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Bioactive components in the marsupial pouch and milk

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Abstract

Marsupials give birth to immunologically naïve young after a relatively short gestation period compared to eutherians. Consequently, the joey significantly relies 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, investigating their mode of action, dynamics, stability, and ability to penetrate the gut epithelium ensuring systemic actions.

Keywords: Immune cells, Immunoglobulins, S100 family proteins, Lysozymes, Whey proteins, Antimicrobial peptides

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 days, after which they give birth to altricial young that undergo the majority of development ex-utero within the pouch[1]. 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[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 is 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 around 9 months[6,7].

Marsupial young are immunologically naïve at birth and lack mature immune tissues and cells for up to 120 days postpartum[8]. Given 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 (Lillelund Pty Ltd, NSW, Australia) and Marsupial Milk Replacer (Exotic Nutrition, Virginia, United States), which are tailored milk substitutes for kangaroos, wombats,

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possums and koalas of varying ages. A colostrum substitute is also commercially available (Woombaroo Food Products, SA, Australia) that contains bovine colostrum powder. While 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 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 to 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 (AMPs)[18,20], marsupial-specific growth factors[24], as well as 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 for advances in the development of antimicrobials and the targeted development of marsupial-specific milk substitutes.

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Bioactive compounds

Immune cells

Marsupial milk contains numerous immune cell types that provide passive immunity to the developing pouch young[28]. Macrophages, neutrophils, and lymphocytes have been identified in 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]. 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-time points 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 to eutherian colostrum[34,35]. In possums, IgG was significantly elevated during late lactation compared to early lactation[36]. While in koalas,

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IgG levels remained constant through early and late lactation, although the neonatal Fc receptor was only expressed 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 to early lactation in the koala and brushtail possum[20,41]. In the koala milk proteome, IgA comprised 2% of peptides in late lactation, compared to only 0.08% in early lactation. Conversely, IgM was only identified during early lactation in the koala and was not found in a late lactation milk proteome[20]. Tasmanian devil milk has only been investigated 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 A9 are highly expressed in colostrum and at lower levels in mature milk[45]. Gene knockout experiments in mice have shown that S100A8/A9 is absorbed across the neonatal gut, enters peripheral circulation, and may protect against neonatal sepsis via direct antimicrobial activity[45,46]. In marsupials, S100A8 and A9 are expressed in the milk and mammary gland[17], while A9 and A15 are expressed in the pouch skin[18,20,42]. Interestingly, S100A8 and A9 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 7th most highly expressed immune transcript in the Tasmanian devil mid lactation milk transcriptome (0.07%)[18]. Similarly, S100A8 and A9 were highly abundant in the koala late lactation milk proteome, being the 8th (1.67%) and 25th (0.59%) most abundant protein respectively[18]. The high abundance of S100A8 and A9 indicates these proteins may have important functions in marsupial milk, likely having anti-inflammatory and antimicrobial properties similar to human S100A8/A9. The marsupial S100A8/A9 complex may also enter peripheral circulation in the young and protect against systemic infection, as in humans[45].

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

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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.

Lysozyme

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 in humans, four in the gray 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], 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]. Upregulation 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, -β, and -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 anti-carcinogenic activity[59]. Furthermore, enzymatic cleavage of the N-lobe of lactoferrin yields crucial peptides lactoferricin and lactoferrampin, which exhibit

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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.

Figure 1

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]. Elevated levels of transferrin have also been observed at the 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 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]. 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.

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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]. 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. While 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]. Therefore, 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, comprising 9% and 6.6% of all proteins in koala and Tasmanian devil milk, respectively[18,20]. These include immune receptors such as the major histocompatibility complex proteins (MHC) class I and II, toll-like receptors (TLRs) and natural killer (NK) cell receptors[18,20,76]. The presence of these

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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 -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, comprising 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 defense 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 prepropeptides 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 only found in old-world primates, 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 also immunomodulatory;

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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. This differs from eutherians, such as humans and mice, that only have a single cathelicidin peptide[89]. Cathelicidins have only been characterised in the gray short-tailed opossum, koala, Tasmanian devil, and tammar wallaby, with between six and 19 cathelicidin genes identified in a single species[17,39,90–92]. Similar to human cathelicidins, marsupial peptides have broad-spectrum antimicrobial activity against Gram-negative and -positive bacteria, including methicillin-resistant *S. aureus*, and fungi (Fig. 2)[17,90,91]. 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 anti-cancer 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[18,20,90,93]. This is unlike other immune proteins that are generally only expressed in two periods as discussed in previous sections[12]. In the tammar wallaby and koala, individual cathelicidins were differentially expressed during early and late lactation[7,20,94]. Some of these peptides had antimicrobial activity, while others were not active against the strains tested and may have immunomodulatory functions[17,94]. In addition, cathelicidins may also be involved in mammary gland proliferation and remodelling during involution in the tammar wallaby (Fig. 2)[94]. Cathelicidins are also expressed in the pouch skin[50,90] 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[90].

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, gray short-tailed opossum, koala, tammar wallaby and Tasmanian devil[92,95,96] . Marsupials encode multiple α-and β-defensin genes

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within their genomes, similar to eutherians. However, some α-and β-defensin lineages are specific to marsupials and hence may have novel functions[95]. 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[18,20,95]. A small number of β-defensins were expressed during early lactation in the koala[20,95] 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[96]. Human defensins are expressed in the mammary gland and provide antibacterial defence against infections such as mastitis, as well as neonatal gastrointestinal infections[97,98]. 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. Some are unique to marsupials, such as marsupial milk 1 (MM1), while others are orthologs of eutherian proteins, such as very early lactation protein (VELP)[20].

VELPs have 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 only present in some eutherians and is a pseudogene in humans[99]. 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, comprising 13.3% of all peptides, which continues throughout late lactation[20]. The closely related eutherian protein glycam1 is also expressed in milk[100], where it has antibacterial and antiviral functions[101,102]. 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.

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Marsupial milk 1 (MM1) (previously called *novel gene 1*) is a putative antimicrobial peptide unique to marsupials. MM1 has only been identified 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 27th most highly abundant transcript in the devil mammary gland, and 26th 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 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[103]. 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[104]. 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[103]. 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[104].

The diversity and complexity of antimicrobial peptides (AMPs) discovered thus far highlight their pivotal role in aiding immune protection to the developing pouch young. The array of capabilities AMPs 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).

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Casein

Compared to bovine milk, where protein is approximately 80% casein and 20% whey proteins[105], marsupial milk has relatively less casein and so this ratio is approximately 50:50. This is generally constant throughout lactation, though concentration tends to increase[106]. The most abundant caseins are β-casein, followed by α-casein, then κ-casein[20,107]. κ-casein was once thought to be absent in marsupials until the genes encoding κ-casein were discovered in the brushtail possum[108]. 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[109]. 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 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[110]. 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[111]. 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[112]. However, a focus on milk peptides derived from caseins in marsupials has been notably insufficient or absent. Given 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 joey's survival.

Vitamins & 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], though the marsupial-specific literature on milk vitamins is limited and varies across species.

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Kangaroo milk contains a similar diversity and concentration of vitamins to cow milk[113], including lipophilic (vitamin A) and hydrophilic (B1, B2, B3, B5, B6, B7 and B12) vitamins[114]. Similar to other milk nutrients, the concentration of vitamins differed between lactation phases 2 and 3[113]. 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 lead to perinatal morbidity and mortality, signifying their importance[115]. In marsupials, neural development occurs *ex-utero* within the pouch, hence folic acid is delivered via the milk. This finding could explain the reason red and grey kangaroos' (*Macropus giganteus*) early lactation milk contains elevated levels of folic acid, which then decreases throughout the lactation period[113]. The fluctuation of these vitamins based on their lactation stages has been descriptively studied in Poole's 1982 study[113].

Oligosaccharides

The carbohydrate fraction of milk contains oligosaccharides, which are present in higher proportions than lactose in marsupials compared to eutherians[116]. Human milk oligosaccharides have demonstrated prebiotic, anti-inflammatory, and immunomodulatory activity[26]. Milk oligosaccharides in humans have proven to be incredibly resistant to being hydrolysed 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[117]. The beneficial microflora's metabolic activity further lowers gut pH, which hinders pathogen proliferation[118]. 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 attach to joey tissue (Fig. 4)[119]. 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[120], there remains a strong likelihood that oligosaccharides found in marsupial species will exhibit similar behaviour to human milk oligosaccharides in this context.

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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 of triacylglycerides, diacylglycerides, saturated and polyunsaturated fatty acids, and phospholipids, which have been shown to have anticancer, antibacterial, anti-inflammatory, and immunosuppressive traits[121].

Lipid content in marsupial milk gradually increases during early lactation, before rising substantially in late lactation to a peak during weaning[25,122–124]. 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[125]. However, in arboreal folivorous marsupials, like koalas, brushtail possums, and ringtail possums, lipid concentration peaks in mid lactation[126,127]. Koala milk has the highest milk lipid content of these three arboreal folivores[122,125,127]. Triacylglycerides are the major lipid type in both marsupial and bovine milk. Hydrolysis of tryglycerides has yielded adequate levels of antimicrobial fatty acids and monoacylglycerides in human milk. Resulted monoacylglycerides are known to exert antiviral, antibacterial, and antiprotozoal effects[121].

Fatty acids are known to have distinct biological roles[112]. 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[128]. 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[129] and koala[130] As a result, LA in milk may be digested within the pouch young gut to generate CLA, which may have similar bioactive functions 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*[131]. Arachidonic acids are a type of polyunsaturated fatty acid that have been identified in varying amounts in the milk of many marsupial species[132,133]. 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[134]. Arachindonic acid in marsupial milk may have comparable bioactive functions.

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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[122,133,135]. Given the importance of EPA and DHA to the development of the nervous systems of other mammals, their evasiveness from detection in marsupial milk warrants further investigation[136–138].

Milk fat is carried in globules that are encapsulated by a phospholipid bilayer called milk fat globular membrane (MFGM). Lipids and embedded MFGM proteins are of particular interest due to their health-promoting functionalities in mammals[139]. However, the functionalities of MFGM proteins and their ability to generate bioactive peptides in marsupial milk are largely unexplored.

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 milk miRNAs' capacity to tolerate low pH and RNAse activity due to the presence of exsosomes 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[140,141].

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 either be consistent throughout lactation or differentially

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expressed and many show increased expression around birth and when the joey starts exiting the pouch at the 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 these compounds' resilience and efficacy 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, while 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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Declaration of interests

The authors declare none.

Authorship

M.W.J.M and C.E.G led the conceptualisation and writing of the original draft, with M.W.J.M also handling the reviewing, editing, and visualisation of the information. M.G.N and K.A.F contributed to conceptualisation, reviewing, editing, and supervision. E.P assisted with conceptualisation, reviewing, and editing, while A.J was responsible for reviewing, editing, and supervision. K.B. supported the project through conceptualisation and funding acquisition. C.J.H and M.L.C provided essential support in conceptualisation, reviewing, editing, supervision, project administration, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

References

1. Tyndale-Biscoe C (2005) *Life of marsupials*. CSIRO publishing.

2. Martin R & Handasyde K (1999) *The koala: natural history, conservation and management*. UNSW press.

3. Kuruppath S, Bisana S, Sharp JA *et al*. (2012) Monotremes and marsupials: comparative models to better understand the function of milk. *J Biosci* **37**, 581–588.

4. Stannard HJ, Miller RD & Old JM (2020) Marsupial and monotreme milk – a review of its nutrient and immune properties. *PeerJ* **8**, 1-35.

5. Sharp J, Lefèvre C & Nicholas KR (2019) The comparative genomics of monotremes, marsupials, and pinnipeds: models to examine functions of milk proteins. In *Milk Proteins*, 3rd ed., pp. 99-141. Academic press.

6. Demmer J, Ross IK, Ginger MR *et al*. (1998) Differential expression of milk protein genes during lactation in the common brushtail possum (Trichosurus vulpecula). *J Mol Endocrinol* **20**, 37-44.

7. Joss JL, Molloy MP, Hinds L *et al*. (2009) A longitudinal study of the protein components of marsupial milk from birth to weaning in the tammar wallaby (Macropus eugenii). *Dev Comp Immunol* **33**, 152-161.

8. Old JM & Deane EM (2000) Development of the immune system and immunological protection in marsupial pouch young. *Dev Comp Immunol* **24**, 445-454.

9. Maidment TI, Bryan ER, Pyne M *et al*. (2023) Characterisation of the koala (Phascolarctos cinereus) pouch microbiota in a captive population reveals a dysbiotic compositional profile associated with neonatal mortality. *Microbiome* **11**, 75.

10. Weiss S, Taggart D, Smith I *et al*. (2021) Host reproductive cycle influences the pouch microbiota of wild southern hairy-nosed wombats (Lasiorhinus latifrons). *Anim Microbiome* **3**, 1-14.

11. Cheng Y & Belov K (2017) Antimicrobial protection of marsupial pouch young. *Front Microbiol* **8**, 354.

12. Edwards MJ, Hinds LA, Deane EM *et al*. (2012) A review of complementary mechanisms which protect the developing marsupial pouch young. *Dev Comp Immunol* **37**, 213-220.

13. Doherty TS, Geary WL, Miritis V *et al*. (2023) Multiple threats affecting the marsupials of Australasia: impacts and management. In *American and Australasian Marsupials*, 1st ed., pp. 1531-1554, Cham: Springer International Publishing.

14. Taylor-Brown A, Booth R, Gillett A *et al*. (2019) The impact of human activities on Australian wildlife. *PloS one* **14**, 1-28.

15. Luo ZX, Yuan CX, Meng QJ *et al*. (2011) A Jurassic eutherian mammal and divergence of marsupials and placentals. Nature **476**, 442-445.

16. Peel E, Cheng Y, Djordjevic JT *et al*. (2016) Cathelicidins in the Tasmanian devil (Sarcophilus harrisii). *Sci Rep* **6**, 1-9.

17. Peel E, Cheng Y, Djordjevic JT *et al*. (2021) Koala cathelicidin PhciCath5 has antimicrobial activity, including against Chlamydia pecorum. *PLoS One* **16**, 1-20.

18. Hewavisenti RV., Morris KM, O'Meally D *et al*. (2016) The identification of immune genes in the milk transcriptome of the Tasmanian devil (Sarcophilus harrisii). *PeerJ* **4**, 1-23.

19. Lefèvre CM, Digby MR, Whitley JC *et al*. (2007) Lactation transcriptomics in the Australian marsupial, Macropus eugenii: transcript sequencing and quantification. *BMC Genomics* **8**, 1-14.

20. Morris KM, O'Meally D, Zaw T *et al*. (2016) Characterisation of the immune compounds in koala milk using a combined transcriptomic and proteomic approach. *Sci Rep* **6**, 1-14.

21. Ambatipudi K, Joss J, Raftery M *et al*. (2008) A proteomic approach to analysis of antimicrobial activity in marsupial pouch secretions. *Dev Comp Immunol* **32**, 108-120.

22. Bobek G & Deane EM (2001) Possible antimicrobial compounds from the pouch of the koala, Phascolarctos cinereus. *Lett Pept Sci* **8**, 133-137.

23. Baudinette RV, Boontheung P, Musgrave IF *et al*. (2005) Eugenin: an immunomodulator used to protect young in the pouch of the tammar wallaby, Macropus eugenii. *FEBS J* **2**, 433-443.

24. Sharp JA, Wanyonyi S, Modepalli V *et al*. (2017) The tammar wallaby: a marsupial model to examine the timed delivery and role of bioactives in milk. *Gen Comp Endocrinol* **244**, 164-177.

25. Crowleyac HM, Woodward DR & Rose RW (1988) Changes in milk composition during lactation in the potoroo, Potorous tridactyius (Marsupialia: Potoroinae). *Aust J Bioi Sci* **41**, 289-296.

26. Durham SD, Wei Z, Lemay DG *et al*. (2023) Creation of a milk oligosaccharide database, MilkOligoDB, reveals common structural motifs and extensive diversity across mammals. *Sci Rep* **13**, 1-26.

27. Pharo EA (2019) Marsupial milk: a fluid source of nutrition and immune factors for the developing pouch young. *Reprod Fertil Dev* CSIRO **31**, 1252-1265.

28. Smith KK & Keyte AL (2020) Adaptations of the marsupial newborn: birth as an extreme environment. *Anat Rec* **303**, 235-249.

29. Young L, Basden K, Cooper DW *et al*. (1997) Cellular components of the milk of the tammar wallaby (Macropus eugenii). *Aust J Zool* **45**, 423-433.

30. Young LJ & Deane EM (2001) Cellular composition of the late milk of the koala (Phascolarctos cinereus). *Aust J Zool* **49**, 195-202.

31. Cockson A & McNeice R (1980) Survival in the pouch: the role of macrophages and maternal milk cells. *Comp Biochem Physiol A Comp Physiol* **66**, 221-225.

32. Morris K, Prentis PJ, O'Meally D *et al*. (2014) The koala immunological toolkit: sequence identification and comparison of key markers of the koala (Phascolarctos cinereus) immune response. *Aust J Zool* **62**, 195-199.

33. Hurley WL & Theil PK (2011) Perspectives on immunoglobulins in colostrum and milk. *Nutrients* **3**, 442-274.

34. Deane EM & Cooper DW (1984) Immunology of pouch young marsupials. I. Levels of immunoglobulin transferrin and albumin in the blood and milk of euros and wallaroos (hill kangaroos: Macropusrobustus, marsupialia). *Dev Comp Immunol* **8**, 863-876.

35. Deane EM, Cooper DW & Renfreec MB (1990) Immunoglobulin G levels in fetal and newborn tammar wallabies (Macropus eugenii). *Reprod Fertil Dev* **2**, 369-375

36. Adamski FM & Demmer J (2000) Immunological protection of the vulnerable marsupial pouch young: two periods of immune transfer during lactation in Trichosurus vulpecula (brushtail possum). *Dev Comp Immunol* **24**, 491-502.

37. Daly KA, Digby M, Lefèvre C *et al* (2007) Analysis of the expression of immunoglobulins throughout lactation suggests two periods of immune transfer in the tammar wallaby (Macropus eugenii). *Vet Immunol Immunopathol* **120**, 187-200.

38. Yadav M (1971) The transmissions of antibodies across the gut of pouch-young marsupials. *Immunology* **21**, 839-851.

39. Daly KA, Digby MR, Lefévre C *et al*. (2008) Identification, characterization and expression of cathelicidin in the pouch young of tammar wallaby (Macropus eugenii). *Comp Biochem Physiol* **149**, 524-533.

40. Woof JM & Kerr MA (2006) The function of immunoglobulin A in immunity. *J Pathol* **208**, 270-282.

41. Adamski FM & Demmer J (1990) Two stages of increased IgA transfer during lactation in the marsupial, Trichosurus vulpecula (brushtail possum). *J Immunol* **162**, 6009-6015.

42. Kwek JHL, Wynne A, Lefèvre C *et al*. (2006) Molecular evolution of a novel marsupial S100 protein (S100A19) which is expressed at specific stages of mammary gland and gut development. *Mol Phylogenet Evol* **69**, 4-16.

43. Ryckman C, Vandal K, Rouleau P *et al*. (2003) Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. *J Immunol* **170**, 3233-3242.

44. Büchau AS, Hassan M, Kukova G *et al*. (2007) S100A15, an antimicrobial protein of the skin: regulation by E. coli through Toll-like receptor 4. *J Invest Dermatol* **127**, 2596-2604.

45. Pirr S, Richter M, Fehlhaber B *et al*. (2017) High amounts of S100-alarmins confer antimicrobial activity on human breast milk targeting pathogens relevant in neonatal sepsis. *Front Immunol* **8**, 1-10.

46. Wang S, Song R, Wang Z *et al*. (2018) S100A8/A9 in inflammation. *Front Immunol* **9**, 1-14.

47. Gläser R, Harder J, Lange H *et al*. (2005) Antimicrobial psoriasin (S100A7) protects human skin from Escherichia coli infection. *Nat Immunol* **6**, 57-64.

48. Ragland SA & Criss AK (2017) From bacterial killing to immune modulation: recent insights into the functions of lysozyme. *PLoS Pathog* **13**, 1-22.

49. Irwin DM, Biegel JM & Stewart CB (2011) Evolution of the mammalian lysozyme gene family. *BMC Evol Biol* **11**, 1-16.

50. Peel E, Silver L, Brandies P *et al*. (2021) A reference genome for the critically endangered woylie, Bettongia penicillata ogilbyi. *GigaByte* **2021**, 1-15.

51. Johnson RN, O'Meally D, Chen Z *et al*. (2018) Adaptation and conservation insights from the koala genome. *Nat Genet* **50**, 1102-1111.

52. Stammnitz MR, Gori K, Kwon YM *et al*. (2023) The evolution of two transmissible cancers in Tasmanian devils. *Science* **380**, 283-293.

53. Joss J, Molloy M, Hinds L *et al*. (2007) Proteomic analysis of early lactation milk of the tammar wallaby (Macropus eugenii). *Comp Biochem Physiol Part D Genomics Proteomics* **2**, 150-164.

54. Nicholas K, Loughnan M, Messer M *et al*. (1989) Isolation, partial sequence and asynchronous appearance during lactation of lysozyme and alpha-lactalbumin in the milk of a

marsupial, the common ringtail possum (Pseudocheirus peregrinus). *Comp Biochem Physiol B* **94**, 775-778.

55. Piotte CP, Marshall CJ, Hubbard MJ *et al*. (1997) Lysozyme and α-lactalbumin from the milk of a marsupial, the common brush-tailed possum (Trichosurus vulpecula). *Biochim Biophys Acta Gen Subj* **1336**, 235-242.

56. Vander Jagt CJ, Whitley JC, Cocks BG *et al*. (2016) Gene expression in the mammary gland of the tammar wallaby during the lactation cycle reveals conserved mechanisms regulating mammalian lactation. *Reprod Fertil Dev* **28**, 1241-1257.

57. Kuy S, Kelly VC, Smit AM *et al*. (2007) Proteomic analysis of whey and casein proteins in early milk from the marsupial Trichosurus vulpecula, the common brushtail possum. *Comp Biochem Physiol Part D Genomics Proteomics* **2**, 112-120.

58. Ambatipudi K, Joss J & Deane E (2007) A comparative proteomic analysis of skin secretions of the tammar wallaby (Macropus eugenii) and the wombat (Vombatus ursinus) *Comp Biochem Physiol Part D Genomics Proteomics* **2**, 322-331.

59. Kell DB, Heyden EL & Pretorius E. (2020) The biology of lactoferrin, an iron-binding protein that can help defend against viruses and bacteria. *Front Immunol* **11**, 1-15.

60. Barber MF, Kronenberg Z, Yandell M *et al*. (2016) Antimicrobial functions of lactoferrin promote genetic conflicts in ancient primates and modern humans. *PLOS Genet* **12**, 1-15.

61. Hughes AL & Friedman R (2014) Evolutionary diversification of the vertebrate transferrin multi-gene family. *Immunogenetics* **66**, 651-661.

62. Grigor MR, Bennett BL, Carne A *et al*. (1991) Whey proteins of the common brushtail possum (Trichosurus vulpecula): isolation, characterization and changes in concentration in milk during lactation of transferrin, α-lactalbumin and serum albumin. *Comp Biochem Physiol B* **98**, 451-459.

63. Wilkinson TS, Roghanian A, Simpson AJ *et al*. (2011) WAP domain proteins as modulators of mucosal immunity. *Biochem Soc Trans* **39**, 1409-1415.

64. Wiesner J & Vilcinskas A (2010) Antimicrobial peptides: the ancient arm of the human immune system. *Virulence* **1**, 440-464.

65. Iwamori T, Nukumi N, Itoh K *et al*. (2010) Bacteriostatic activity of whey acidic protein (WAP). *J Vet Med Sci* **72**, 621-625.

66. Topcic D, Auguste A, De Leo AA *et al*. (2009) Characterization of the tammar wallaby (Macropus eugenii) whey acidic protein gene; new insights into the function of the protein. *Evol Dev* **11**, 363-375.

67. Sharp JA, Lefèvre C & Nicholas KR (2007) Molecular evolution of monotreme and marsupial whey acidic protein genes. *Evol Dev* **9**, 378-392.

68. Nicholas KR, Fisher JA, Muths E *et al*. (2001) Secretion of whey acidic protein and cystatin is down. *Comp Biochem Physiol A Mol Integr Physiol* **129**, 851-858.

69. Demmer J, Stasiuk SJ, Adamski FM *et al*. (1999) Cloning and expression of the transferrin and ferritin genes in a marsupial, the brushtail possum (Trichosurus vulpecula). *Biochim Biophys Acta* **1445**, 65-74.

70. Watt AP, Sharp JA, Lefevre C *et al*. (2012) WFDC2 is differentially expressed in the mammary gland of the tammar wallaby and provides immune protection to the mammary gland and the developing pouch young. *Dev Comp Immunol* **36**, 584–590.

71. Roth-Walter F, Pacios LF, Gomez-Casado C *et al*. (2014) The major cow milk allergen Bos d 5 manipulates T-helper cells depending on its load with siderophore-bound iron. *PLoS One* **9**, 1–8.

72. Brew K (2003) alpha-Lactalbumin. *Adv Dairy Chem* **1**, 388–419.

73. Wang X & Zhang B (2014) Integrating genomic, transcriptomic, and interactome data to improve peptide and protein identification in shotgun proteomics. *J Proteome Res* **13**, 2715–2723.

74. Pellegrini A, Thomas U, Bramaz N *et al*. (1999) Isolation and identification of three bactericidal domains in the bovine α-lactalbumin molecule. *Biochim Biophys Acta Gen Subj* **1426**, 439-448.

75. Pellegrini A, Dettling C, Thomas U *et al*. (2001) Isolation and characterization of four bactericidal domains in the bovine β-lactoglobulin *Biochim Biophys Acta Gen Subj* **1526**, 131-140.

76. Lefèvre CM, Digby MR, Whitley JC *et al*. (2007) Lactation transcriptomics in the Australian marsupial, Macropus eugenii: transcript sequencing and quantification. *BMC Genomics* **8**, 1-14.

77. Abe S, Onoda R, Furushima D *et al*. (2023) Detection of CCL25 and the correlation between CCL25, CCL28, IL-7, and TSLP in human breast milk. *J Reprod Immunol* **155**, 1-5.

78. Uehara S, Hayes SM, Li L *et al*. (2006) Premature expression of chemokine Receptor CCR9 impairs T cell development. *J Immunol* **176**, 75–84.

79. Wilson E & Butcher EC (2004) CCL28 controls immunoglobulin (Ig) A plasma cell accumulation in the lactating mammary gland and IgA antibody transfer to the neonate. *J Exp Med* **200**, 805-809.

80. Bowman EP, Kuklin NA, Youngman KR, *et al*. (2002) The intestinal chemokine thymus-expressed chemokine (CCL25) attracts IgA antibody-secreting cells. *J Exp Med* **195**, 269-275.

81. Hieshima K, Ohtani H, Shibano M *et al*. (2003) CCL28 has dual roles in mucosal immunity as a chemokine with broad-spectrum antimicrobial activity. *J Immunol* **170**. 1452-1461.

82. Janeway C, Travers P, Walport M *et al*. (2001) *Immunobiology: the immune system in health and disease*. Garland Science.

83. Kościuczuk EM, Lisowski P, Jarczak J *et al*. (2012) Cathelicidins: family of antimicrobial peptides. a review. *Mol Biol Rep* **39**, 10957–10970.

84. Yang D, Biragyn A, Hoover DM *et al*. (2004) Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. *Annu Rev Immunol* **22**, 181–215.

85. Petrohilos C, Patchett A, Hogg CJ *et al*. (2023) Tasmanian devil cathelicidins exhibit anticancer activity against Devil Facial Tumour Disease (DFTD) cells. *Sci Rep* **13**, 1-11.

86. Bals R & Wilson JM (2003) Cathelicidins - a family of multifunctional antimicrobial peptides. *Cell Mol Life Sci* **60**, 711–720.

87. Ganz T (2003) Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* **3**, 710-720.

88. Hazlett L & Wu M (2011) Defensins in innate immunity. *Cell Tissue Res* **343**, 175-188.

89. van-Harten RM, van-Woudenbergh E, van-Dijk A *et al*. (2018) Cathelicidins: immunomodulatory antimicrobials. *Vaccines* **6**, 1-23.

90. Peel E, Cheng Y, Djordjevic JT *et al*. (2016) Cathelicidins in the Tasmanian devil (Sarcophilus harrisii). *Sci Reports* **6**, 1-9.

91. Peel E, Cheng Y, Djordjevic JT *et al*. (2017) Marsupial and monotreme cathelicidins display antimicrobialactivity, including against methicillin-resistant Staphylococcus. *Microbiology* **163**, 1457-1465.

92. Belov K, Sanderson CE, Deakin JE *et al*. (2007) Characterization of the opossum immune genome provides insights into the evolution of the mammalian immune system. *Genome Res* **17**, 982-991.

93. Carman RL, Simonian MR, Old JM *et al*. (2008) Immunohistochemistry using antibodies to the cathelicidin LL37/hCAP18 in the tammar wallaby, Macropus eugenii. *Tissue Cell* **40**, 459-466.

94. Wanyonyi SS, Sharp JA, Khalil E *et al*. (2011) Tammar wallaby mammary cathelicidins are differentially expressed during lactation and exhibit antimicrobial and cell proliferative activity. *Comp Biochem Physiol A Mol Integr Physiol* **160**, 431–439.

95. Jones EA, Yuanyuan C, O'Meally D *et al*. (2017) Characterization of the antimicrobial peptide family defensins in the Tasmanian devil (Sarcophilus harrisii), koala (Phascolarctos cinereus), and tammar wallaby (Macropus eugenii). *Immunogenetics* **69**, 133–143.

96. Peel E, Hogg C & Belov K (2024) Characterisation of defensins across the marsupial family tree. *Dev Comp Immunol*. **158,** 1-11.

97. Baricelli J, Rocafull MA, Vázquez D *et al*. (2015) β-defensin-2 in breast milk displays a broad antimicrobial activity against pathogenic bacteria. *J Pediatr* **91**, 36–43.

98. Tunzi CR, Harper PA, Bar-Oz B *et al*. (2000) β-defensin expression in human mammary gland epithelia. *Pediatr Res* **48**, 30-35.

99. Rasmussen LK, Johnsen LB, Petersen TE *et al*. (2002) Human GlyCAM-1 mRNA is expressed in the mammary gland as splicing variants and encodes various aberrant truncated proteins. *Immunol Lett* **1**, 73-75.

100. Groenen MAM, Dijkhof RJM & ban-der-Poel JJ (1995) Characterization of a GlyCAM1-like gene (glycosylation-dependent cell adhesion molecule 1) which is highly and specifically expressed in the lactating bovine mammary gland. *Gene* **158**, 189–195.

101. Campagna S, Mathot AG & Fleury Y *et al* (2004) Antibacterial activity of lactophoricin, a synthetic 23-residues peptide derived from the sequence of bovine milk component-3 of proteose peptone. *J Dairy Sci* **87**, 1621-1626.

102. Inagaki M, Nagai S, Yabe T *et al*. (2010) The bovine lactophorin C-terminal fragment and PAS6/7 were both potent in the inhibition of human rotavirus replication in cultured epithelial cells and the prevention of experimental gastroenteritis *Biosci Biotechnol Biochem* **74**, 1386-1390.

103. Schittek B, Hipfel R, Sauer B *et al* (2001) Dermcidin: a novel human antibiotic peptide secreted by sweat glands. *Nat Immunol.* **2**, 1133–1137.

104. Schittek B (2012) The multiple facets of dermcidin in cell survival and host defense. *J Innate Immun* **4**, 349–360.

105. Jäkälä P & Vapaatalo H (2010) Antihypertensive peptides from milk proteins. *Pharmaceuticals* **3**, 251-272.

106. Nicholas KR (1988) Asynchronous dual lactation in a marsupial, the tammar wallaby (Macropus eugenii). *Biochem Biophys Res Commun* **154**, 529–533.

107. Vercruysse L, Van Camp J, Dewettinck K *et al*. (2009) Production and enrichment of bioactive peptides derived from milk proteins. In *Dairy-Derived Ingredients*, 1st ed., pp. 51-67. Woodhead Publishing.

108. Stasiuk SJ, Summers EL & Demmer J (2000) Cloning of a marsupial k-casein cDNA from the brushtail possum (Trichosurus vulpecula). *Reprod Fertil Dev* **12**, 215-222.

109. Horne DS, Anema S, Zhu X *et al*. (2007) A lactational study of the composition and integrity of casein micelles from the milk of the tammar wallaby (Macropus eugenii). *Arch Biochem Biophys* **467**, 107–118.

110. Silva SV & Malcata FX (2005) Caseins as source of bioactive peptides. *Int Dairy J* **15**, 1–15.

111. Nielsen SDH, Liang N, Rathish H *et al*. Bioactive milk peptides: an updated comprehensive overview and database. *Crit Rev Food Sci Nutr*. Published online: 28 Jul 2023. doi.org/10.1080/10408398.2023.2240396.

112. Fox PF, Uniacke-Lowe T, McSweeney PLH *et al*. (2015) Biologically active compounds in milk. In *Dairy Chem Biochem*, 1st ed., pp. 415–497. Cham: Springer International Publishing.

113. Poole WE, Sharman GB, Scott KJ *et al*. (1982) Composition of milk from red and grey kangaroos with particular reference to vitamins. *Aust J BioI Sci* **35**, 607-616.

114. Gaucheron F (2014) Milk and dairy products: a unique micronutrient combination. *J Am Coll Nutr* **30**, Suppl. 5, 400S-409S.

115. Denny KJ, Jeanes A, Fathe K *et al*. (2013) Neural tube defects, folate, and immune modulation. *Birth Defects Res A Clin Mol Teratol* **97**, 602–609.

116. Urashima T, Horiuchi R, Sakanaka M *et al*. (2023) Lactose or milk oligosaccharide: which is significant among mammals? *Anim Front* **13**, 14-23.

117. Engfer MB, Stahl B, Finke B *et al*. (2000) Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. *Am J Clin Nutr* **71**, 1589–1596.

118. Urashima T, Fukuda K & Messer M (2012) Evolution of milk oligosaccharides and lactose: a hypothesis. *Animal* **6**, 369–374.

119. Newburg DS (1996) Oligosaccharides and glycoconjugates in human milk: their role in host defense. *J Mammary Gland Biol Neoplasia* **1**, 271-283.

120. Hume ID (1982) The digestive physiology of marsupials. *Comp Biochem Physiol A Physiol* **71**, 1-10.

121. German JB & Dillard CJ (2006) Composition, structure and absorption of milk lipids: a source of energy, fat-soluble nutrients and bioactive molecules. *Crit Rev Food Sci Nutr* **46**, 57–92.

122. Green B, Merchant J & Newgrain K (1987) Milk composition in the eastern quoll, Dasyurus viverrinus (Marsupialia : Dasyuridae). *Aust J BioI Sci* **40**, 379-388.

123. Ikonomopoulou MP, Smolenski AP & Rose RW (2005) Changes in milk composition during lactation in the eastern barred bandicoot (Perameles gunnii) (Marsupialia: Peramelidae). *Aust J BioI Sci* **53**, 59–65.

124. Muths E (1996) Milk composition in a field population of red kangaroos, Macropus rufus (Desmarest) (Macropodidae: Marsupialia). *Aust J Zool* **44**, 165-175.

125. Rose RW, Shetewi AD & Flowers K (2005) Milk composition of the Tasmanian pademelon (Thylogale billardierii Desmarest) (Macropodoidea: Marsupialia) in captivity. *Aust J Zool* **53**, 67-71.

126. Cowan PE (1989) Changes in milk composition during lactation in the common brushtail possum, Trichosurus vulpecula (Marsupialia: Phalangeridae). *Reprod Fertil Dev* **1**, 325-335.

127. Krockenberger AK (1996) Composition of the milk of the koala, Phascolarctos cinereus, an arboreal folivore. *Phsiol Zool* **69**, 701-718.

128. Parodi PW (1999) Conjugated linoleic acid and other anticarcinogenic agents of bovine milk fat. *J Dairy Sci* **82**, 1339–1349.

129. Li M, Jin W, Li Y *et al*. (2016) Spatial dynamics of the bacterial community structure in the gastrointestinal tract of red kangaroo (Macropus rufus). *World J Microbiol Biotechnol* **32**, 1-9.

130. Alfano N, Courtiol A, Vielgrader H *et al*. (2015) Variation in koala microbiomes within and between individuals: effect of body region and captivity status. *Sci Rep* **5**, 1-12.

131. Welsh JK & May JT (1979) Anti-infective properties of breast milk. *J Pediatr* **94**, 1-9.

132. Green B, Griffiths M & Leckie RM (1983) Qualitative and quantitative changes in milk fat during lactation in the tammar wallaby (Macropus eugenii). *Aust J BioI Sci* **36**, 455-462.

133. Green B & Merchant JC (1988) The composition of marsupial milk. In *The developing marsupial: models for biomedical research*, 1st ed., pp. 41-54. Berlin, Heidelberg: Springer Berlin Heidelberg.

134. Tallima H & El-Ridi R (2018) Arachidonic acid: physiological roles and potential health benefits – a review. *J Adv Res* **11**, 33–41.

135. Griffiths M (1978) *The Biology of Monotremes*, Elsevier.

136. Harris F, Dennison SR & Phoenix DA (2009) Anionic antimicrobial peptides from eukaryotic organisms. *Curr Protein Pept Sci* **10**. 585-606.

137. Milte CM, Parletta N, Buckley JD *et al*. (2012) Eicosapentaenoic and docosahexaenoic acids, cognition, and behavior in children with attention-deficit/hyperactivity disorder: a randomized controlled trial. *Nutrition* **28**, 670–677.

138. Swanson D, Block R & Mousa SA (2012) Omega-3 fatty acids EPA and DHA: health benefits throughout life. *Adv Nutr* **3**, 1–7.

139. Xu D, Zhou S, Liu Y *et al*. (2024) Complement in breast milk modifies offspring gut microbiota to promote infant health. *Cell* **187**, 750-763.

140. Modepalli V, Kumar A, Hinds LA *et al*. (2014) Differential temporal expression of milk miRNA during the lactation cycle of the marsupial tammar wallaby (Macropus eugenii). *BMC Genomics* **15**, 1-18.

141. Sharp JA, Modepalli V, Enjapoori AK *et al*. (2015) Bioactive functions of milk proteins: a comparative genomics approach. *J Mammary Gland Biol Neoplasia* **19**, 289–302.

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 Milk Pouch	Antimicrobial Anti-inflammatory	Peel[17]; Hewavisenti[18]; Morris[20]; Kwek[42]
Lysozyme	Mammary gland Milk Pouch	Antimicrobial Immunomodulatory	Demmer[6]; Vander-Jagt[56]; Kuy[57]; Ambatipudi[58]
Transferrin and lactoferrin	Mammary gland Milk	Antimicrobial Anti-inflammatory Anticarcinogenic	Lefèvre[19]; Morris[20]; Adamski[36]; Joss[53]; Nicholas[54]
Whey proteins • WAP • WFDC2 • β-lactoglobulin • α-lactalbumin	Mammary gland Milk Pouch	Pro-proliferative effects on mammary gland epithelial cells Antimicrobial	Hewavisenti[18]; Ambatipudi[21]; Topcic[66]; Watt[70]
Other immune proteins • Major histocompatibility	Mammary gland	Aid in phagocytosis by immune cells	Hewavisenti[18]; Morris[20]; Lefèvre[76]; Abe[77];

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complex proteins • Toll-like receptors • Natural killer cell receptors • Chemokines and cytokines • Complement cascade proteins	Milk	Immune signalling and immunomodulatory Antigen recognition and handling	Wilson[79]; Hieshima[81]; Hazlett[88]
Major AMP families • Cathelicidin • Defensin	Mammary gland Milk Pouch	Pro-proliferative effects on mammary gland Antimicrobial Immunomodulatory Anticancer	Peel[17]; Hewavisenti[18]; Morris[20]; van-Harten[89]; Peel[90]; Peel[91]; Wanyonyi[94]; Jones[95]
Other AMP families • MM1 • VELP • Dermicidin	Mammary gland Milk Pouch	Antimicrobial	Hewavisenti[18]; Morris[20]; Joss[53]; Piotte[55]; Ambatipudi[58]; Schittek[103]

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

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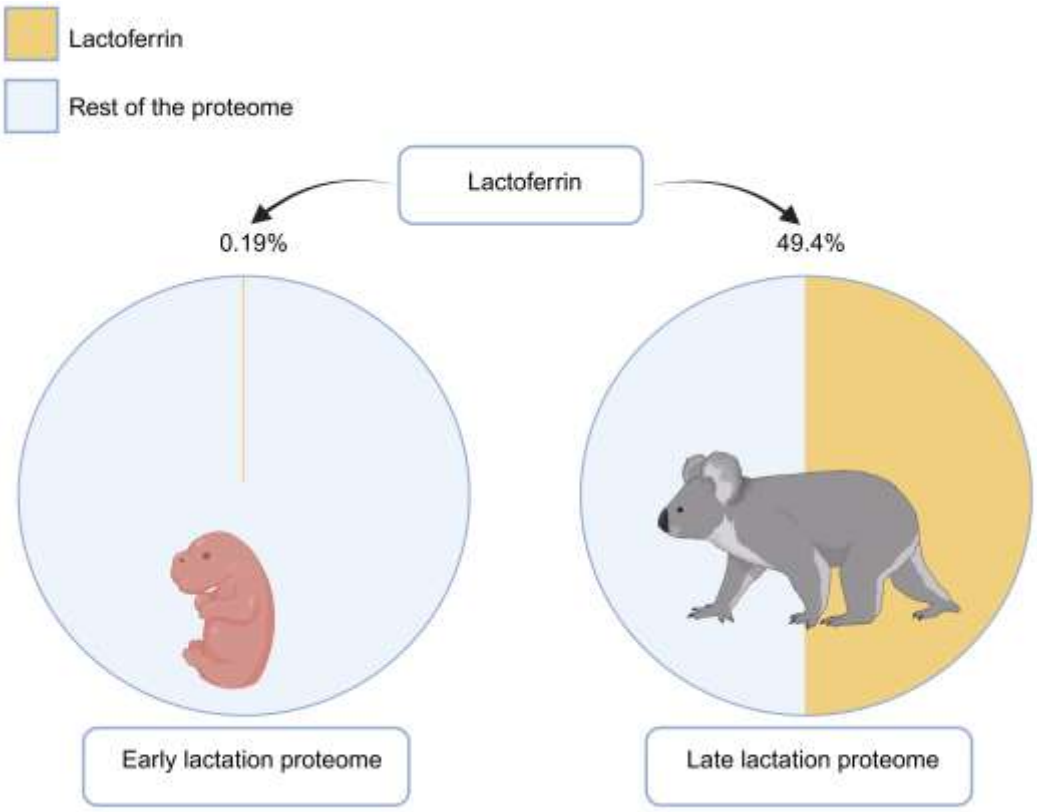

Fig. 1. Differential expression of lactoferrin between early and late proteomes. Lactoferricin accounted for 49.4% of all transcripts expressed in koala milk during late lactation, but just 0.19% of transcripts produced during early lactation.

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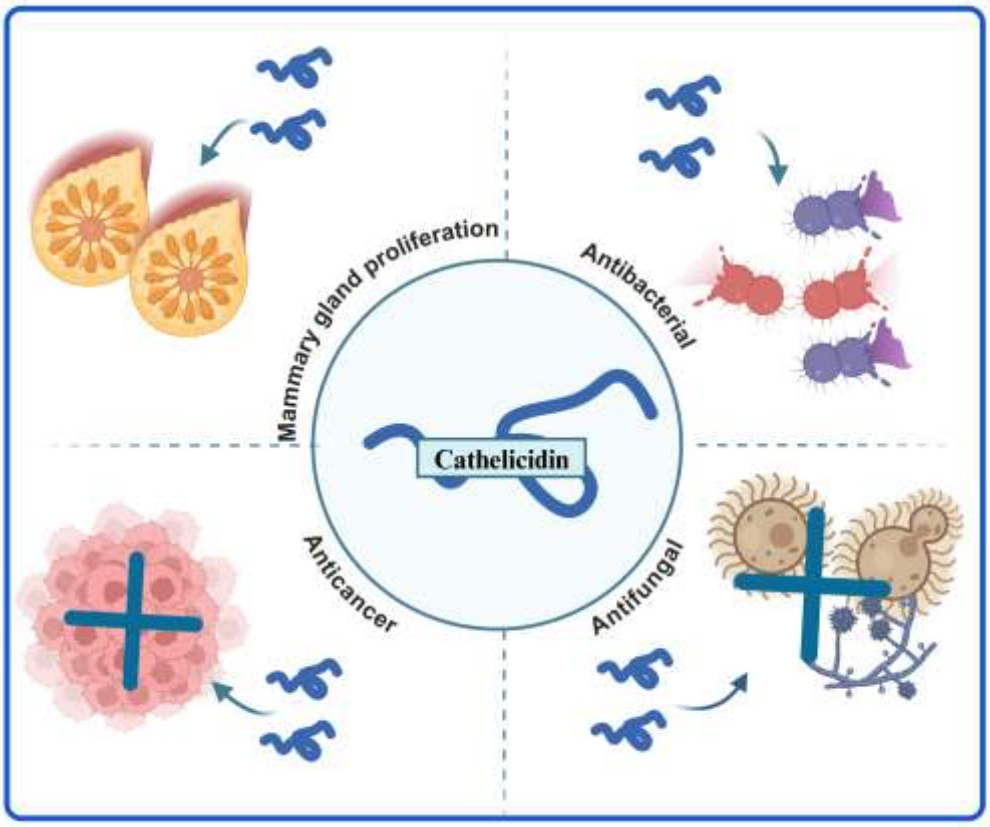

Fig. 2. Bioactive roles of cathelicidin.

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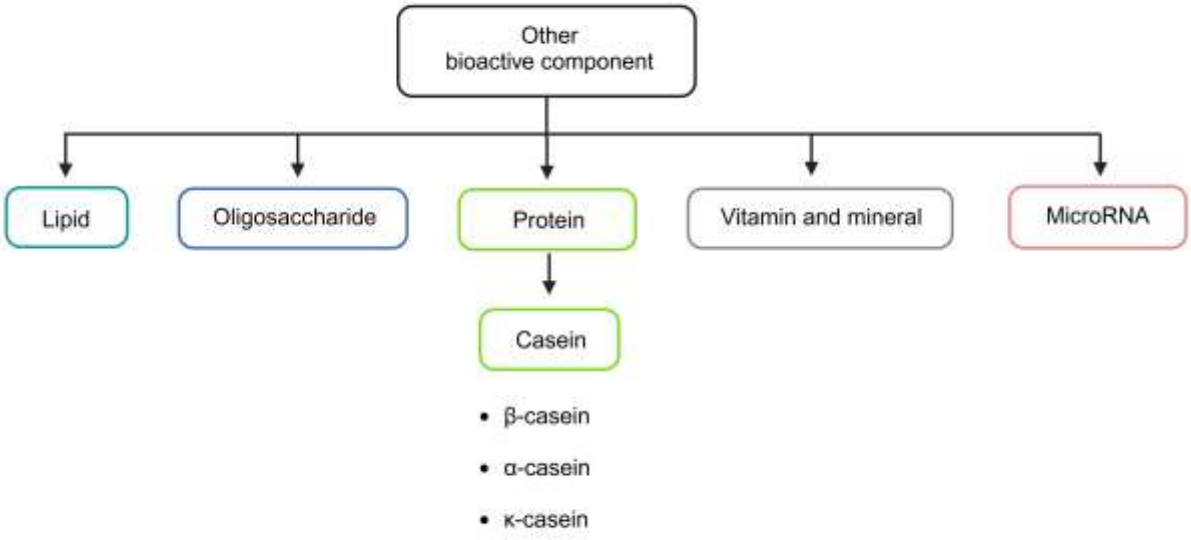

Fig. 3. Other potentially bioactive maternal compounds.

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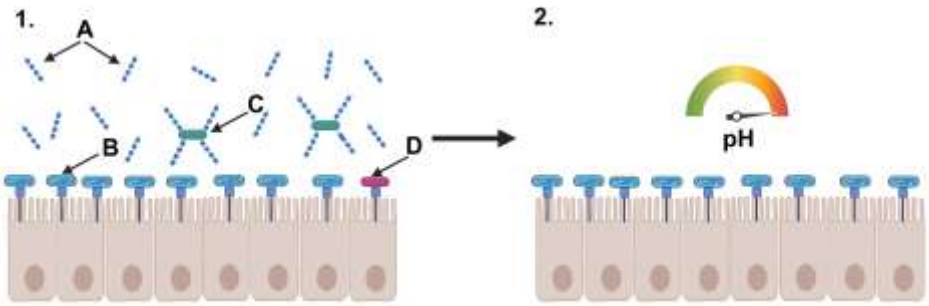

Fig. 4. 1. Oligosaccharides (A) promote the proliferation of beneficial bacteria (B) while serving as receptor analogues and binding to invading pathogens (C), blocking their attachment to the gut epithelium. 2. Proliferation of beneficial microflora results in lower pH levels, which leads to competitive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

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