaroo Food Products, SA, Australia), Di-Vetelact

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(Lillelund Pty Ltd, NSW, Australia) and Marsupial Milk Replacer (Exotic Nutrition, Virginia, United States), which are tailored milk substitutes for kangaroos, wombats, possums and koalas of varying ages. A colostrum substitute is also commercially available (Woombaroo Food Products, SA, Australia) that contains bovine colostrum powder. Although these milk formulae meet the nutritional requirements (carbohydrate, protein, lipid) of joeys during development, they do not contain the diversity of bioactive immune compounds within marsupial milk. Given the essential role of milk immune proteins in marsupial development, it is not surprising that many orphaned joeys fail to thrive in care. For example, at one wildlife hospital (QLD, Australia), 31% of orphaned marsupials and 35·5% of orphaned koalas do not survive, compared with 25·7% mortality across all admitted orphaned wildlife species[14]. Given the long evolutionary divergence between marsupials and eutherians[15], there is a need for marsupial-specific knowledge of their milk's bioactive components. In addition, the important role of the pouch in marsupial development compels investigation of possible bioactivity in pouch secretions.

Bioactive compounds in the milk, mammary gland and pouch of marsupials have been investigated in multiple species. These include the koala, tammar wallaby, Tasmanian devil (*Sarcophilus harrisii*), brushtail possum, ringtail possum (*Pseudocheirus peregrinus*), woylie (*Bettongia penicillata*), quokka (*Setonix brachyurus*) and red kangaroo (*Macropus rufus*). A range of methods have been used to investigate bioactive compounds, including mammary gland and pouch skin transcriptomes[16–19]; proteomes from milk[7,20,21]; pouch secretion proteomes; and antimicrobial testing of pouch washes[21–23].

Marsupial milk contains many bioactive compounds, including bioactive proteins, antimicrobial peptides (AMP)[18,20], marsupial-specific growth factors[24] and lipids[25], oligosaccharides[26] and microRNAs[5]. Here, we expand upon recent milk reviews[4,11,27] by summarising research specific to components of the marsupial milk and pouch with demonstrable bioactive roles. We also highlight compounds with suspected bioactive roles based on evidence in other species as areas for future investigation in marsupials. A thorough understanding of bioactive components in marsupial milk will allow advances in the development of antimicrobials and the targeted development of marsupial-specific milk substitutes.

Bioactive compounds

Immune cells

Marsupial milk contains numerous immune cell types that provide passive immunity to the developing pouch young[28] (Table 1). Macrophages, neutrophils and lymphocytes have been identified in the colostrum and throughout lactation in the tammar wallaby, quokka and koala[29–31]. Neutrophils are the most abundant immune cells in koala milk, and their numbers increase until the end of lactation, highlighting their importance to phagocytic protection of the joey even at this late stage[29,30]. Preference for a high level of neutrophils may be due to their superior capacity to deal with pathogenic bacteria and fungi[29]. A range of other immune cells are likely to be present in milk, given the identification of immune receptors and cell markers in milk transcriptomes and proteomes[18,20]. These include dendritic cells, helper (CD4) and cytotoxic (CD8) T lymphocytes, and other granulocytes such as eosinophils and basophils[18,20].

Immunoglobulins

Immunoglobulins (Igs) play an important role in adaptive immunity. There are four types of Igs in marsupials (IgA, IgE, IgM and IgG) that have different functions[32] (Table 1). IgA is involved in mucosal immunity, IgE is involved in defence against parasites and hypersensitivity, IgG is the most abundant immunoglobulin in the extracellular fluid and blood, and IgM is the first immunoglobulin produced after exposure to an antigen[32]. All four Ig types are present in marsupial milk and mammary glands. Once transferred through the milk, they can provide passive immunity to the altricial young[18,20,33]. IgA and IgG are the predominant Igs in marsupial milk, which is also the case in eutherians[34–36].

Immunoglobulins are generally transferred via the milk during two periods in marsupials: in the colostrum immediately after birth at the start of phase 2A, and when the young first emerges from the pouch at the onset of phase 3[36,37]. At these times, high levels of Igs provide passive protection as young are exposed to new environments and microorganisms[11]. However, the expression of Ig types at these two timepoints can differ between species. IgG is absorbed across the pouch young gut epithelium, facilitated by the neonatal Fc receptor, and enters peripheral circulation, thereby protecting against infection[38]. IgG was detected in wallaby, kangaroo and possum colostrum, although the concentration was low compared with eutherian colostrum[34,35]. In possums, IgG was significantly elevated during late lactation compared with early lactation[36], whereas in koalas, IgG levels remained constant through early and late lactation, although the neonatal Fc receptor was expressed only during early lactation[18,36,39].

IgA and IgM are not absorbed across the gut epithelium but are involved in immune defence at these sites[40]. IgA was identified in the colostrum of the tammar wallaby and brushtail possum[35,41]. Higher IgA levels were observed during late lactation compared with early lactation in the koala and brushtail possum[20,41]. In the koala milk proteome, IgA accounted for 2% of peptides in late lactation, compared with only 0·08% in early lactation. Conversely, IgM was identified only during early lactation in the koala and was not found in a late lactation milk proteome[20]. Tasmanian devil milk has been investigated only during mid-lactation, and IgA levels were the highest of the four Ig types present[18].

The S100 family of calcium-binding proteins

The S100 protein family consists of S100A1–S100A16, S100A19, S100B, S100G, S100P and S100Z proteins[42]. These proteins have well-known antimicrobial and immunomodulatory functions in eutherians[43,44]. In humans, S100A8 and S100A9 are highly expressed in colostrum and at lower levels in mature milk[45]. Gene knockout experiments in mice have shown that S100A8 and S100A9 are absorbed across the neonatal gut, enter peripheral circulation and may protect against neonatal sepsis via direct antimicrobial activity[45,46]. In marsupials, S100A8 and S100A9 are expressed in the milk and mammary gland[17], whereas S100A9 and S100A15 are expressed in the pouch skin[18,20,42] (Table 1). Interestingly, S100A8 and S100A9 were not present in the koala early lactation milk transcriptome or proteome and have not been investigated in the colostrum of any marsupial species. Instead, these proteins were highly abundant in mid- and late lactation in devils and koalas[18,20]. S100A9 was the seventh most highly expressed immune transcript in the Tasmanian devil mid-lactation milk transcriptome (0·07%)[18]. Similarly, S100A8 and S100A9 were highly abundant in the koala late lactation milk proteome,

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Table 1. Bioactive components present in mammary gland, milk and pouch environment

Bioactive component	Source	Potential role	Reference first author
Immune cells • Macrophage • Neutrophil • Lymphocyte • Dendritic cell • Eosinophil • Basophil	Milk	Phagocytic protection	Hewavisenti[18]; Morris[20]; Young[29]; Young[30]
Immunoglobulin • IgA • IgE • IgM • IgG	Mammary gland Milk	Passive immunity through milk	Hewavisenti[18]; Morris[20]; Baudinette[23]
S100 family • S100A8 • S100A9 • S100A15 • S100A19	Mammary gland	Antimicrobial Anti-inflammatory	Peel[17]; Hewavisenti[18]; Morris[20]; Kwek[42]
	Milk		
	Pouch		
Lysozyme	Mammary gland	Antimicrobial Immunomodulatory	Demmer[6]; Vander-Jagt[56]; Kuy[57]; Ambatipudi[58]
	Milk		
	Pouch		
Transferrin and lactoferrin	Mammary gland	Antimicrobial Anti-inflammatory	Lefèvre[19]; Morris[20]; Adamski[36]; Joss[53]; Nicholas[54]
	Milk		
		Anticarcinogenic	
Whey proteins • WAP • WFDC2 • β-Lactoglobulin • α-Lactalbumin	Mammary gland	Pro-proliferative effects on mammary gland epithelial cells	Hewavisenti[18]; Ambatipudi[21]; Topcic[66]; Watt[70]
	Milk	Antimicrobial	
	Pouch		
Other immune proteins • Major histocompatibility complex proteins • Toll-like receptors • Natural killer cell receptors • Chemokines and cytokines • Complement cascade proteins	Mammary gland	Aid in phagocytosis by immune cells	Hewavisenti[18]; Morris[20]; Lefèvre[76]; Abe[77]; Wilson[79]; Hieshima[81]; Hazlett[88]
	Milk	Immune signalling and immunomodulatory	
		Antigen recognition and handling	
Major AMP families • Cathelicidin • Defensin	Mammary gland	Pro-proliferative effects on mammary gland	Peel[17]; Hewavisenti[18]; Morris[20]; van-Harten[89]; Peel[16]; Peel[90]; Wanyonyi[93]; Jones[94]
		Antimicrobial	
	Milk	Immunomodulatory	
	Pouch	Anticancer	
Other AMP families • MM1 • VELP • Dermicidin	Mammary gland	Antimicrobial	Hewavisenti[18]; Morris[20]; Joss[53]; Piotte[55]; Ambatipudi[58]; Schittek[102]
	Milk		
	Pouch		

IgA, immunoglobulin A; IgE, immunoglobulin E; IgG, immunoglobulin G; IgM, immunoglobulin M; MM1, marsupial milk 1; VELP, very early lactation protein; WAP, whey acidic protein; WFDC2, WAP four-disulphide domain protein-2.

being the eighth (1·67%) and twenty-fifth (0·59%) most abundant protein, respectively[18]. The high abundance of S100A8 and S100A9 indicates that these proteins may have important functions in marsupial milk, likely having anti-inflammatory and antimicrobial properties similar to human S100A8 and S100A9. The marsupial S100A8–S100A9 complex may also enter peripheral circulation in the young and protect against systemic infection, as in humans[45].

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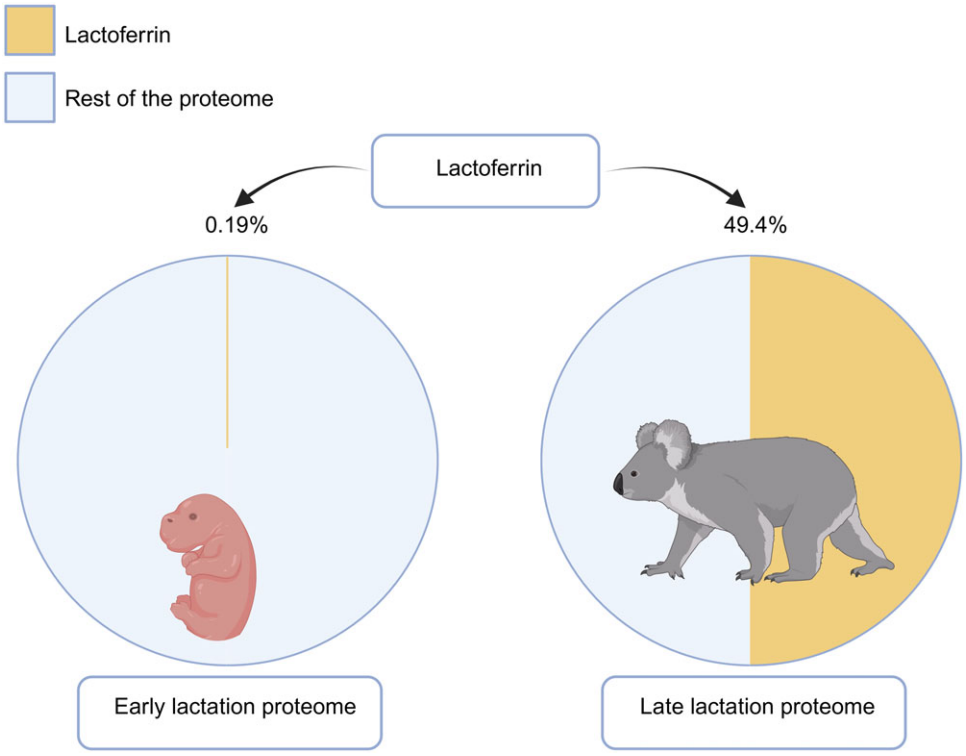


Figure 1. Differential expression of lactoferrin between early and late proteomes. Lactoferricin accounted for 49·4% of all transcripts expressed in koala milk during late lactation, but just 0·19% of transcripts produced during early lactation.

Marsupials also have a unique S100 protein not found in eutherians, S100A19. This protein is expressed in the tammar wallaby mammary gland during pregnancy and involution but not lactation[42]. S100A19 is also expressed in the foregut of joeys during early pouch life when their diet consists only of milk[42]. Marsupial S100A19 is closely related to eutherian S100A15 and A7[42], both of which are antimicrobial[44,47]. As such, S100A19 likely provides antimicrobial protection to the mammary gland and pouch young gut during development.

Lysozymes

The lysozyme family comprises lysozyme C and calcium-binding lysozyme. Among these, lysozyme C is the most prevalent across mammals, while calcium-binding lysozyme is less common[48,49]. Lysozyme is widely expressed in milk and is renowned for its antibacterial activity and immunomodulatory roles within the innate immune system[48]. Variation exists between mammalian species in the number of lysozyme C genes, with one gene identified in humans, four in the grey short-tailed opossum (*Monodelphis domestica*) and two complete and six incomplete genes in the tammar wallaby[49]. Gene duplications in the lysozyme family have not been well characterised across marsupials despite the recent release of numerous high-quality reference genomes across the marsupial tree[50–52]. In marsupial milk, lysozyme has been identified in the early milk proteome of the tammar wallaby[53], mid-lactation milk transcriptome of the Tasmanian devil[18], mid-lactation milk of the ringtail possum[54] and late lactation milk proteome of the koala[20], and in the whey proteins and mammary gland RNA of all lactation stages of the brushtail possum[55] (Table 1). Up-regulation of lysozyme gene expression in the mammary gland and greater lysozyme C abundance in the milk as the lactation cycle progresses[6,56,57] suggests an important role of lysozyme C as the joey detaches from the teat and starts to move out of the pouch. Lysozyme has also been identified by proteomics in post-reproductive tammar wallaby pouch secretions[58], and lysozyme C was the most highly abundant transcript in the woylie pouch skin[50]. Although lysozyme has well-characterised antibacterial activity in eutherians[49], possible antimicrobial and immunomodulatory roles of lysozyme in marsupials remain to be confirmed.

Transferrin and lactoferrin

The glycoproteins transferrin and lactoferrin are both integral components of the transferrin family and major components of milk. Lactoferrin exists in three isoforms: lactoferrin-α, lactoferrin-β and lactoferrin-γ. Lactoferrin-α is the only isoform capable of binding iron. The iron-binding and sequestration capabilities of transferrin and lactoferrin-α are crucial in inhibiting bacterial growth[59]. Lactoferrin has demonstrated antibacterial, antiviral, antifungal, anti-inflammatory and anticarcinogenic activity[59] (Table 1). Furthermore, enzymatic cleavage of the N-lobe of lactoferrin yields crucial peptides lactoferricin and lactoferrampin, which exhibit antimicrobial activity and are under positive selection in primates[60]. Marsupial N lobe sequences from the opossum and Tasmanian devil cluster separately to eutherians[61], but neither antimicrobial function nor diversity within marsupials has been explored.

Lactoferrin was the most abundant protein in the koala late lactation milk proteome (Fig. 1), accounting for almost half of all transcripts expressed[20]. Possible antimicrobial or other functions should be investigated to better understand the role of lactoferrin in marsupials.

Transferrin has been detected around the time of birth in the brushtail possum[36] and tammar wallaby[21] (Table 1). Elevated levels of transferrin have also been observed at mid-lactation, when the joey begins exiting the pouch, in both the ringtail[54] and brushtail possums[36,41,62]. Transferrin was also identified in the

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early lactation milk proteome[53] and mid-lactation mammary gland transcriptome[19] of the tammar wallaby. Evidence for increased expression of transferrin coinciding with birth and first pouch exit suggests that it may play an important role in the immunological protection of marsupial young.

Whey proteins

The whey acidic protein (WAP) is a type of whey protein characterised by cysteine-rich domains, known as disulfide core (4-DSC) domains[63]. The WAP is classified as antimicrobial and involved in inhibiting neutrophil serine proteases[64]. WAP inhibits the growth of *Staphylococcus aureus*[65] and also has non-immune functions, such as stimulating the proliferation of mammary epithelial cells[66]. WAP has been identified as a major whey protein in the milk of numerous eutherian and marsupial species, as well as monotremes[67] (Table 1). Marsupial WAP has an additional 4-DSC domain to the two 4-DSCs in eutherian WAP[67]. WAP was one of the top 200 most highly expressed transcripts in the Tasmanian devil mid-lactation milk transcriptome[18] and has also been detected at mid-lactation in the red kangaroo[68] and brushtail possum[69]. The tammar wallaby mammary gland WAP four-disulphide domain protein-2 (WFDC2) has demonstrated bactericidal activity against *Salmonella enterica*, *Pseudomonas aeruginosa* and *S. aureus* but no activity against commensal bacteria[70]. In koalas, WFDC2 was abundant in the early lactation milk proteome and present in the late lactation proteome[20]. The abundance of WFDC2 in early lactation may coincide with the need for increased immune protection around birth, while the expression of WAP in mid-lactation suggests a possible immunological role in protecting the joey as it begins to exit the pouch.

Whey proteins with specific primary roles can also have secondary bioactive functions as pre-proteins, aside from contributing to the macronutrient profile of milk. Prominent preproteins in marsupial milk include β-lactoglobulin, which is a lipocalin protein that transports iron and other hydrophobic molecules[71], and α-lactalbumin, which is intimately involved in lactose metabolism[72] and iron absorption[73]. Evidence from eutherians suggests possible bioactivity of these two whey proteins. Proteolytic digestion of bovine β-lactoglobulin and α-lactalbumin resulted in numerous sequences with activity against Gram-positive bacteria[74,75] (Table 1). β-Lactoglobulin and α-lactalbumin have been widely identified in marsupial milk: both were among the top 200 most highly expressed transcripts in the Tasmanian devil milk transcriptome[18] and were the predominant proteins in the mature reproductively active tammar wallaby pouch[58]. α-Lactalbumin was expressed in the mammary gland of the brushtail possum at all phases of lactation and increased in phase 3[6,55] and was present in all samples of ringtail possum milk tested[54]. β-Lactoglobulin was the most abundant transcript in the koala mammary gland transcriptome[20]; was secreted at constant levels in red kangaroo mid-lactation[68]; was present in the early milk protein and expressed in the mammary gland at constant levels throughout lactation in the brushtail possum[6,57]; and was present in the tammar wallaby mid- and late lactation mammary gland transcriptomes[19]. The main chain of β-lactoglobulin was also identified in the milk proteins of the tammar wallaby throughout lactation, with a second isoform present in phase 2A and a third isoform at a high concentration in phase 2B[7]. In pouch secretions of the tammar wallaby, β-lactoglobulin was present in high concentrations. Although there was no evidence of direct antimicrobial activity of β-lactoglobulin against *Escherichia coli* or *S. aureus*, the antimicrobial potential of cleaved peptides has been hypothesised[21]. Both α-lactalbumin and β-lactoglobulin are present in the milk, mammary glands and pouches of numerous marsupials. It is possible they provide immune protection when cleaved, but given that they have other functions, their expression likely has other effects, too, and further investigation is required to confirm their bioactivity.

Additional immune proteins

Marsupial milk is also a rich source of other immune proteins, accounting for 9% and 6·6% of all proteins in koala and Tasmanian devil milk, respectively[18,20] (Table 1). These include immune receptors such as the major histocompatibility complex proteins (MHC) classes I and II, toll-like receptors (TLRs) and natural killer (NK) cell receptors[18,20,76]. The presence of these receptors indicates the diversity of immune cells present in the milk and mammary gland, and potential protective functions involving antigen recognition and binding.

Immune signalling molecules, such as cytokines and chemokines, are also present in marsupial milk and mammary glands. The chemokine CCL25 was one of the most highly expressed immune signalling molecules in both koala and Tasmanian devil milk[18,20]. CCL25 has only recently been identified in human milk and is highly expressed in colostrum[77]. In humans, CCL25 is essential for thymocyte development[78] and may play a similar role in pouch young. Other chemokines, such as CCL28, were also identified in the devil mammary gland transcriptome[20]. In humans, CCL28 and CCL25 facilitate the transfer of IgA into the milk by attracting IgA-antibody-producing immune cells to the mammary gland epithelium[79,80]. CCL28 is also antibacterial against Gram-negative and Gram-positive bacteria, and the fungus *Candida albicans*[81]. As such, these chemokines may be involved in both direct and indirect protection of the pouch young gastrointestinal tract.

Complement factors are present in both marsupial milk and the mammary gland. Proteins involved in the classical complement pathway (C2, C3 and C4A) were identified in the koala milk proteome and mammary gland transcriptome[20]. These factors were highly abundant during early lactation, accounting for 2·4% of peptides. Complement proteins bind to pathogens, thereby enabling phagocytosis by immune cells, and some are also bactericidal[82].

Antimicrobial peptides

Cathelicidins and defensins

Cathelicidins and defensins are two major families of antimicrobial peptides in mammals. They form part of the innate immune system through antimicrobial and immunomodulatory activity and may also be involved in immune defence against cancer[83–85]. Cathelicidins and defensins are small, cationic, amphipathic peptides that are expressed in immune and epithelial cells[83,86]. Both cathelicidins and defensins are encoded as pre-propeptides that undergo enzymatic cleavage to release the C-terminal active mature peptide[83,87]. Defensin mature peptides contain six highly conserved cysteine residues that form three disulphide bonds that define the α-, β- and θ-subfamilies[84,88]. Theta defensins are found only in Old World primates and so are not discussed further in this review. Both cathelicidins and defensins from eutherian mammals have antimicrobial activity against fungi, enveloped viruses, Gram-positive and Gram-negative bacteria and parasites[84,89]. They are

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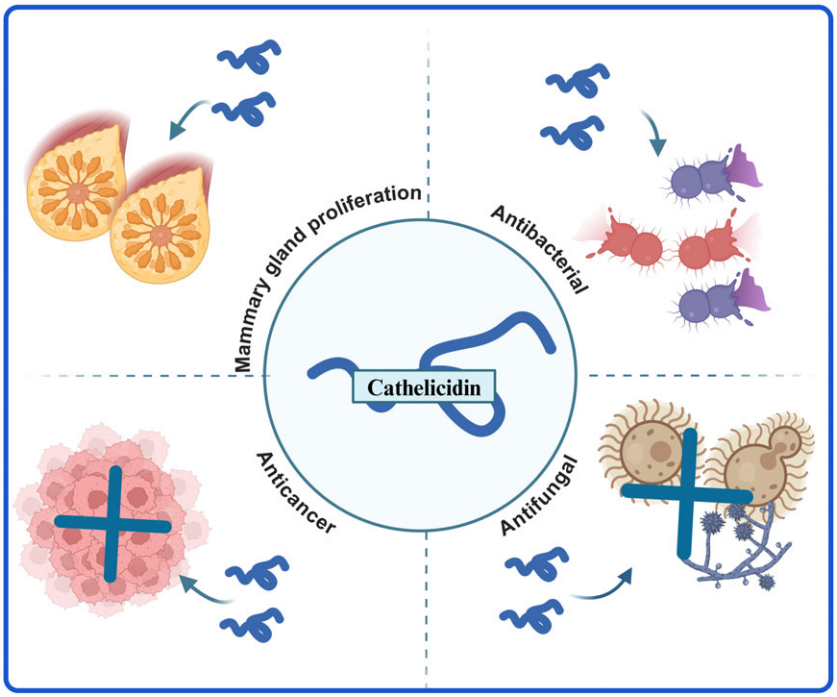


Figure 2. Bioactive roles of cathelicidin.

also immunomodulatory; they enhance phagocytosis, are chemotactic, induce degranulation of neutrophils and mast cells, and induce migration of epithelial cells[84,89].

Marsupials have a large repertoire of diverse cathelicidins, likely driven by the need for additional immune protection of altricial young during development in the pouch (Table 1). This differs from eutherians, such as humans and mice, that have only a single cathelicidin peptide[89]. Cathelicidins have only been characterised in the grey short-tailed opossum, koala, Tasmanian devil and tammar wallaby, with between six and nineteen cathelicidin genes identified in a single species[16,17,39,90,91]. Similar to human cathelicidins, marsupial peptides have broad-spectrum antimicrobial activity against Gram-negative and Gram-positive bacteria, including methicillin-resistant *S. aureus*, and fungi (Fig. 2)[16,17,90]. Koala cathelicidin PhciCath5 was active against *Chlamydia pecorum*, one of the aetiological agents that cause chlamydiosis that has severely impacted koala populations[17]. Recent work has shown that Tasmanian devil cathelicidins have anticancer activity against devil facial tumour disease (DFTD) cells and may also have immunomodulatory functions (Fig. 2)[85].

Marsupial cathelicidins are expressed in the milk and mammary gland throughout lactation[16,18,20,92]. This is unlike other immune proteins that are generally expressed in only two periods as discussed in previous sections[12]. In the tammar wallaby and koala, individual cathelicidins were differentially expressed during early and late lactation[7,20,93]. Some of these peptides had antimicrobial activity, whereas others were not active against the strains tested and may have immunomodulatory functions[17,93]. In addition, cathelicidins may also be involved in mammary gland proliferation and remodelling during involution in the tammar wallaby (Fig. 2)[93]. Cathelicidins are also expressed in the pouch skin[16,50] and may provide direct protection. Tasmanian devil cathelicidins expressed in the pouch were active against bacteria identified in the pouch microbiome. Cathelicidins may also indirectly protect pouch young by contributing to modulation of bacterial communities in the pouch, although this hypothesis has not been proven[16].

Defensins are the second major family of antimicrobial peptides in mammals. Defensins have been characterised in the greater bilby (*Macrotis lagotis*), brown antechinus (*Antechinus stuartii*), common wombat (*Vombatus ursinus*), fat-tailed dunnart (*Sminthopsis crassicaudata*), eastern barred bandicoot (*Perameles gunnii*), red kangaroo, western ringtail possum, numbat (*Myrmecobius fasciatus*), woylie, grey short-tailed opossum, koala, tammar wallaby and Tasmanian devil[91,94,95] (Table 1). Marsupials encode multiple α- and β-defensin genes within their genomes, similar to eutherians. However, some α- and β-defensin lineages are specific to marsupials and, hence, may have novel functions[94]. α-Defensins have not been detected in any marsupial milk, mammary gland or pouch skin transcriptome or proteome to date, so their bioactive role at these sites is unknown[18,20,94]. A small number of β-defensins were expressed during early lactation in the koala[20,94] and mid-lactation in the Tasmanian devil[18]. β-Defensins were also found in the transcriptomes of the koala's lactating mammary gland, the bilby's non-lactating mammary gland and the pouch skin of both bilby and the woylie[95]. Human defensins are expressed in the mammary gland and provide antibacterial defence against infections such as mastitis as well as neonatal gastrointestinal infections[96,97]. Given the function of marsupial defensins is unknown, future studies should focus on understanding the antimicrobial and immunomodulatory functions of these peptides, and their potential role in protecting marsupial pouch young.

Other antimicrobial peptides

Numerous antimicrobial peptides have been identified in marsupial milk, pouch and mammary gland, many due to availability of genomic and proteomic datasets and the comprehensive and in-depth nature of bioinformatic analysis (Table 1). Some are unique to marsupials, such as marsupial milk 1 (MM1),

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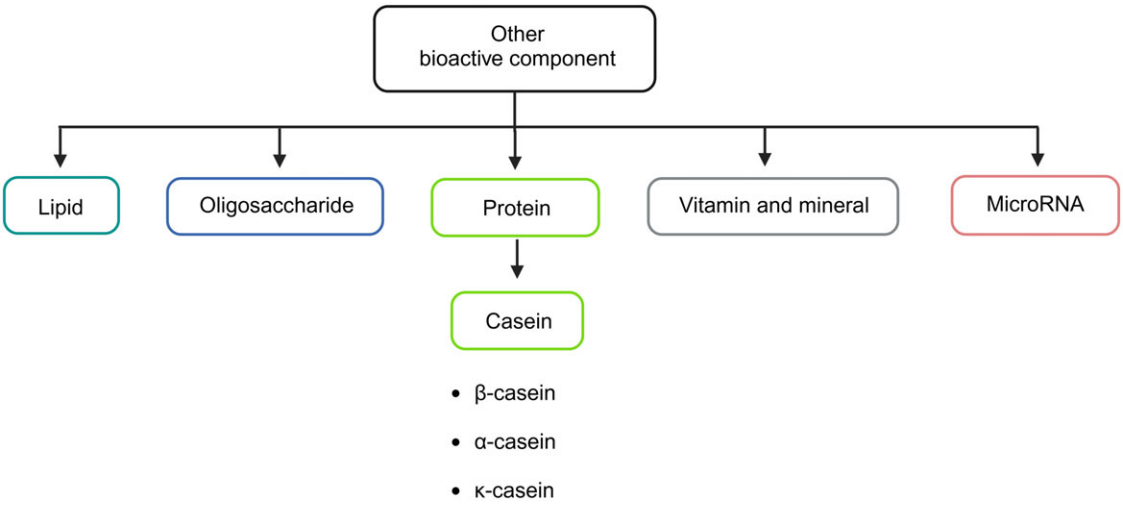


Figure 3. Other potentially bioactive maternal compounds.

whereas others are orthologs of eutherian proteins, such as very early lactation protein (VELP)[20].

VELP has been identified in the milk and mammary glands of the brushtail possum, tammar wallaby, koala and Tasmanian devil[18,20,53,57]. VELP was initially thought to be unique to marsupials but has since been identified as an ortholog of the eutherian protein glycam 1[18]. Glycam 1 is present in only some eutherians and is a pseudogene in humans[98]. VELP abundance changes throughout lactation and differs between species. In the tammar wallaby, VELP is expressed in the mammary gland from pregnancy through to mid-lactation, but not in late lactation[7]. VELP is also expressed at low levels during mid-lactation in the Tasmanian devil, being the only lactation phase investigated in this species[18]. In koalas, VELP is highly abundant in early lactation, accounting for 13·3% of all peptides, which continues throughout late lactation[20]. The closely related eutherian protein glycam1 is also expressed in milk[99], where it has antibacterial and antiviral functions[100,101]. The function of VELP has not been tested, but it likely provides antimicrobial protection to the pouch young given the close evolutionary relationship to glycam 1.

Marsupial milk 1 (MM1) (previously called *novel gene 1*) is a putative antimicrobial peptide unique to marsupials. MM1 has been identified only in mammary gland transcriptomes and milk proteomes of the koala, devil and tammar wallaby and is specifically expressed in these tissues[18,20]. MM1 was highly expressed in the koala and devil, being the twenty-seventh most highly abundant transcript in the devil mammary gland, and twenty-sixth most highly abundant peptide in the koala early lactation proteome[18,20]. The function of MM1 is unknown but is likely antimicrobial given genomic evidence. In the koala genome, MM1 is encoded in a genomic region that is syntenic to the region encoding antimicrobial proteins such as glycam 1 and dermcidin in humans[20]. The abundance and tissue specificity of MM1 suggests that this peptide may play an important role in the antimicrobial protection of the mammary gland and pouch young.

Dermcidin is an AMP secreted from sweat glands in the skin and is the major antimicrobial compound in human sweat[102]. Dermcidin has broad-spectrum antimicrobial activity against bacteria and fungi across a range of pH and salt concentrations, which attenuate the activity of many other AMPs[103]. The pouch is essentially a fold of skin that undergoes significant structural changes leading up to birth and during lactation[1]. As such, it is not surprising that dermcidin is present in the pouch secretions of tammar wallabies and wombats[58]. Pouch wash protein fractions, which included dermcidin, displayed antibacterial activity[102]. Dermcidin was not identified in woylie pouch skin, although this sample was from a female without pouch young[50]. As such, dermcidin in marsupials may be involved in regulating the pouch microbiome and preventing infections, similar to human dermcidin[103].

The diversity and complexity of antimicrobial peptides (AMP) discovered thus far highlight their pivotal role in aiding immune protection to the developing pouch young. The array of capabilities AMP possess shows their relevance in governing immune protection and represents marsupials' adaptive evolutionary methods to maintain the survival and health of their young.

Plurifunctional components with bioactive potential

Beyond their essential nutritive functions, milk components such as caseins and their derived peptides, as well as oligosaccharides, lipids, vitamins, minerals and microRNAs, demonstrate additional bioactivities in mammals (Fig. 3).

Casein

Compared with bovine milk, where protein is approximately 80% casein and 20% whey proteins[104], marsupial milk has relatively less casein with a ratio of approximately 50:50. This is generally constant throughout lactation, although concentration tends to increase[105]. The most abundant caseins are β-casein, followed by α-casein, then κ-casein[20,106]. κ-Casein was once thought to be absent in marsupials until the genes encoding κ-casein were discovered in the brushtail possum[107]. The amount of casein increases throughout lactation, predominantly owing to β-casein while α-casein remains relatively stable. In wallabies, caseins form micelles similar in structure to the well-studied bovine milk, despite the notable evolutionary distance[108]. Although caseins and whey proteins are well-known sources of nourishment, the peptides generated from these proteins can also play key roles in supporting an array of biological processes. Bioactive peptides generated from eutherian milk caseins have demonstrated an array

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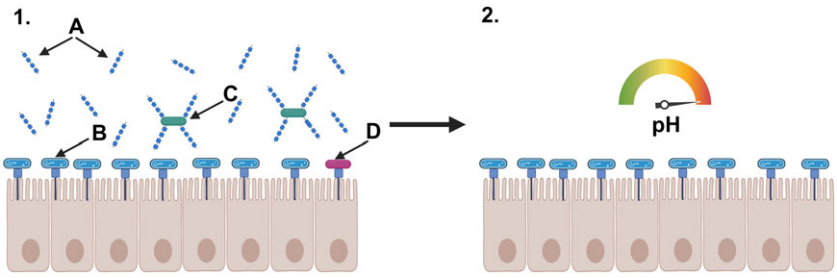


Figure 4. (1) Oligosaccharides (A) promote the proliferation of beneficial bacteria (B) while serving as receptor analogues and binding to invading pathogens (C), blocking their attachment to the gut epithelium. (2) Proliferation of beneficial microflora results in lower pH levels, which leads to competitive inhibition of potentially pathogenic microflora (1. D).

of immunomodulatory and antimicrobial functionalities. Casocidin-1 generated from α_{s2}-casein in cow has potent *in vitro* antimicrobial activity against *E. coli* and *Staphylococcus carnosus*. Isracidin is another casein (α_{s1}-casein)-derived peptide that has displayed antibacterial activity *in vivo* against *S. aureus* and *Candida albicans* in cow and sheep[109]. Apart from these direct antimicrobial roles, β- and κ-casein-derived peptides in cow milk may promote lymphocyte proliferation, whereas β-casein identified from human milk induces chemokine production[110]. Peptides derived from casein have been found in peripheral circulation in neonates but not in adults, indicating that they can cross the gastrointestinal barrier and enter systemic circulation in infants. This finding highlights the possible age-specific functions that these peptides may have in promoting juvenile health and development[111]. However, a focus on milk peptides derived from caseins in marsupials has been notably insufficient or absent. Given that they likely represent a large proportion of the milk proteome in marsupials, a deeper understanding of casein-derived peptides will be essential for the development of tailored marsupial immune milk supplements to ensure orphaned joeys' survival.

Vitamins and minerals

Milk provides essential dietary vitamins and minerals that are required for a variety of bioactive functions, including bone mineralisation, enzyme cofactors and hormonal/nutritive signalling. Marsupial milk mineral content has been reviewed recently[4], although the marsupial-specific literature on milk vitamins is limited and varies across species.

Kangaroo milk contains a similar diversity and concentration of vitamins to cow milk[112], including lipophilic (vitamin A) and hydrophilic (B1, B2, B3, B5, B6, B7 and B12) vitamins[113]. Similar to other milk nutrients, the concentration of vitamins differs between lactation phases 2 and 3[112]. Folic acid (vitamin B9) is the most stable form of folate, which is an essential bioactive vitamin for neural tube closure and guides spinal cord development in mammals. In eutherians, this occurs early in gestation as folic acid is delivered via maternal blood. Suboptimal uptake of folic acid during pregnancy in eutherians leads to perinatal morbidity and mortality, signifying its importance[114]. In marsupials, neural development occurs *ex utero* within the pouch; hence, folic acid is delivered via the milk. This finding could explain why red and grey kangaroos' (*Macropus giganteus*) early lactation milk contains elevated levels of folic acid, which then decreases throughout the lactation period[112]. The fluctuation of these vitamins based on their lactation stages has been descriptively studied in Poole's 1982 study[112].

Oligosaccharides

The carbohydrate fraction of milk contains oligosaccharides, which are present in higher proportions than lactose in marsupials compared with eutherians[115]. Human milk oligosaccharides have demonstrated prebiotic, anti-inflammatory and immunomodulatory activity[26]. Milk oligosaccharides in humans have proven to be incredibly resistant to hydrolysis by digestive enzymes in the small intestine and are known to pass through it unaltered. When they reach the colon, the endogenous microflora is known to secrete enzymes that allow them to utilise oligosaccharides as a source of energy, which leads to competitive inhibition of pathogenic microbial proliferation as illustrated in Fig. 4, thereby performing the prebiotic role[116]. The beneficial microflora's metabolic activity further lowers gut pH, which hinders pathogen proliferation[117]. They also function as receptor analogues that inhibit the attachment of pathogens to epithelial cells by facilitating their binding to oligosaccharides in the gut lumen. Pathogens may mistake free oligosaccharides for those bound to cell membranes and bind to them rather than attaching to joey tissue (Fig. 4)[118]. A database of oligosaccharides across mammals, MilkOligoDB, revealed predominantly linear oligosaccharide structures in the koala, wombat and brushtail possum[26]. Despite the variety of digestive systems and distinct microflora observed among marsupials due to their diverse dietary preferences[119], there remains a strong likelihood that oligosaccharides found in marsupial species will exhibit similar behaviour to human milk oligosaccharides in this context.

Lipids

Although milk fat has a reputation as a primary source of energy, it also has the potential to be bioactive. Bioactive lipids in mammalian milk comprise triacylglycerols, diacylglycerols, saturated and polyunsaturated fatty acids, and phospholipids, which have been shown to have anticancer, antibacterial, anti-inflammatory and immunosuppressive traits[120].

Lipid content in marsupial milk gradually increases during early lactation, before rising substantially in late lactation to a peak during weaning[25,121–123]. Lipid content during late lactation can be five-fold higher than early lactation, which indicates a switch from carbohydrates to lipids as the major energy source[124]. However, in arboreal folivorous marsupials, such as koalas, brushtail possums and ringtail possums, the lipid concentration peaks in mid-lactation[125,126]. Koala milk has the highest milk lipid content of these three arboreal folivores[121,124,126]. Triacylglycerols are the major lipid type in both marsupial and bovine milk. Hydrolysis of triacylglycerol has yielded adequate levels of antimicrobial fatty acids and monoacylglycerols in human milk. The monoacylglycerols are known to exert antiviral, antibacterial and antiprotozoal effects[120].

Fatty acids are known to have distinct biological roles[111]. Conjugated linoleic acid (CLA) is a geometric isomer of linoleic acid (LA) present in bovine milk and has anticarcinogenic, growth-stimulating, antiatherogenic and immunomodulatory activity[127].

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Actinobacteria within the human gut microbiome convert LA to CLA. LA, a precursor for the formation of CLA, has been identified in potoroo (*Potorous tridactyius*) and kangaroo milk. Bacteria from the phylum Actinobacteria have also been discovered in the gastrointestinal tracts of kangaroos[128] and koala[129]. As a result, LA in milk may be digested within the pouch young gut to generate CLA, which may have bioactive functions similar to the human counterpart. To date, minimal research has been conducted to link the marsupial gut microbiotas and LA metabolism.

Fatty acids from marsupial milk, such as linoleic, oleic and palmitic acids[25], have antiviral properties *in vitro*[130]. Arachidonic acids are a type of polyunsaturated fatty acid that have been identified in varying amounts in the milk of many marsupial species[131,132]. In humans, many biological processes and tissues depend on arachidonic acid and its metabolites to develop and function, including skeletal muscle, immunological, brain and neural functions[133]. Arachidonic acid in marsupial milk may have comparable bioactive functions. Unlike in bovine milk, omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have not yet been detected in red kangaroo, eastern quoll (*Dasyurus viverrinus*) nor potoroo milk[121,132,134]. Given the importance of EPA and DHA for the development of the nervous systems of other mammals, their evasiveness from detection in marsupial milk warrants further investigation[135–137].

Milk fat is carried in globules that are encapsulated by a phospholipid bilayer called the milk fat globular membrane (MFGM). Lipids and embedded MFGM proteins are of particular interest due to their health-promoting functionalities in mammals[138]. However, the functionalities of MFGM proteins and their ability to generate bioactive peptides in marsupial milk are largely unexplored.

MicroRNAs

MicroRNAs (miRNAs) are known to have an array of biological activities, including organogenesis and morphogenesis, through gene regulation. They are most widely known in mammals for the role they play in lactation stage-dependent mammary gland growth. Recent research on the variable abundance of miRNA in milk suggests that they may also play a function in directing the development and immunological protection of joeys. Milk is thought to accomplish this by functioning as a carrier of miRNA and other bioactive components to growing young, resulting in exogenously generated miRNAs contributing to gene regulation in joeys. This hypothesis is supported further by the capacity of milk miRNAs to tolerate low pH and RNAse activity owing to the presence of exosomes that shield them from such extreme conditions. These miRNAs can enter the bloodstream alongside exosomes via the juvenile joey's gut, or they might be digested, resulting in the release of miRNAs and subsequent absorption by gut cells[139,140].

Conclusion

Recent advances in genomics, transcriptomics, lipidomics and proteomics have shed light on the array of bioactive components found in marsupial milk, mammary gland and pouch environment. Some of these are unique to marsupials and may support the healthy development of pouch young. Among several functions of these bioactive components, providing immunological protection to the young is of critical importance. These components play a pivotal role in safeguarding the developing marsupial against pathogens, thereby facilitating healthy growth and development within the pouch environment. Expression of these bioactive components can be either consistent throughout lactation or differentially expressed, and many show increased expression around birth and when the joey starts exiting the pouch at mid-lactation. Given their pivotal role, the uncharacterised potential bioactive components found in marsupials, which have demonstrated activity in eutherians, should be a focus for future studies. It is essential to conduct more investigations on both defined and uncharacterised beneficial compounds to determine their stability and functionality in the environmental conditions of the marsupials' pouch and gastrointestinal tract. This approach will help us gain a better understanding of the resilience and efficacy of these compounds in the biological environments in which they function. However, large-scale studies on marsupial milk are not always feasible. Hence, methods require modification (down-scaling) to maximise the utility of rare samples. Current milk substitutes for orphaned joeys lack marsupial maternal immune compounds. This review examined current knowledge on maternal immune protection in joeys and identified potential avenues for future research to discover further potent bioactive components. These components could enhance existing milk supplement formulae, increasing the survival rates of orphaned, hand-reared marsupial joeys. Furthermore, different substitutes optimised for the different phases of lactation would be needed to meet the changing needs of the developing joeys. Therefore, although this review offers a preliminary understanding of the requirements of milk substitutes, further work is needed to design milk substitutes that meet marsupial joeys' immune needs.

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