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Preventing atopic dermatitis with probiotic supplementation - the role of selected breast milk components

The Probiotics in the Prevention of Allergy among Children in Trondheim (ProPACT) study

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
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Department of Public Health and Nursing

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Doctoral thesis

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Norsk sammendrag

Probiotika i forebygging av atopisk eksem –

rollen til utvalgte morsmelk faktorer

Probiotikatilskudd i siste del av svangerskapet og de første levemåneder forebygger utvikling av atopisk dermatitt hos barn. Denne effekten har blitt observert i samlestudier som har undersøkt probiotikatilskudd i forebygging av eksem. Dette til tross for mange ulikheter mellom de randomiserte kontrollerte studiene. Likevel er de biologiske mekanismene bak den forebyggende effekten ukjent. Dette doktorgradsarbeidet undersøker rollen til probiotika og utvalgte morsmelk faktorer i forebygging av atopisk eksem ved å benytte morsmelksprøver innsamlet under studien – «the Probiotics in the Prevention of Allergy among Children in Trondheim (ProPACT) study». Gravide kvinner ble rekruttert til ProPACT, randomisert til å få et kommersielt tilgjengelig syrnet melkeprodukt (Biola®) som inneholder tre probiotiske bakteriearter, eller syrnet placebo melk, fra svangerskapsuke 36 til 3 måneder etter fødselen. Biola® inneholder *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus acidophilus* La-5 (La-5) og *Bifidobacterium animalis* subsp. *lactis* Bb-12 (Bb-12). Ved to års alder ble det påvist ca. 40 % reduksjon i den kumulative forekomsten av atopisk eksem i probiotikagruppen sammenliknet med placebogruppen. Morsmelk gir først og fremst ernæring og væske til nyfødte barn, men morsmelk beskytter også mot sykdomsfremkallende mikrober, og anses å ha langvarige helseeffekter fordi morsmelken påvirker etableringen av mikrobene i tarmen (tarmmikrobiota) og utviklingen av mage/tarm- og immunsystemet. Blant faktorene i morsmelk som kan bidra til langvarige immunologiske effekter er mikrober assosierte med amming (undersøkt i Paper I), cytokiner og vekstfaktorer (Paper II) og det nylig beskrevne mikroRNA (miRNA; Paper III). De overordnede målene i denne avhandlingen var å undersøke om probiotikatilskudd til mor påvirket disse morsmelk faktorene, og om de bidro til den forebyggende effekten av probiotika mot atopisk eksem som ble observert i ProPACT studien.

Alle tre artiklene benytter morsmelksprøver som ble innsamlet fra deltagende kvinner i ProPACT studien ved 10 dager og 3 måneder. I artikkel I, undersøkte vi om de tre probiotiske bakteriene kunne påvises i morsmelk, og om mikrobesammensetningen i morsmelken hadde

blitt påvirket. Kun 10 av 472 morsmelksprøvene inneholdt de gitte probiotiske bakteriene og mikrobesammensetningen var uendret hos mødrene som fikk probiotikatilskudd. Om probiotiskatilskudd påvirket konsentrasjonen av cytokinene tymisk stromal lymphopoietin (TSLP) og transformerende vekstfaktorer β_1 (TGF- β_1), TGF-β_2, og TGF-β_3 ble undersøkt i artikkel II. Nivået av TSLP 10 dager etter fødsel viste en tendens til å være høyere blant kvinnene i probiotikagruppen, men dette så ikke ut til å ha bidratt i betydelig grad i den forebyggende effekten probiotika hadde mot eksem. Konsentrasjonene av TGF- β_1, TGF-β_2, og TGF-β_3 var ikke forskjellig mellom gruppene 10 dager og 3 måneder etter fødselen, og det var heller ikke forskjeller mellom gruppene mht. TSLP ved 3 måneder. I artikkel III undersøkte vi miRNA profilen 3 måneder etter fødsel i en undergruppe av deltagende kvinner (n = 54). Vi fant en gruppe med 5 miRNA som var høyt uttrykt i alle prøvene. Funksjonelle analyser av predikerte mål mRNA antydet et bredt spekter av potensielle biologiske effekter. Ingen individuelle miRNA viste en overbevisende sammenheng med hverken probiotisktilskudd eller utvikling av atopisk eksem.

Oppsummert; vi fant at tilskudd av LGG, La-5 og Bb-12 i 4 måneder til mor hadde minimal effekt på tilstedeværelsen av disse bakteriene i morsmelken. Det var også minimal effekt på konsentrasjonene av TLSP og tre ulike TGF-β og det relative uttrykket av miRNA i morsmelk. Amming resulterte ikke i nevneverdig overføing av de gitte bakteriene og ingen av de undersøkte morsmelkfaktorene syntes å ha bidratt i betydelig grad i forebyggingen av atopisk eksem. Imidlertid kan probiotika gi en forbigående økning i nivået av TSLP i morsmelk. Resultatene i artiklene I, II og III gir innsikt i sammensetningen av morsmelk, spesielt med hensyn til mikrobesammensetningen i morsmelk og miRNA profil.

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Overnevnte avhandling er funnet verdig til å forsvares offentlig for graden ph.d. i samfunnsmedisin.

Disputas finner sted i Auditorium MTA, Medisinsk teknisk forskningssenter, Fakultet for medisin og helsevitenskap, NTNU, onsdag 25. april 2018, kl. 12.15.

Summary

Probiotic supplementation during the later stages of pregnancy and during the first months of life prevents the development of atopic dermatitis in infancy. This effect has been observed in meta-analyses despite the use of differing bacterial strains and administration regimes across randomised controlled trials. However, the biological mechanisms behind this effect are incompletely understood. This doctoral work investigated the contribution of selected breast milk components to the preventative effect of probiotics using samples collected during one of the published randomised controlled trials – the Probiotics in the Prevention of Allergy among Children (ProPACT) trial. In the ProPACT trial, women were randomised to receive a commercially available fermented milk containing three probiotic bacterial strains (Biola®), or a placebo fermented milk, from 36 weeks gestation until 3 months after birth. Biola® contains *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus acidophilus* La-5 (La-5) and *Bifidobacterium animalis* subsp. *lactis* Bb-12 (Bb-12). At 2 years of age, the cumulative incidence of atopic dermatitis was approximately 40 % lower in the probiotic arm of the ProPACT trial.

The primary role of breast milk is to nourish and hydrate newborn infants. However, breast milk also provides protection against pathogens and is considered to have long lasting health consequences due its early influence on the establishment of the intestinal microbiota and developing gastrointestinal and immune systems. Among the breast milk components implicated in the long-term immunological effects of breast milk are the breastfeeding-associated microbiota (assessed in Paper I), cytokines and growth factors (Paper II), and the more recently described microRNAs (Paper III). The overriding aims of the papers presented in this thesis were to assess if maternal supplementation had influenced each of these breast milk components, and if they may have mediated the observed preventative effect of the ProPACT regime on the development of atopic dermatitis in infants.

The breast milk samples analysed in all three presented papers were collected from women participating in the ProPACT trial at 10 days and 3 months postpartum. In Paper I, we assessed if breastfeeding was a source of the administered bacteria and if the general breastfeeding-associated microbiota was affected by probiotic supplementation. The administered bacteria were only recovered in 10 of 472 breast milk samples and the breastfeeding-associated microbiota was unaltered. The influence of probiotic supplementation on the breast milk

concentrations of thymic stromal lymphopoietin (TSLP) and transforming growth factor-β_1 (TGF- β_1), TGF-β_2, and TGF-β_3 were assessed in Paper II. Whilst TSLP levels at 10 days postpartum tended to be higher among women in the probiotic arm of the trial, this does not appear to have significantly contributed to the preventative effect of probiotics. The concentration of TGF-β isotypes did not differ between the probiotic and placebo groups at either time point, nor did TSLP at 3 months. In Paper III, we assessed the microRNA profile at 3 months postpartum in a subgroup of women in the ProPACT trial (n = 54). We observed a group 5 miRNAs that were highly expressed in all samples, and functional analysis of predicted targets suggested a broad range of potential biological consequences. No individual miRNA was convincingly associated with either probiotic supplementation or the development of atopic dermatitis.

In conclusion, we found that a four-month period of supplementation with LGG, La5 and Bb-12 had a minimal effect on the composition of human milk with respect to the presence of these bacteria, concentrations of TSLP and TGF-β isotypes, and the relative expression of miRNAs. Breastfeeding does not result in substantial transfer of these bacteria and none of the investigated breast milk components appeared to significantly contribute to the preventative effect of maternal supplementation on the development of atopic dermatitis. However, probiotic supplementation may lead to a transient increase in TSLP levels in breast milk. More generally, the results of these studies provide insights into the human breast milk components, particularly with respect to the breastfeeding-associated microbiota and human milk miRNA profile.

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Supervisors:	Torbjørn Øien (main supervisor) and Ola Storrø (co-supervisor)

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This work has spanned the fields of epidemiology, microbiology, immunology and nucleic acid research, and I am extremely grateful to have worked with so many experienced co-authors. Your knowledge and advice has been invaluable. Particular thanks goes to Gaute Brede, who invited me into the laboratory and introduced me to the curious world of extracellular vesicles and microRNAs.

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My dear husband, Tomas, thank you for your patience, your support and making sure I "stay on target". And to our wonderful children, Frida and Torgeir, thank you for all the laughter and love that you bring. You three mean the world to me.

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List of papers

This thesis is based on the following three papers:

Paper I

Simpson MR, Avershina E, Storrø O, Johnsen R, Rudi K, Øien T. Breastfeeding-associated microbiota in human milk following supplementation with *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* La-5, and *Bifidobacterium animalis* supspecies *lactis* Bb-12. *J. Dairy Sci.* 2018. 101(2): Article in press; https://doi.org/10.3168/jds.2017-13411.

Paper II

Simpson MR, Rø AD, Grimstad Ø, Johnsen R, Storrø O, Øien T. Atopic dermatitis prevention in children following maternal probiotic supplementation does not appear to be mediated by breast milk TSLP or TGF-β. *Clin Transl Allergy*. 2016 Jul 22;6:27.

Paper III

Simpson MR, Brede G, Johansen J, Johnsen R, Storrø O, Sætrom P, Øien T. Human Breast Milk miRNA, Maternal Probiotic Supplementation and Atopic Dermatitis in Offspring. *PLoS One*. 2015 Dec 14;10(12):e0143496.

List of papers not included in the thesis

1. Dotterud CK, Storrø O, Simpson MR, Johnsen R, Øien T. The impact of pre- and postnatal exposures on allergy related diseases in childhood: a controlled multicentre intervention study in primary health care. *BMC Public Health*. 2013 Feb 8;13:123

2. Rø AD, Simpson MR, Storrø O, Johnsen R, Videm V, Øien T. The predictive value of allergen skin prick tests and IgE tests at pre-school age: the PACT study. *Pediatr Allergy Immunol*. 2014 Nov;25(7):691-8.

3. Simpson MR, Dotterud CK, Storrø O, Johnsen R, Øien T. Perinatal probiotic supplementation in the prevention of allergy related disease: 6 year follow up of a randomised controlled trial. *BMC Dermatol*. 2015 Aug 1;15:13.

4. Dotterud CK, Avershina E, Sekelja M, Simpson MR, Rudi K, Storrø O, Johnsen R, Øien T. Does Maternal Perinatal Probiotic Supplementation Alter the Intestinal Microbiota of Mother and Child? *J Pediatr Gastroenterol Nutr*. 2015 Aug;61(2):200-7.

5. Simpson MR. Systems biology: impressions from a newcomer graduate student in 2016. *Adv Physiol Educ*. 2016 Dec;40(4):443-445.

6. Rø AD, Simpson MR, Rø TB, Storrø O, Johnsen R, Videm V, Øien, T. Reduced Th22 cell proportion and prevention of atopic dermatitis in infants following maternal probiotic supplementation. *Clinical & Experimental Allergy*. 2017 Apr;47(8):1014-21.

7. Avershina E, Slangsvold S, Simpson M, Storrø O, Øien T, Johnsen R, Rudi K. Diversity of vaginal microbiota increases by the time of labor onset. *Scientific Reports* 7(1):17558.

Acronyms and abbreviations

AGO	Argonaute protein
APC	Antigen presenting cell
Bb-12	*Bifidobacterium animalis* subsp. *lactis* Bb-12
CFU	Colony forming units
CI	Confidence interval
DNA	Deoxyribonucleic acid
EAACI	European Academy of Allergy & Clinical Immunology
FcεRI	Fc epsilon receptor I (high affinity IgE receptor)
GWAS	Gene wide association studie(s)
HMO	Human milk oligosaccharide
IL	Interleukin
IQR	Interquartile range
ISAAC	International Study of Asthma and Allergies in Childhood
La-5	*Lactobacillus acidophilus* La-5
LGG	*Lactobacillus rhamnosus* GG
lncRNA	Long non-coding RNA
miRNA	MicroRNA
NDE	Natural direct effect
NIE	Natural indirect effect
OR	Odds ratio
OTU	Operational taxonomic unit
PACT	Prevention of Allergy among Children in Trondheim
PCR	Polymerase chain reaction

ProPACT	Probiotics in the Prevention of Allergy among Children in Trondheim
PUFA	Polyunsaturated fatty acids
qPCR	Quantitative real-time polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
sIgE	Specific IgE
SPT	Skin prick test
TGF-β	Transforming growth factor-β
tRNA	Transfer RNA
TSLP	Thymic stromal lymphopoietin
WAO	World Allergy Organisation

1 Background

The prevalence of allergy related diseases, such as atopic dermatitis (eczema), asthma and allergic rhinoconjunctivitis (hay fever), has increased over the past decades[1,2]. Whilst this increase was initially seen in developed countries, the continued worldwide increase in prevalence is now largely due to increasing rates in developing countries[1]. Epidemiological studies have identified a number of potential risk factors, although it is the so-called "hygiene hypothesis" and its refinements that have prompted the research in this thesis.

Modern interpretations of the "hygiene hypothesis" suggest that recent changes in the patterns and diversity of microbial exposure in early infancy may be partially responsible for the increased prevalence of allergy related diseases[3]. The notion that microbial exposure is particularly influential has led to a number of trials investigating the possibility of preventing and treating allergy related diseases using live microorganisms presumed to have beneficial health effects, or "probiotics". To date, the most promising results for probiotics have been in the prevention of atopic dermatitis in infants[4,5]. The Probiotics in the Prevention of Allergy among Children in Trondheim (ProPACT) study was one such trial. The ProPACT study demonstrated a nearly 40% reduction in the cumulative incidence of atopic dermatitis at 2 years of age after maternal supplementation with three probiotic bacterial strains[6]. The analyses presented in this thesis are based on breast milk samples collected from participants in the ProPACT study.

There are a number of interrelated research fields covered in this thesis, including allergy related diseases, probiotics as a preventative measure, and the composition of breast milk. This background section starts with the nomenclature, clinical presentations and epidemiology of allergy related diseases (§§1.1 - 1.2). Drawing on epidemiological observations, I elaborate on the "hygiene hypothesis", its revisions, and the associations that have been observed between atopic dermatitis and the microbiotas of the skin and the gut (§1.3). Next, I present evidence for the use of probiotics in the primary prevention of allergy related diseases and for atopic dermatitis in particular (§1.4). Whilst there is growing evidence for the use of probiotics in the prevention of atopic dermatitis, the biological mechanisms behind this effect are incompletely understood. Current understanding of the mechanisms of probiotics are introduced after first returning to the immunopathology of atopic dermatitis (§1.5 and §1.6). The background concludes with a section on breast milk, breastfeeding and an introduction to the selected

breast milk components that have been analysed in this thesis; namely the breastfeeding-associated microbiota, cytokines and microRNAs (§1.6). Each of these components has been assessed as a potential mediator of the preventative effect of probiotics on atopic dermatitis.

1.1 Definitions and nomenclature in allergy

Historically, the terms "hypersensitivity", "allergy", "atopy", "allergic diseases" and "atopic diseases" have been defined inconsistently. Sometimes "allergy" and "atopy" have been used synonymously. At other times, their use has implied specific underlying pathophysiology.

In an effort to standardise the use of these terms, the European Academy of Allergology and Clinical Immunology (EAACI)[7] and the World Allergy Organisation (WAO)[8] have proposed definitions for the terms "hypersensitivity", "allergy" and "atopy" as follows: A "hypersensitivity" reaction is defined by WAO as "objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons". The term "allergy" is limited to hypersensitivity reactions that are "… initiated by specific immunological mechanisms". Allergies can be divided into antibody- and cell-mediated allergies, with Immunoglobulin E (IgE) mediated reactions predominantly responsible for the former type of allergic reaction. In contrast, "atopy" is defined as

> "a personal and/or familial tendency, usually in childhood or adolescence, to become sensitised and produce IgE antibodies in response to ordinary exposures to allergens, usually proteins. As a consequence, these persons can develop typical symptoms of asthma, rhinoconjunctivitis, or eczema."

Allergens are molecules that are capable of inducing an immediate (or type I) allergic reaction as a result of their binding to specific IgE. In healthy individuals, exposure to these allergens is harmless. WAO/EAACI suggested that the description "atopic" should only be used after demonstrated IgE sensitisation through skin prick testing or serum analysis.

Under this proposed nomenclature, using the term "allergic" or "atopic" in conjunction with disease names, such as in "allergic asthma" or "atopic dermatitis", implies that there is a defined stimulus and or specific immunological mechanism at play. However, the use of these terms remains less stringent in both research publications and clinical arenas. In particular, "atopic dermatitis" or "atopic eczema" remains a commonly used name for skin inflammation with a typical clinical presentation, regardless of whether IgE sensitisation has been

demonstrated. Atopic dermatitis is used throughout this thesis to describe the clinical disease, and the terms IgE-associated or non-IgE associated atopic dermatitis are used to indicate the presence of allergic sensitisation when appropriate.

It is also worth pointing out that, the majority of cases of atopic dermatitis do not have any hypersensitivity to a defined stimulus and should not technically be considered an allergic disease under the WAO/EAACI definitions. Additionally, there is a high degree of comorbidity between atopic dermatitis, asthma and rhinoconjunctivitis in children both with or without IgE sensitisation, and with or without a family history[9]. Since atopic dermatitis, asthma and allergic rhinoconjunctivitis tend to coexist, even in the absence of IgE sensitisation, the term "allergy related diseases" is used to describe this group of diseases throughout the thesis and Papers I and II. In Paper III, the terms "allergy related diseases" and "allergic diseases" are used synonymously. Each of these diseases have been diagnosed based on clinical signs and symptoms, independently of evidence of IgE sensitisation, and are described in more detail in the coming sections.

1.2 Allergy related diseases and the atopic march

Atopic dermatitis, asthma and allergic rhinoconjunctivitis are chronic, inflammatory diseases that represent the most common non-communicable diseases in childhood and throughout life[10]. Whilst the majority of children with allergy related diseases will only have one of these diseases, there is a well described pattern of comorbidity between atopic dermatitis, allergic sensitisation, asthma and allergic rhinoconjunctivitis. In such cases, atopic dermatitis is usually the first to present with symptoms in infancy, followed by the subsequent development of sensitisation and food allergy, asthma, and allergic rhinoconjunctivitis. This typical progression of disease development is referred to as the "atopic march" (Figure 1). Originally, the coexistence of the allergy related diseases was thought to be primarily due to an underlying immunological predisposition with organ-specific symptoms arising at different ages. A dysfunctional epidermal barrier and atopic dermatitis were considered to be the cutaneous expression of this underlying allergic tendency. More recently, it has been postulated that the dysfunctional epidermal barrier seen in atopic dermatitis may be the initial pathology that leads to an aberrant immune response in susceptible individuals[11-13].

The clinical presentation and epidemiology of atopic dermatitis, asthma, allergic rhinoconjunctivitis and allergic sensitisation are introduced in the coming sections. Other related conditions, such as food allergies, urticaria and anaphylaxis, are briefly presented as clinical consequences of allergic sensitisation. The study of these as clinical outcomes is beyond the scope of this thesis.

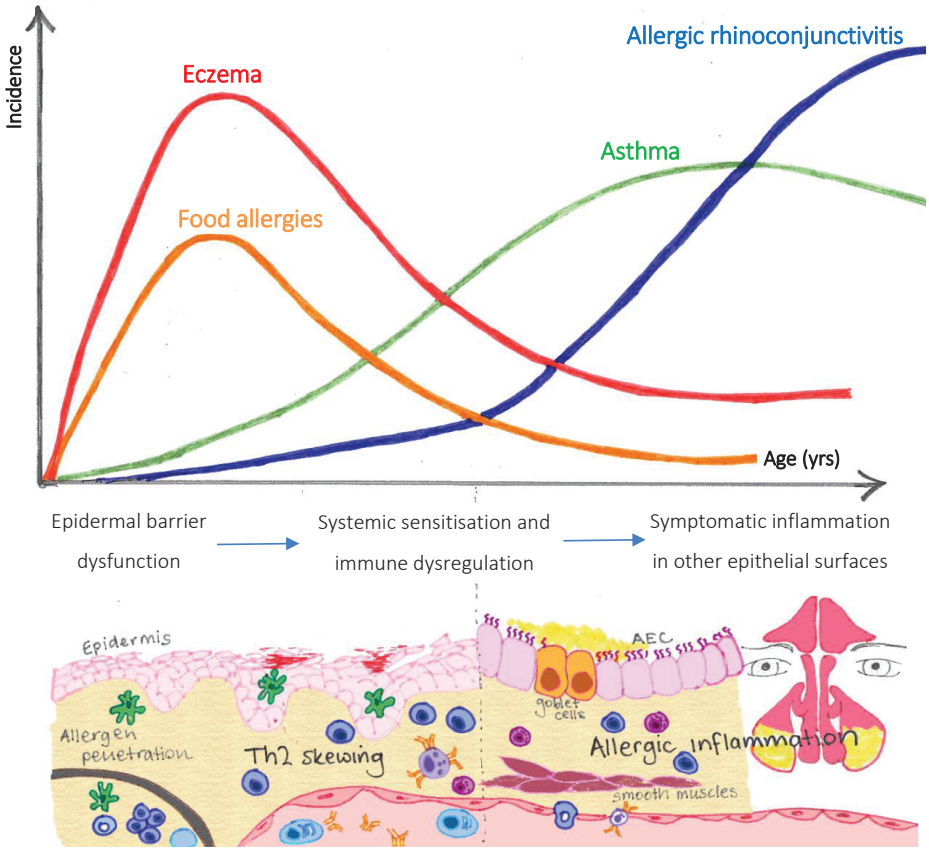


Figure 1: Illustration of the atopic march and the hypothesised causal link between atopic dermatitis and later asthma and allergic rhinoconjunctivitis. Incidence of allergy related diseases adapted from Figure 1 in Barnetson, RS and Rogers M. 2002. Childhood atopic eczema. *BMJ* (Clinical research ed.) 324(7350):1376. Immunopathology schematic adapted from Weidinger and Novak (2015)[13] and Papadopoulos et al (2012)[26].

1.2.1 Atopic dermatitis

Atopic dermatitis is a chronic, relapsing, inflammatory skin disease that often begins in infancy, and typically presents with dry, erythematous skin on the face and extensor surfaces. Around 60 % of patients with atopic dermatitis reportedly develop symptoms within the first year of life[13]. Whilst many children outgrow their atopic dermatitis, others have persistent problems throughout childhood, and or recurrence in adolescence[14,15]. The flexures of the elbows and knees are more commonly affected in these older children, as well as in children who first develop atopic dermatitis after infancy. Atopic dermatitis in adulthood tends to be characterised by persistent, localised inflammation and often involves the face, upper body and hands. In all age groups, there is a spectrum of severity from mild intermittent lesions, to widespread disease with substantially impaired quality of life for patients and their carers, and high direct and indirect costs[13,14,16]. A German birth cohort study found that severe disease in infancy, early allergic sensitisation, parental history of atopic dermatitis and concomitant symptoms of recurrent wheeze were factors that predicted persistence of atopic dermatitis from infancy to 7 years of age[15].

Worldwide, the prevalence of atopic dermatitis varies by country and is reported to range from 2 – 24 % in school children[1,17,18]. Norwegian estimates have tended to be at the higher end of this range[18-20]. As mentioned, a significant proportion of children who develop atopic dermatitis will present with symptoms in the first year of life. Using questionnaire based assessment of disease, Norwegian and Danish cohorts have reported a cumulative incidence of atopic dermatitis of 13 – 17 % at 18 to 24 months of age[21-24]. Between 2000 and 2008 in Trondheim, the local cumulative incidence of atopic dermatitis in 2-year-old infants remained around 16 – 17 % throughout the community-based intervention Prevention of Allergy among Children in Trondheim (PACT) study[24]. Also using questionnaire based data, the Mechanisms of the Development of Allergy (MeDALL) research team pooled results from 12 European cohorts and estimated the lifetimes prevalence of atopic dermatitis to be 27 % and 34 % at 4 and 8 years, respectively[25].

As the most common and earliest presentation of the allergy related diseases, atopic dermatitis is a convenient outcome in prevention studies. For this reason, atopic dermatitis is the primary health outcome of interest in this thesis. Under the theory that the epidermal barrier dysfunction seen in atopic dermatitis is the forerunner to the atopic march, the prevention of

atopic dermatitis may be an important first step in efforts to prevent other allergy related diseases.

1.2.2 Asthma

Asthma is a chronic lung disease characterised by intermittent symptoms of wheezing, chest tightness, coughing and shortness of breath[26]. The symptoms of asthma are a result of bronchial hyperresponsiveness and airway inflammation leading to reversible airflow obstruction. Exercise, pollens, cold air and weather changes, tobacco smoke, and respiratory tract infections classically trigger episodes of asthma. Symptoms may also be worse at night and early in the morning[26]. Before puberty, boys are more commonly affected than girls, and this trend is reversed after puberty[27].

The diagnosis of asthma is difficult, and controversial, in young children. Objective spirometry evidence of reversible airway obstruction is the preferred method of diagnosis. However, spirometry is generally only possible after 5 years of age when children are more able to cooperate[28]. Using questionnaire based estimates of asthma prevalence, the International Study of Asthma and Allergies in Childhood (ISAAC) reported that the prevalence of asthma in 6-7 years olds ranged from <5% to over 20% in 13-14 year olds[28,29]. European estimates from the MeDALL study suggest that the lifetime risk of asthma is 8 % and 15 % at 4 and 8 years, respectively[25].

In infants, the most common clinical expression of asthma is recurrent wheezing. Whilst many infants with recurrent wheezing will not develop asthma, it represents a major cause of morbidity and is a risk factor for later asthma[28,29]. A survey of over 30,000 infants in two continents estimated that the prevalence of recurrent wheezing during the first year of life was 22% and 15 % in Latin American and European infants, respectively[29]. In Trondheim, 26 % of infants were reported to have had at least one episode of wheezing at 2 years of age in the PACT study[23,24]. Also in the PACT study, the prevalence of doctor diagnosed asthma among 2 year olds reduced from 7 % to 5 % after families received structured advice during routine clinical follow-up aimed at reducing cigarette smoke exposure, improving indoor dampness, and increasing dietary intake of fish and n-3 polyunsaturated fatty acids (PUFAs)[24].

1.2.3 Allergic rhinoconjunctivitis

Allergic rhinoconjunctivitis is characterised by nasal congestion, sneezing and itchy, watering eyes. Symptoms may only be present, or worsened, during the pollen season. This pattern of symptoms inspired the colloquial term "hay fever". Other people experience perennial nasal congestion, often due to other airborne allergens such as dust mite or household pets[30]. Allergic rhinoconjunctivitis tends to develop later in childhood or adolescence compared to atopic dermatitis and asthma. In the PACT study, less than 3 % of children had parentally reported allergic rhinoconjunctivitis at 2 years of age[23]. This is similar to the results from the MeDALL study which estimate the lifetime prevalence of allergic rhinitis to be nearly 4 % at 4 years. By 8 years of age, 16 % of the children in the MeDALL study had developed allergic rhinitis[25]. Epidemiological studies have variously focussed on allergic rhinitis (i.e. without ocular symptoms) or allergic rhinoconjunctivitis, although it is reported that most children experience both ocular and nasal symptoms[30].

1.2.4 Allergic sensitisation

Allergic sensitisation refers to the production of IgE molecules to specific allergens, also known as specific-IgE (sIgE). The process of sensitisation in susceptible individuals starts with an initial exposure to an allergen and ends with activated mast cells coated with sIgE molecules (Figure 2)[31]. After ingestion, inhalation or contact, an allergen is taken up and processed by antigen presenting cells (APCs), such as dendritic cells and B cells. Antigen-activated dendritic cells migrate to regional lymph nodes, or local lymphoid tissues, where they present the peptide fragments. In the presence of a pro-allergenic cytokine milieu, presentation of peptide fragments to naïve T cells results in differentiation of these cells into T helper cells type 2 (Th2). Interleukin 4 (IL-4) is the primary cytokine implicated in the promotion of Th2 differentiation, and is expressed by other Th2 cells, mast cells, basophils and eosinophils[32,33]. In turn, Th2 cells promote the differentiation of B cells into sIgE producing plasma cells. Mast cells and basophils express an Fc receptor with high affinity to IgE (FcεRI) such that the sIgE molecules produced by the plasma cells bind to mast cells and basophils, and are expressed on their cell surface. The next time the individual is exposed to this allergen, cross-binding of the FcεRI-IgE complexes results in mast cell degranulation and the release of histamines, tryptases, prostaglandins and leukotrines. The release of these molecules results in local or systemic

vasodilation and increased vascular permeability. In the airways, they also cause bronchoconstriction and increased mucus production. Clinically this may result in immediate local or systemic symptoms including urticaria, angioedema and anaphylaxis[31].

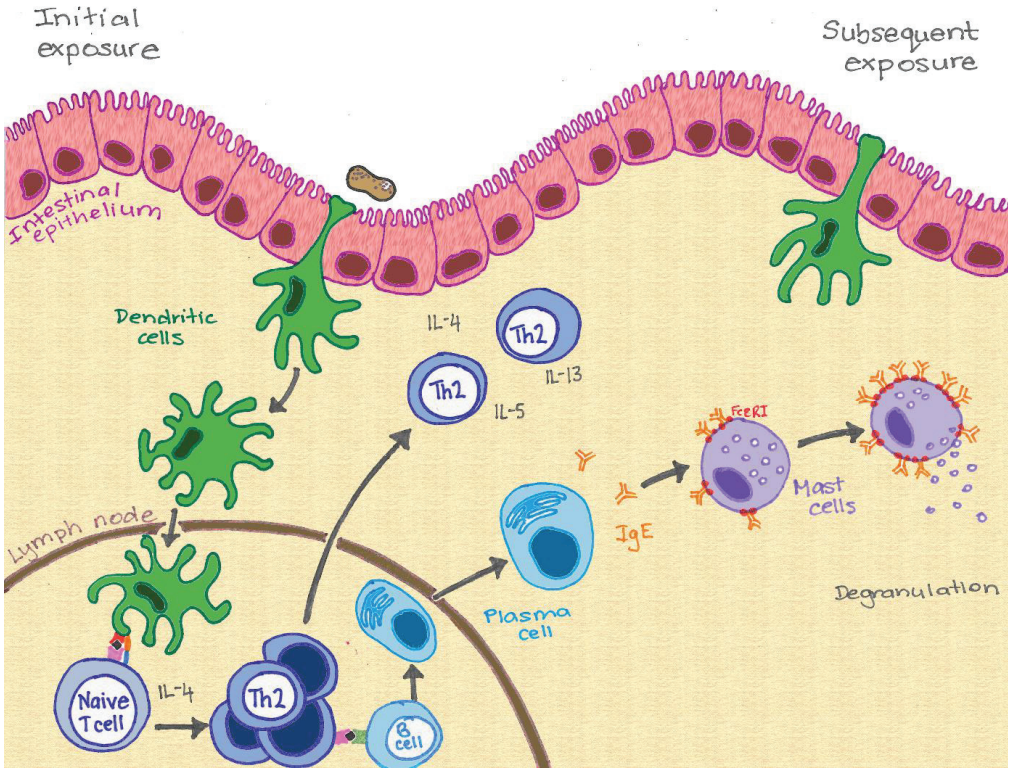


Figure 2: The process of allergic sensitisation from initial exposure to subsequent re-exposure. Figure adapted from: Galli and Tsai (2012)[31]

Allergic sensitisation is usually determined by demonstrating the presence of sIgE molecules through skin prick testing (SPT) or measurement of serum sIgE to common food and airborne allergens. Results from the MeDALL group suggest that 21 % and 39 % of children are sensitised to at least one common allergen by 4 and 8 years of age, respectively, and that sensitisation is more common in children with allergy related disease symptoms[9,25]. However, not all individuals that produce sIgE have clinical symptoms of an allergic reaction on exposure to the relevant allergen and not all individuals with allergy related diseases are found to have sIgE to common allergens. Indeed, estimates from the Swedish BAMSE study suggest that 23 % of children who have a positive sIgE test sometime before the age of 16 do not develop eczema, asthma or rhinitis[34]. On the other hand, 61 % and 67 % children who had ever developed atopic dermatitis or asthma were sensitised at some point during the study, respectively. Rhinitis was more commonly associated with IgE sensitisation with 78 % of children having been sensitised at least once during the study[34]. These proportions are dependent on the population, definition of allergic sensitisation and age of follow-up.

1.2.5 Trends in allergy related diseases

The worldwide prevalence of allergy related diseases is high and increasing[1,2]. During the 1970's and 1980's the prevalence of atopic dermatitis, asthma and allergic rhinoconjunctivitis were reported to be on the rise in several high-income countries, including Great Britain, Australia, New Zealand and Japan[35,36]. Correspondingly, increasing prevalence of all three diseases were observed in Finnish and Swedish national service recruits between the 1950's and 1980's[37,38]. In a follow-up study, Finnish researchers observed an ongoing increase in the prevalence of asthma and allergy up until 2003, and a stabilisation of the prevalence of atopic dermatitis[39]. More recently, the ISAAC study has substantially contributed to our understanding of the epidemiology and trends of allergy related disease, also in low- and middle-income countries. Data from ISAAC suggests that there is an ongoing global increase in prevalence, but this may be predominantly due to increases in low- and middle-income countries. The prevalence of allergy related diseases in high-income countries appears to have plateaued[1]. These observations are partially consistent with Norwegian data which suggested there has been a stabilisation of the prevalence of atopic dermatitis, but a continued increase in asthma or allergic rhinoconjunctivitis, in school aged children after 1995[19,20].

There is undoubtedly a strong genetic component to these diseases, although the recent rise in prevalence suggests that environmental factors also play a substantial role[10,13]. It also appears likely that the risk factors for these three diseases are intrinsically linked since the pattern of comorbidity has been stable across time and similar between high-, middle- and low-income countries[40]. Observational and ecological studies have identified geographical, urban-rural and socioeconomic trends in addition to the time trends. Urbanisation and westernisation is an important contributor to several risk factors that have been associated with allergy related diseases including decreasing family size, dietary habits (low dietary fibre, high saturated fat, relatively lower n-3 PUFAs), reduced exposure to household and farm animals and increased antibiotic use[10,41-43]. Families and children are also spending more time indoors in well insulated houses, resulting in a more sedentary lifestyle, reduced sunlight exposure, vitamin D deficiency, and increased exposure to house dust mite and mould. These factors have variously been linked to allergy related disease, along with environmental tobacco smoke exposure, persistent organic pollutants and other chemical toxins. Many of the risk factors mentioned here are also implicated in other non-communicable diseases that present later in life, such as cardiovascular disease, metabolic disease, cancer and chronic lung disease. Each of these have an underlying low-grade inflammation. Whilst the inflammatory processes in allergy related diseases and late-onset non-communicable diseases are not identical, there is growing evidence that increasing standards of hygiene may be driving these "modern" diseases by reducing microbial exposures and changing intestinal colonisation patterns[10,44].

1.3 The evolving hygiene hypothesis

The leading theory of why the prevalence of allergy related diseases has risen over recent decades is the "hygiene hypothesis". The hygiene hypothesis is widely attributed to Professor David Strachan and his 1989 publication where he suggested that the increasing incidence of allergy related diseases may be a consequence of reduced rates of infection during childhood[45]. In a British birth cohort of over 17 000 young adults, Strachan observed that there was an inverse relationship between the prevalence of hay fever and eczema, and the number of older siblings in the family. Strachan remarked that "These observations … could be explained if allergic diseases were prevented by infection in early childhood, transmitted by unhygienic contact with older siblings…"[45]. A similar observation had been made by Gerrard et al in 1976, when they described an association between an "unhygienic lifestyle" and lower rates of allergy

related diseases in indigenous Canadians living in rural communities compared to urban Caucasian Canadians. Gerrard et al also suggested that "… atopic disease is the price paid by some members of the white community for their relative freedom from diseases due to viruses, bacteria and helminths" (as cited in Lambrecht and Hammad, 2017[41]). Furthermore, Gerrard et al linked the type of microbial exposure to the development of immune responses observing that the indigenous Canadians had persistent high levels of IgE throughout life, probably due to helminth infection. On the other hand, the common infections "… encountered in the developed, hygienic world…" did not result in ongoing production of IgE and instead some atopic individuals developed allergen specific IgE production[46]. Since the publication of these early observations, the colloquial term "hygiene hypothesis" has emerged and a number of extensions, revisions, reformulations, updates and refinements have been proposed.

Today, there is a greater focus placed on the patterns and characteristics of early microbial exposure, rather than the absence of pathogenic infections, as the underlying cause of the increase in allergy related diseases. This change in thinking has been presented with varying emphasis on the pattern and stability of intestinal colonisation[47-49], the types of microbes[50], the diversity of these microbes[47] and biodiversity in general[51]. Whilst a number of observational studies have investigated the association between specific infections and the development of allergy related diseases, the epidemiological evidence for a causal preventative effect is considered to be weak[43,49]. Instead, it has been suggested that the more hygienic existence of the developed world has not only resulted in fewer pathogenic infections in childhood, but also an "abnormally" stable intestinal microbiota[48,49]. It is hypothesised that a more stable intestinal microbiota does not promote, and may even prevent, the development of oral tolerance. Reduced levels of Enterobacter and increased levels of Staphylococcus and Clostridium species have been highlighted as potentially detrimental shifts in the intestinal microbiota of infants and children in industrialised countries[49]. Antibiotic use and changing dietary habits have also been implicated as drivers of this shift[48]. Similarly, the "Old Friends Hypothesis" suggests that modern urbanisation has resulted in the loss of key microorganisms which have co-evolved with our immune system[50,52]. Helminth infections, non-pathogenic environmental bacteria and gut commensals are counted among the "Old Friends" and it is hypothesised that these microorganisms have promoted immunoregulation and tolerance throughout a mutual evolutionary process. Rather than the loss of specific microorganisms, others suggest that a

decrease in overall microbial diversity[47], or even the biodiversity of our immediate environments more generally[51], may be responsible for the increasing prevalence of allergy related diseases. The hygiene hypothesis has also been broadened to encompass other immune-mediated diseases, including inflammatory bowel disease, multiple sclerosis and type I diabetes mellitus, since these also appear to be associated with industrialisation and affluence[53].

These hypotheses have a common underlying notion that an increasingly hygienic lifestyle has led to a critical change in our microbial exposures early in life. In turn, this has altered the initial development of the foetal and neonatal immune system and resulted in the aberrant immune responses seen in allergy related and autoimmune diseases. The relationship between the intestinal microbiota and the development of immunological tolerance of harmless common allergens and self-antigens is a central theme in these hypotheses. The nature of this critical change and the exact immunological consequences are topics of ongoing investigation.

1.3.1 Human microbiota and the development of allergy related diseases

Trillions of microbes colonise our skin and the mucosal surfaces of our gastrointestinal and respiratory systems. The composition of the human microbiota varies across body sites, with the gastrointestinal tract harbouring the greatest number and diversity of microbes. Humans have come to live in symbiosis with microbes and together have developed both mutualistic and commensalistic relationships throughout more than 200 million years of mammalian-microbe coevolution[54]. In recent years, there has been increasing research into associations between the human microbiota and a wide range of diseases and conditions from infancy to old age. The implication is that a harmful shift in the composition of the human microbiota, referred to as a dysbiosis, is partially responsible for a range of disease states from gastrointestinal and respiratory conditions to neuro-psychological and metabolic disease[55].

In the study of allergy related disease and the human intestinal microbiota, no single species or genus has been consistently associated with a preventative or causative effect on allergy related diseases[53]. However, there have been several reports of increased risk of allergy related disease among children who had reduced levels of *Bifidobacterium* and *Lactobacillus* in infancy[53,56]. Other studies have suggested that high relative abundance of *Clostridium difficile* may be related to later allergy related disease. Interestingly, delayed colonisation and a low

relative abundance of *Bifidobacterium* and or *Bacteroides* have also been associated with other risk factors for allergy related disease, including the absence of siblings, preterm birth and caesarean section delivery[53]. The potentially protective effect of breastfeeding on allergy related diseases is discussed in more detail below (§1.7.1), however it is worth noting here that breastfeeding supports the growth of *Bifidobacterium* species.

With the advancement of sequencing technologies and other culture-independent techniques, recent studies have been able to assess the overall diversity of the intestinal microbiota and have observed that infants with a low diversity were more likely to develop atopic dermatitis in childhood[57,58] and to have more severe symptoms[59]. The diversity of the *Bacteroidetes* phylum was found to be particularly predictive of later atopic dermatitis in one of these studies[57,60]. A lower intestinal microbiota diversity in infancy has also been associated with the development of allergic sensitisation[61], asthma[60] and allergic rhinoconjunctivitis[61]. Although, the associations between microbiota diversity and each allergy related disease has not been consistently observed between these studies.

Fewer studies have investigated the role of the skin microbiota in the development of atopic dermatitis and other allergy related diseases[62]. The skin microbiota of patients with established atopic dermatitis differs to healthy controls, particularly with increased colonisation with *Staphylococcus aureus* and reduced microbial diversity. However, it is unclear if these changes contribute to or are a result of the skin barrier dysfunction in atopic dermatitis. Similar to the interplay between the intestinal microbiota and the immune system, it is postulated that the skin microbiota have systemic effects through its influence on the innate and adaptive immune responses in the skin[62].

1.4 Probiotics in the primary prevention of allergy related diseases

Probiotics are defined as "Live organisms which, when administered in adequate amounts, confer health benefit on the host."[63]. The most widely used probiotics include species of the *Bifidobacterium* and *Lactobacillus* genera and these are now found in a range dietary supplements and foods, particularly fermented dairy products. With rising interest in the health implications of dysbiosis in the human intestinal microbiota there has also been a similar increase in the number of clinical trials investigating the use of probiotics as a means of restoring a more beneficial microbial composition. The idea of correcting a dysbiosis with probiotics is appealing and they have been employed in a broad range of diseases from infancy to old age (Figure 3). However, the evidence for the use of probiotics in most situations is limited.

In the field of allergy research, probiotics have been trialled in both the prevention and treatment of allergy related diseases with partially conflicting results[5,64-70]. The most promising results have come from randomised controlled trials (RCTs) investigating the use of probiotics in the prevention of atopic dermatitis. Meta-analyses including 14 – 17 of these RCTs suggest that there is a statistically significant preventative effect of probiotic supplementation on atopic dermatitis for infants when the probiotics are given around the time of birth[5,64,65]. By and large, each of these meta-analyses included the same RCTs although there were slight differences due to their individual inclusion and exclusion criteria, and method of analysis. Overall, probiotic supplementation is estimated to reduce the relative risk of developing atopic dermatitis by 20-30%[5,64,65].

In line with these findings, the World Allergy Organisation (WAO) recently amended their recommendations to support the use of probiotics in the primary prevention of atopic dermatitis in infants at high risk of developing allergy related diseases because of a positive family history. Specifically, they recommend that mothers of high risk infants take probiotics during pregnancy and whilst breastfeeding, and that supplementation is also given to these infants after birth[4]. Other national and international authorities are reluctant to advise the use of probiotics because of the high degree of heterogeneity between the current clinical trials. Also for this reason, WAO describes their recommendations as "conditional" and of "very low quality".

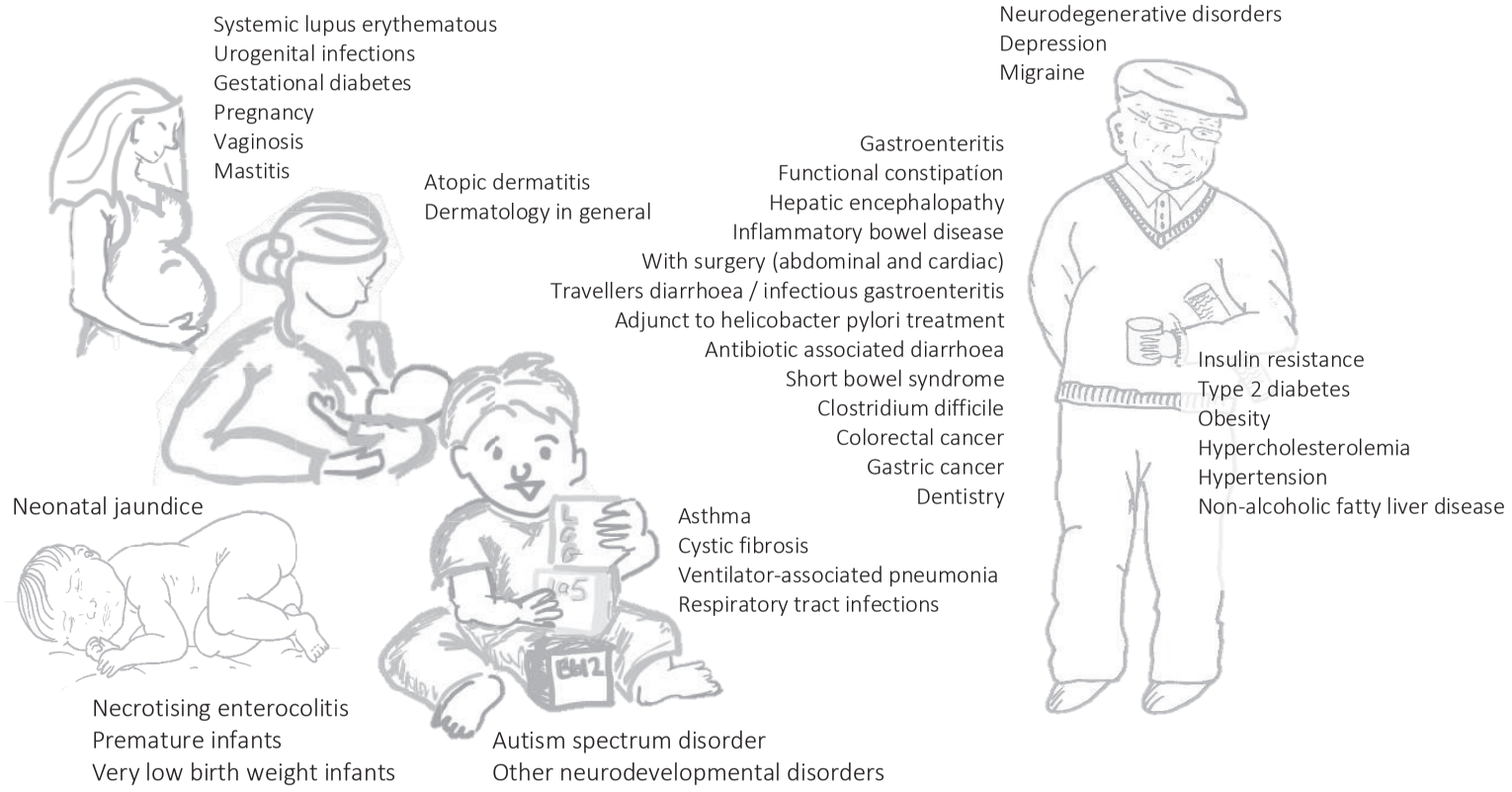


Figure 3: Probiotics in health and disease from infancy to old age. Probiotics have been considered and or trailed in a wide range of diseases and conditions. The list displayed here was constructed by searching for probiotics in the title of review articles in PubMed (December 2017). There were over 3,700 entries in this search and this list is undoubtable incomplete.

The heterogeneity in current trials is a result of differences in the administered strain(s), as well as the timing, duration and recipient of the supplementation, and the age and diagnostic criteria at follow-up. As such, there is a high degree of uncertainty about the optimal regime. Only two trials have conducted head-to-head comparisons of probiotic strains[71-74]. Wickens et al [71-73] observed that mother-infant supplementation with *Lactobacillus rhamnosus* HN001, but not *Bifidobacterium longum* subsp. *lactis* HN019, resulted in a clinically and statistically significant reduction in the cumulative incidence of atopic dermatitis at 2, 4 and 6 years of age compared to a placebo supplement. This study supports the theory that the preventative effect of probiotics may be strain specific. In the other head-to-head trial, Rautava et al[74] found an equivalent effect of two different probiotic regimes compared to placebo. In this trial, they observed an approximately 60% relative reduction in the cumulative incidence of atopic dermatitis at 2 years of age following maternal supplementation with a combination of *Bifidobacterium longum* BL999 together with either *Lactobacillus rhamnosus* LPR or *L. paracasei* ST11. In addition to these two head-to-head trials, comparison of the effectiveness of particular strains or combinations of strains is limited to subgroup analyses within meta-analyses. The largest preventative effect appears to be associated with regimes that employed combinations of probiotics[5,64,75].

With respect to the timing and recipient(s) of the probiotic supplementation, no head-to-head trials have been performed. However, subgroup analyses performed as a part of meta-analyses suggest that regimes with pre- and postnatal supplementation are more effective than regimes that gave probiotics only before or after birth[5,76,77]. It is important to note that there is only one trial employing prenatal supplementation alone[78], and four trials using postnatal supplementation alone[79-85]. In addition to establishing whether the optimal regime involves supplementation for mothers and or infants, it is important to determine if these results are also applicable to a general population. Most of the trials have included only infants considered to be at a high risk of developing allergy related diseases. For this reason, WAO limited their recommendations to high risk infants. Our own study, the ProPACT study, is the only study to investigate the effect of pre- and postnatal supplementation in a general population[6]. Two additional studies have investigated the effect of probiotics in a general population when given directly to infants after weaning or introduction of formula feeding[79,80,85]. Overall, pooled

results from these three trials suggest that there may be a beneficial effect also in general populations[5].

Probiotics also appear to have an ongoing preventative effect on atopic dermatitis into childhood. A meta-analysis that specifically included studies assessing the effect of probiotics at 5 years or older reported a pooled relative risk for atopic dermatitis of 0.86 (95% CI 0.77 – 0.96). Subsequent to this meta-analysis, 2 additional studies have published longer term follow-up including the 6-year follow-up of the ProPACT trial[86] and a 10 year follow-up[87]. Both of these studies report an ongoing reduction in the cumulative incidence of atopic dermatitis.

Turning our attention to the prevention of other allergy related diseases, the benefit of probiotics is less convincing. If atopic dermatitis is a causal forerunner of the atopic march, one would expect to see a subsequent preventative effect on allergic sensitisation, asthma and rhinoconjunctivitis. However, there is less certain protective effect on allergic sensitisation[65,88,89] and no observed statistically or clinically significant effect on asthma, wheeze[64,65,88,90] or allergic rhinoconjunctivitis[64,65] according to meta-analyses assessing these endpoints. The timeline of the atopic march (Figure 1) would suggest that any preventative effect of probiotics on allergic sensitisation should also be most apparent in infancy. Indeed, a recent meta-analysis reported an overall preventative effect on any allergic sensitisation in infancy (RR 0.78, 95% CI 0.66 – 0.92), and that this effect appeared to be greatest when pre- and postnatal supplementation was combined (RR 0.71, 95% CI 0.57 – 0.89)[89]. Another meta-analysis including the same group of studies found a slightly weaker association between probiotic ingestion and any allergic sensitisation (RR 0.90, 95% CI 0.80 – 1.00), however this meta-analysis opted to use the most recently published follow-up from each included trial, such that the pooled results represent allergic sensitisation in a mix of infants and school aged children[88]. Whilst probiotics may prevent allergic sensitisation, their effect on the development of food allergy is unclear. Both increased and decreased prevalence of food allergy after probiotic supplementation have been reported from meta-analyses[65,89] and current studies have not employed objective assessment of food allergies.

There is no apparent effect of probiotics on asthma and allergic rhinoconjunctivitis in current meta-analyses, although this observation should be interpreted cautiously for a number of

reasons. Each of the RCTs were designed using samples size calculations that revolved around the prevalence and prevention of atopic dermatitis, and they are therefore underpowered to individually assess other allergy related disease outcomes, with the possible exception of allergic sensitisation. We must therefore rely on pooled estimates from meta-analyses, although few studies have published results for infants beyond 2 years of age. Once again considering the timeline of the atopic march, any preventative effect of probiotics on asthma and allergic rhinoconjunctivitis would be most apparent later in childhood and in adolescence, respectively. The diagnosis of asthma before 2 years of age is highly uncertain, and allergic rhinoconjunctivitis is extremely rare in this age group. We therefore need more studies to follow children over a longer period of time and preferably into adolescence and early adulthood. In addition, the pooled estimates for asthma and allergic rhinoconjunctivitis are more likely to be prone to problems of heterogeneity between studies and misclassification of disease status within studies.

1.4.1 ProPACT study

The ProPACT trial warrants further introduction since this study forms the basis for all three papers presented in this thesis. The ProPACT study is a double-blind trial in which 415 pregnant women were randomised to receive probiotic milk or placebo milk[6]. The probiotic milk was fermented with *Lactobacillus rhamnosus GG* (LGG), *Bifidobacterium animalis* subsp. lactis Bb-12 (Bb-12) and *L. acidophilus* La-5 (La-5). Further details regarding the design of the ProPACT study are presented in the methods section (§3.1).

At 2 years of age, the cumulative incidence of atopic dermatitis among children in the probiotic arm of the ProPACT trial was 21 % compared to 34 % in the placebo arm (risk ratio, RR: 0.61, 95 % CI 0.41 – 0.91, p = 0.013)[6]. The high cumulative incidence observed in this study is presumed to be due to a combination of more intensive follow-up and a degree of self-selection of high risk families.

The preventative effect of the ProPACT regime appears to persist until school age, with an observed 38% reduction in the risk of atopic dermatitis up to 6 years of age in the probiotic arm (RR: 0.62, 95 % CI 0.40 – 0.95, p = 0.025)[86]. Whilst this result is somewhat less certain because of a high dropout rate at 6 years, it implies that there is probably a true primary preventative effect of probiotics rather than a delay in the onset of atopic dermatitis. Similar to other

studies, the ProPACT regime was not observed to have a statistically significant effect on the presence of asthma or allergic sensitisation, or the cumulative incidence of allergic rhinoconjunctivitis, at 2 or 6 years of age. Although, as noted it was underpowered to assess these outcomes, and 6 years of age may still be too young to expect an observable effect on allergic rhinoconjunctivitis.

The secondary aims of the ProPACT study included the use of biological samples to investigate possible mechanisms of the preventative effect of probiotics. The samples collected included blood, faecal and oral mucosa bacterial samples from participating mothers and their children, as well as bacterial samples from the vaginal mucosa and breast milk samples from mothers. Before describing what is known or presumed about the mechanisms of probiotics, a summary of the immunopathology of atopic dermatitis is presented.

1.5 Immunopathology of atopic dermatitis

Atopic dermatitis is considered to result from the combined effects of an impaired epidermal barrier function and cutaneous inflammation. It is unclear which of these abnormalities appears first, and it seems likely that this may vary between individuals. However, once established, impaired epidermal barrier function promotes cutaneous inflammation and vice versa[13]. As noted previously, the recent rise in the prevalence of atopic dermatitis indicates that environmental factors must play an important role, but atopic dermatitis also has a strong genetic component. Family history is the strongest known risk factor and twin studies estimate that genetic factors contribute approximately 80% of the heritability to atopic dermatitis[13,91].

Multiple genes have been implicated in the development of atopic dermatitis and the atopic march. Over 30 genetic loci associated with atopic dermatitis have been identified in gene wide association studies (GWAS). The genes implicated in GWAS studies include ones involved with epidermal barrier integrity and immunologically active genes known to be involved in innate immune signalling and T cell functions[13,92]. Prior to the advent of next generation sequencing technologies and GWAS, candidate genes association studies had naturally focussed on genes associated with both processes. Filaggrin (FLG) is the most widely studied of the genes involved in epidermal barrier function. It is considered the strongest genetic risk factor, yet most atopic dermatitis patients have no FLG mutation. Indeed, FLG and other susceptibility loci account for

less than 20 % of the estimated heritability[13]. Epidermal barrier function is also affected by other environmental factors, partially through their effect on FLG expression. These include scratching and other mechanical damage, low humidity, water hardness, frequent use of detergents and soaps, and exposure to other environmental proteases[13,93]. Additionally, cutaneous cytokine imbalances and inflammation contribute to epithelial barrier dysfunction.

The adaptive immune system is particularly implicated in the cutaneous inflammation of atopic dermatitis. Even before macroscopic lesions appear, non-lesional skin has increased numbers of Th2 and Th22 cells, and pro-Th2 inflammatory cytokines [13]. Th2 cells are a characteristic feature of atopic dermatitis and the other allergy related diseases [32,94], and they play an integral role in the production of IgE, as described above. Differentiation of T cells from naïve to specific Th subtypes requires binding to APCs, co-stimulation through binding of additional APC and T cell surface molecules, a conducive cytokine milieu and transcription factor regulation (Figure 4)[95]. The APCs involved in atopic dermatitis include dermal dendritic cells, Langerhans cells found in the epidermis, and inflammatory dendritic cells which are recruited during acute flare-ups (Figure 5). During these flare-ups, there remains a predominance of Th2 and Th22 cells. As well as infiltration of inflammatory dendritic cells, acute lesions also display increased number of Th2-cytokine-producing type 2 innate lymphoid cells[13,96]. Th1 and Th17 cells are involved in the acute phase to a lesser degree. Chronic lesions display increasing infiltration of Th1 cells. Whilst Th1 is often reported as the dominant inflammatory profile in the chronic phase of atopic dermatitis, there is some evidence that the Th2 predominance prevails[96].

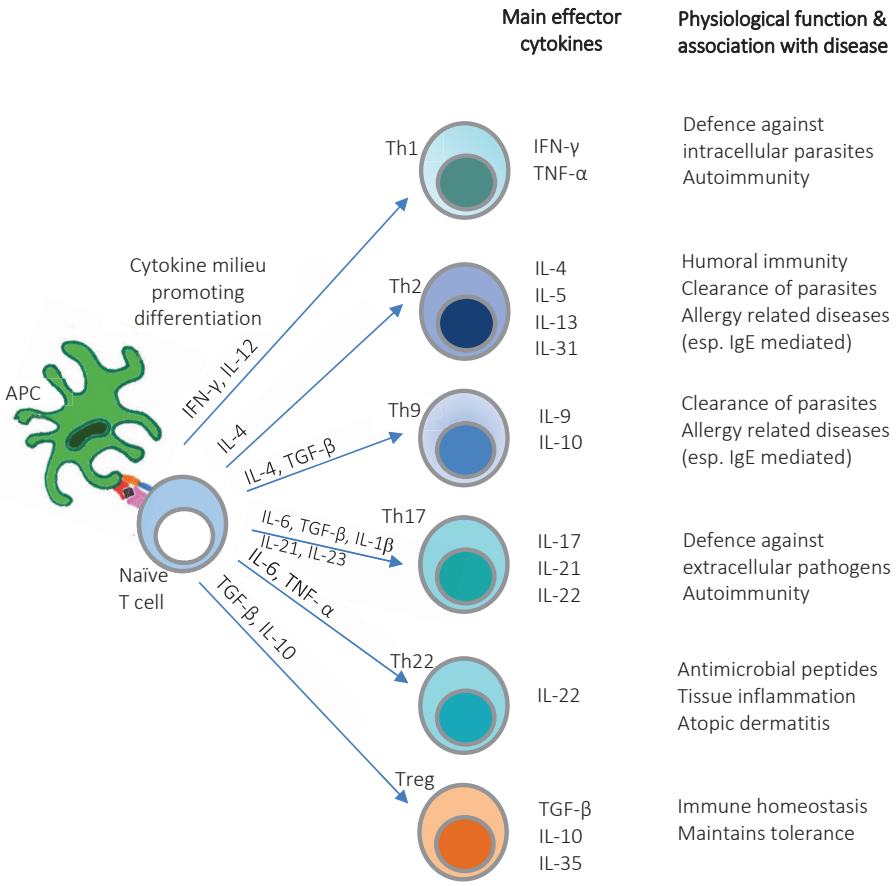


Figure 4: T cell differentiation and the cytokine milieu promoting subset differentiation. Figure is based on Sethi et al (2013)[135] and Berker et al. (2017)[32] APC: antigen presenting cell; IFN: interferon; IL: interleukin; TGF: transforming growth factor; Th: T helper cell; TNF: tumor necrosis factor; Treg: regulatory T cell.

1.5.1 Cytokines

Cytokines are important molecules that orchestrate innate and adaptive immune responses, also in atopic dermatitis. Dysregulation of key cytokines that affect T cell differentiation have been described in non-lesional skin in patients with established atopic dermatitis, as well as acute and chronic lesions (Figure 5). The cytokines classically associated with the Th2 response, including IL-4, IL-5, IL-13 and IL-31, are all increased in acute lesions[92,97-99]. Systemic alterations in these cytokines have been inconsistently reported in patients with established atopic dermatitis, although there is some evidence that elevated levels of these cytokines may precede the development of atopic dermatitis[98,100]. In particular, infants who later develop atopic dermatitis tend to have a more pronounced Th2 skewing already at birth, with higher cord blood levels of IL-4 and IL-13[53,98,100]. Cytokines derived from keratinocytes and other epithelial cells, including IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), can also promote Th2 polarisation and are implicated in atopic dermatitis. The primary effector cytokines of regulatory T (Treg) cells, IL-10 and transforming growth factor (TGF)-β, are thought to attenuate the inflammatory response in atopic dermatitis and other allergy related diseases. However, the role of both of these cytokines appears to be complex and, particularly in the case of IL-10, there is varying reports of increased and decreased levels in lesions and serum from patients with atopic dermatitis lesions[98].

The analysis of cytokines presented in this thesis focussed on levels of TSLP and TGF-β in breast milk. These cytokines and their implications in allergy related disease are discussed in more detail below.

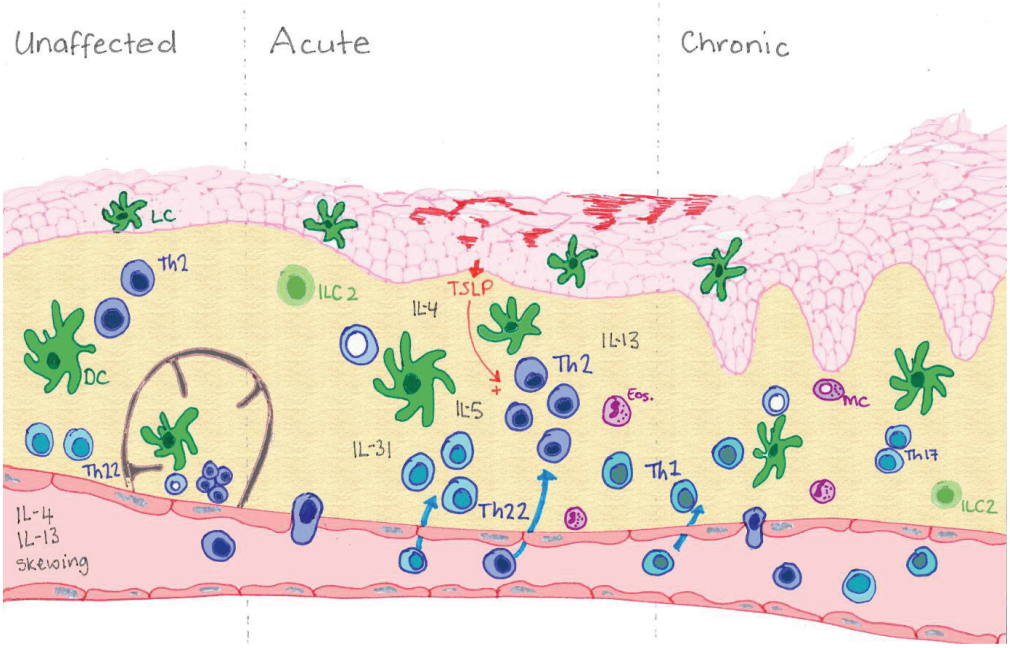


Figure 5: Pathophysiology of atopic dermatitis, focused primarily on the T-helper cell responses. Patients with atopic dermatitis display epidermal barrier dysfunction with structural and gene expression changes even in unaffected skin and an underlying low-grade inflammation and the presence of Th2 and Th22 cells. During acute flare ups, there is an amplification of the Th2 and Th22 response and their associated cytokines, recruitment of eosinophils and mast cells. TSLP secreted from keratinocytes promotes this Th2 skewing. Chronic lesions involve epidermal hyperplasia and greater activation of Th1 and Th17 cells. DC: dendritic cell; Eos.: eosinophil; ILC2: innate lymphoid cell; LC: Langerhans cell; MC: mast cell; Th: T helper cell; TSLP: thymic stromal lymphopoietin.

Figure adapted from Figure 3 in Leung and Bieber (2003)[99] and Figure 4 in Weidinger and Novak (2016)[13].

1.5.1.1 *Thymic stromal lymphopoietin*

Thymic stromal lymphopoietin (TSLP) received its name after it was initially isolated from stromal cells in the mouse thymus. Subsequently, epithelial cells have been identified as the major producers of TSLP[98]. TSLP appears to be related to a range of immunopathological processes in atopic dermatitis including, tissue remodelling, fibrosis and itch, enhanced maturation and proliferation of dendritic cells, promotion of Th2-cell polarisation, and B-cell proliferation and differentiation[13,98].

Through its role in the Th2 response, TSLP has been implicated in the defence against helminthic infections and in the establishment and maintenance of allergy related diseases[101]. Genetic variants of TSLP have been associated with atopic dermatitis and asthma[102] and high levels of epidermal TSLP precede the clinical presentation of childhood atopic dermatitis[103], suggesting that TSLP may be particularly important in the establishment of atopic dermatitis. Chronic atopic dermatitis also appears to be maintained through actions of TSLP through activation of dendritic cells and direct interaction with skin-homing Th2 cells[104,105]. In both instances, TSLP results in greater production of IL-4, IL-5 and IL-13 and ongoing Th2 skewing[94]. Consistent with these studies, higher concentrations of TSLP have been reported in both acute and chronic atopic dermatitis lesions[101]. Furthermore, mouse models have demonstrated that over-expression of TSLP in keratinocytes results in atopic dermatitis-like skin disease and can predispose to allergic airway inflammation after intranasal challenge. It has therefore been suggested that TSLP may play an important role in the subsequent development of asthma and allergic rhinoconjunctivitis seen in the atopic march[101].

1.5.1.2 *Transforming growth factor β isotypes*

In humans, three isoforms of TGF-β are expressed: TGF-β_1, TGF-β_2 and TGF-β_3. All three are produced by most cell types and have been shown to have many, sometimes contradictory, effects on cellular development and behaviour. The effect of TGF-βs appears to be context specific and the study of how the local microenvironment influences TGF-β signalling in different cell types is an area of ongoing investigation. As Massagué stated "It is not clear how TGF-β can inhibit cell proliferation but also promote cell growth, enhance stem cell

pleuripotency but also differentiation, regulate muscle genes in myoblasts and neural genes in neuroblasts, or suppress pre-malignant cells but encourage metastatic ones."[106].

In atopic dermatitis and other inflammatory diseases, TGF-βs appear to be primarily involved in suppression of the immune response and may play a key role in the development and maintenance of tolerance to allergens[107]. Specifically, TGF-β has been shown to inhibit proliferation of both B- and T-lymphocytes, as well as inhibiting both Th1 and Th2 differentiation and attenuating their responses. TGF-βs inhibit Th cell differentiation by limiting antigen presentation, downregulation of FcεR expression on Langerhans cells and others, and suppression of allergen-specific IgE. At the same time, TGF-β promotes Treg differentiation which also moderates both Th1 and Th2 inflammation. In turn, Tregs are considered to be the major source of TGF-βs in the context of allergy related diseases, creating a positive regulatory feedback loop[107]. On the other hand, TGF-βs have pro-inflammatory functions, acting as a chemoattractant for mast cells, macrophages and granulocytes. In the presence of IL-6, TGF-βs induce Th17 differentiation which have been implicated in acute inflammation of atopic dermatitis[108,109]. However, Th17 and its main effector cytokine, IL-17, also appear to have complex roles given that it has been implicated in epidermal barrier dysfunction, but also down regulation of TLSP[109,110]. In both atopic dermatitis and asthma, TGF-βs have been associated with recruitment of fibroblast and tissue remodelling in chronic disease[109,111]. At least in asthma, TGF-β in serum and airway are positively associated with disease severity and negatively associated with lung function[109].

1.5.2 MicroRNAs and non-coding RNAs

The ENCyclopedia of DNA Elements Project (ENCODE) consortium demonstrated that between 74-93% of bases in human DNA are transcribed into RNA, yet less than 2% of these transcripts contribute to sequences which provide coding for proteins[112]. Thus, the vast majority of human RNA transcripts are "non-coding RNA". It has been suggested that these non-coding RNAs contribute more to the biological complexity of eukaryotes, through sophisticated control of gene expression, than the protein coding genes themselves[113]. The most well-known of the non-coding RNAs are transfer RNA (tRNA) and ribosomal RNA (rRNA). These RNA molecules play an integral role in the link between transcribed messenger RNA (mRNA) and the transcription of mRNA to protein. Since the advent of high-throughput sequencing

technologies, advancements in bioinformatics and biochemical approaches, we have seen an increasing number of other non-coding RNA molecules identified and ascribed to regulatory cellular processes, including regulation of chromatin structure, DNA transcription, RNA processing and stability and translation[114]. In the field of allergy related disease research, microRNAs (miRNAs) have been most widely investigated and are the primary non-coding RNA of interest in this thesis. Recent studies have also investigated long non coding RNAs[115] (lncRNA) in asthma[116,117] and allergic rhinoconjunctivitis[118], and more generally in T cell differentiation[119] and immune regulation[120].

MicroRNAs are a group of short, non-coding, RNA molecules that regulate gene expression at the post-transcriptional level[121-123] (Figure 6). Over 2500 unique mature human miRNA have been identified[124,125]. Whilst the majority of miRNAs are found intracellularly, extracellular miRNA have also been identified in several body fluids including serum, breast milk, amniotic fluid and urine, and they have come under particular scientific interest because of their potential role in intercellular communication occurring in a paracrine, or even endocrine, manner[126,127]. The miRNAs in these body fluids tend to be encapsulated within extracellular vesicles (including exosomes)[128], or bound to proteins of the argonaute family (Ago1-4)[129,130] or to high-density lipoproteins (HDL)[131]. These vesicles and proteins protect the miRNA molecules from degradation by the RNases and proteinases found in most body fluids. *In vitro* experiments have demonstrated that miRNAs bound to HDL and those enclosed in extracellular vesicles are capable of regulating gene expression after uptake into another cell[132].

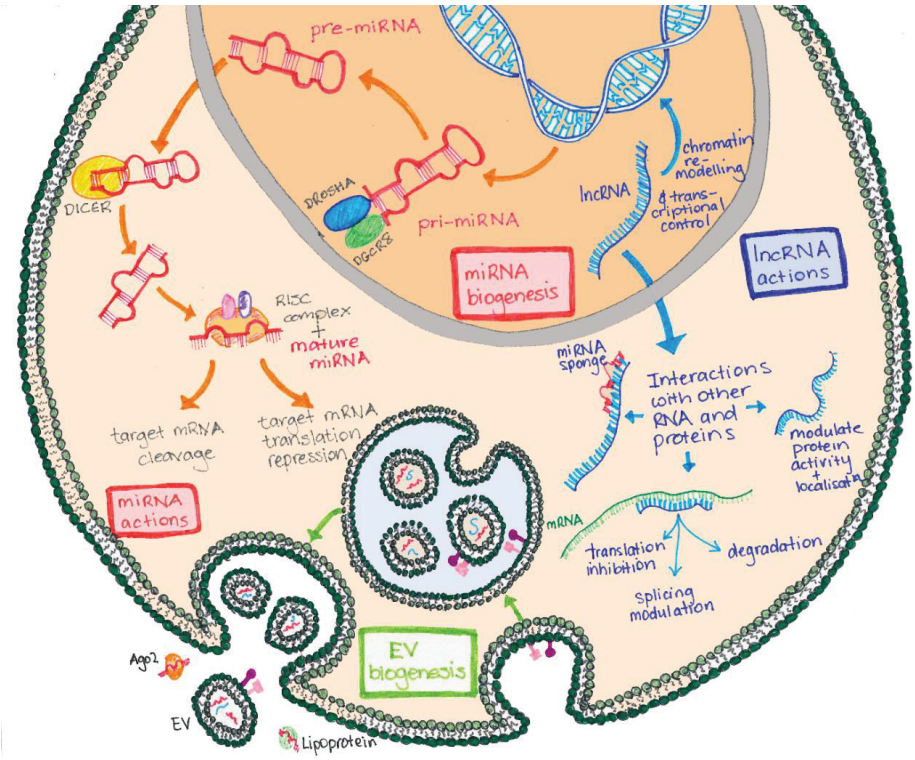


Figure 6: MicroRNAs, extracellular vesicles (EVs) and long non-coding RNA (lncRNA). **MicroRNAs** are derived from both regions between gene coding segments (intergenic regions) and from introns. The primary miRNA (pri-miRNA) transcript is processed within the nucleus and exported as a hairpin structure known as precursor-miRNA (pre-miRNA). They subsequently undergo further processing, whereby the hairpin head is cleaved leaving a double stranded 5'-miRNA:3'-miRNA molecule of approximately 22 base pairs. Either the 5' or 3' strand can then be incorporated into the RNA-induced silencing complex (RISC) which results in prevention of mRNA translation or direct mRNA degradation through complementary binding with the 3' untranslated region (3' UTR) of the target mRNA. The RNA binding argonaute (Ago1-4) proteins are part of the RISC complex, and extracellular miRNAs may protected from degradation through binding with Ago proteins, particularly Ago2. **Extracellular vesicles (EVs)** are another way in which extracellular miRNAs are protected. The biogenesis of a subtype of EVs, known as exosomes, is displayed in this figure. **Long non-coding RNAs (lncRNAs)** are a diffusely defined group of RNAs of more than 200 nt in length. They are implicated in regulation of gene expression through a number of mechanisms, and have been known to interact with miRNAs acting as a sponge or decoy.

Figure adapted figures and text in Ha and Kim (2015)[123] and Mercer, Dinger and Mattick (2012)[115].

A number of miRNAs have been implicated in both allergy related diseases and various skin conditions. To date, four studies have compared miRNA expression levels in patients with atopic dermatitis to healthy individuals using miRNA arrays (Table 1). Two of these studies have been published by Sonkoly et al and each included skin biopsies from three individuals with and four without atopic dermatitis[133,134]. The first of their studies identified 12 upregulated and 10 downregulated miRNAs in atopic dermatitis. In the second study by Sonkoly et al[133], an almost entirely different set of 44 miRNAs were observed to be differentially expressed (10 upregulated and 34 downregulated). MiR-155 was identified for further analysis because it was one of the most highly upregulated miRNAs and had known functions in the organisation of the adaptive immune system. Upregulation of miR-155 was confirmed using PCR analysis in a larger sample (n = 18/29, with and without atopic dermatitis, respectively), and *in vitro* experiments were conducted to further investigate the potential role of miR-155. Within the skin, miR-155 was observed to be predominantly expressed by immune cells including T cells, dendritic cells, fibroblasts and mast cells. More specifically in T cells, they demonstrated that miR-155 was induced during both Th1 and Th2 cell differentiation and that T cell stimulation in atopic dermatitis patients resulted in a greater increase in miR-155 expression. It was proposed that cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) was the primary target responsible for the T cell activities of miR-155. In other experiments, miR-155 expression has been associated with promotion of Th1 and Th17 differentiation and reduced Th2 differentiation[135], so its action is likely to be influenced by other factors present at the time of differentiation.

Table 1: Differentially expressed miRNAs in atopic dermatitis

Sample type	Upregulated	Down regulated
Skin[a] [134] (n = 3 / n = 4)	let-7i, miR-29a, miR-146a, miR-222, miR-24, miR-193a, miR-199a, miR-27a, miR-21, miR-20a, miR-17-5p, miR-105b	miR-122a, miR-133a/133b, miR-326, miR-215, miR-483, miR-519d, miR-335, miR-133b, miR-515-5p, miR-125b
Skin [133] (n = 3 / n = 4)	miR-501, miR-223, miR-155, miR-135b, miR-142-3p, miR-142-5p, miR-362, miR-487b, miR-31, miR-187	miR-204, miR-486, miR-375, miR-383, miR-10a, miR-125b, miR-149, miR-99a, miR-100, miR-328, miR-193b, miR-195, miR-23a, miR-452, let-7b, miR-197, miR-214, miR-30e-3p, let-7a, let-7c, miR-196b, miR-30a-3p, miR-26a, miR-26b, miR-101, miR-199b, let-7f, let-7d, miR-335, miR-143, miR-145, miR-365, miR-196a, miR-615
Keratinocytes [136] (n = 3 / n = 3)	miR-146a, miR-10b, miR-10a, miR-10a*, miR-216, miR-921-1*, miR-454, miR-29b-1*	miR-99a*, miR-34a*, miR34c-5p, miR-30a
Serum [137] (n = 30 / n = 28)	miR-205, miR-539, miR-122, miR-203, miR-483-5p, miR-134, let-7g, miR-495, miR-642	miR-590-5p
Urine [137] (n = 30 / n = 28)	MammU6, miR-142-3p, miR-20a, miR-548c-3p, miR-205, miR-19a, miR-483-5p, miR-222, miR-92a, miR-548a-3p	miR-203, miR-125a-5p, miR-886-3p, miR-184, miR-886-5p, miR-26a, miR-194

Prior to 2011, the 3'-miRNA was commonly annotated with an asterisk and referred to, for example, as "miR-10-star". This was phased out, and largely replace in the miRBase list of mature miRNAs by -3p and -5p naming in miRBase version18 (released November 2011). The 5'-miRNA was annotated without the asterisk.

[a] Upregulation of miR-21, but not miR-146a was confirmed using PCR analysis of skin biopsies from 20 atopic dermatitis patients and 26 healthy controls. Additionally, downregulation of miR-125b was observed using PCR, but not miRNA array.

Another small study focused specifically on miRNA expression in keratinocytes isolated from people with and without atopic dermatitis (n = 3 for each)[136]. In this study, miR-146a proved to be the most abundant of 12 differentially expressed miRNAs and was therefore chosen for further investigation with in vivo, and mouse experiments (Table 1). In these experiments, miR-146a appeared to play a regulatory role in chronic atopic dermatitis, probably through the inhibition of Interferon (IFN)-γ driven inflammation[136]. Looking more generally at the systemic expression of miRNAs, a fourth study assessed the miRNA profile in serum and urine from children with (n = 30) and without (n = 28) established atopic dermatitis[137]. The initial miRNA array identified 10 and 17 differentially expressed miRNAs in serum and urine, respectively (Table 1). Subsequent PCR analysis of selected miRNAs confirmed upregulation of miR-203 and miR-483-5p in serum samples, and down regulation of miR-203 in urine samples. Interestingly, the upregulation of miR-203 in serum and its downregulation in urine were associated with high levels of IgE. Patients with non IgE associated atopic dermatitis did not have altered miR-203 expression when compared to healthy controls. On the other hand, the expression of miR-483-5p in serum was upregulated in atopic dermatitis patients both with and without elevated IgE when compared to controls. Other miRNAs assessed with PCR without confirmation of the differential expression observed in the array analysis included miR-205, miR-134 and miR-122 in serum, and miR-205 and miR-483-5p in urine[137].

Across these four studies there is little overlap between the miRNAs identified as up- or downregulated (Table 1). In particular, the upregulation of miR-155, which was highlighted as biologically important, was not observed in any of the other tissue or samples types and upregulation of miR-146a was only observed in one other study. Furthermore, with the exception of miR-146a which appears to play a regulatory role, it is unclear if the observed differential expressions of miRNAs in skin, keratinocytes, serum or urine are drivers or products of the inflammation seen in atopic dermatitis. Expression of two miRNA, miR-155 and mi-223, prior to the development of atopic dermatitis has been assessed in maternal blood during pregnancy and cord blood at birth[138]. They observed that miR-223 during pregnancy and in cord blood was negatively associated with Treg numbers and that children with lower Tregs had a higher risk of developing atopic dermatitis. However, there was no direct association reported between miR-223 and atopic dermatitis. Nor was there any observed association between miR-155 expression and Tregs or atopic dermatitis[138].

In terms of other allergy related diseases, a number of other miRNAs have been implicated in individual diseases or mechanistic pathways[135,139]. Upregulation of miR-21, miR-142-5p, miR-142-3p, miR-146a, miR-155 and miR-223 and downregulation of the let-7 family and miR-193b have been reported in both atopic dermatitis and asthma. The biological functions of these miRNAs are under ongoing investigation, however experimental evidence suggests that miR-21 may be upregulated by IL-13 and contribute to further promotion of Th2 skewing through inhibition of IL-12 translation. Conversely, the let-7 family of miRNAs appears to downregulate IL-13 production and inhibit Th2 airway inflammation in experimental asthma, although some reports suggest that inhibition of let-7 improves asthma symptoms. Also in mouse models of asthma, miR-126 promotes Th2 inflammation in acute asthma and inhibition of miR-145 and miR-106a improves inflammation and decreases asthma severity[139]. Many of the miRNAs identified in atopic dermatitis and asthma have also been linked to positive and negative regulation of T helper subtype differentiation[135] and cytokine production[140], adding another layer of complexity to the regulation of this process (Figure 7).

1.6 Mechanisms of probiotics in the prevention of atopic dermatitis

The health implications of dysbiosis, particularly of the human intestinal microbiota, and the use of probiotics to restore a more beneficial microbial composition, have received a lot of scientific and public attention. However, the biological mechanisms behind why a dysbiosis may lead to disease or why probiotics may improve health are incompletely understood.

Probiotics are thought to provide beneficial health effects through improved intestinal epithelial barrier function, inhibition of pathogenic bacteria and modulation of the systemic immune system[141]. The presence and adhesion of probiotic bacteria to the intestinal mucosal surface is considered to be particularly important in their ability to improve epithelial barrier function and inhibit pathogenic bacteria. However, only the mothers received the probiotic supplementation in the ProPACT study. As such, the observed preventative effect of probiotics was presumably due to either the transfer of probiotics to the infants and or modulation of the maternal immune system. Transfer would have allowed a direct interaction with the infants' gastrointestinal and immune systems. Whilst immunomodulation in the mother may have then influenced the infant immune system *in utero* and or after birth via breast milk.

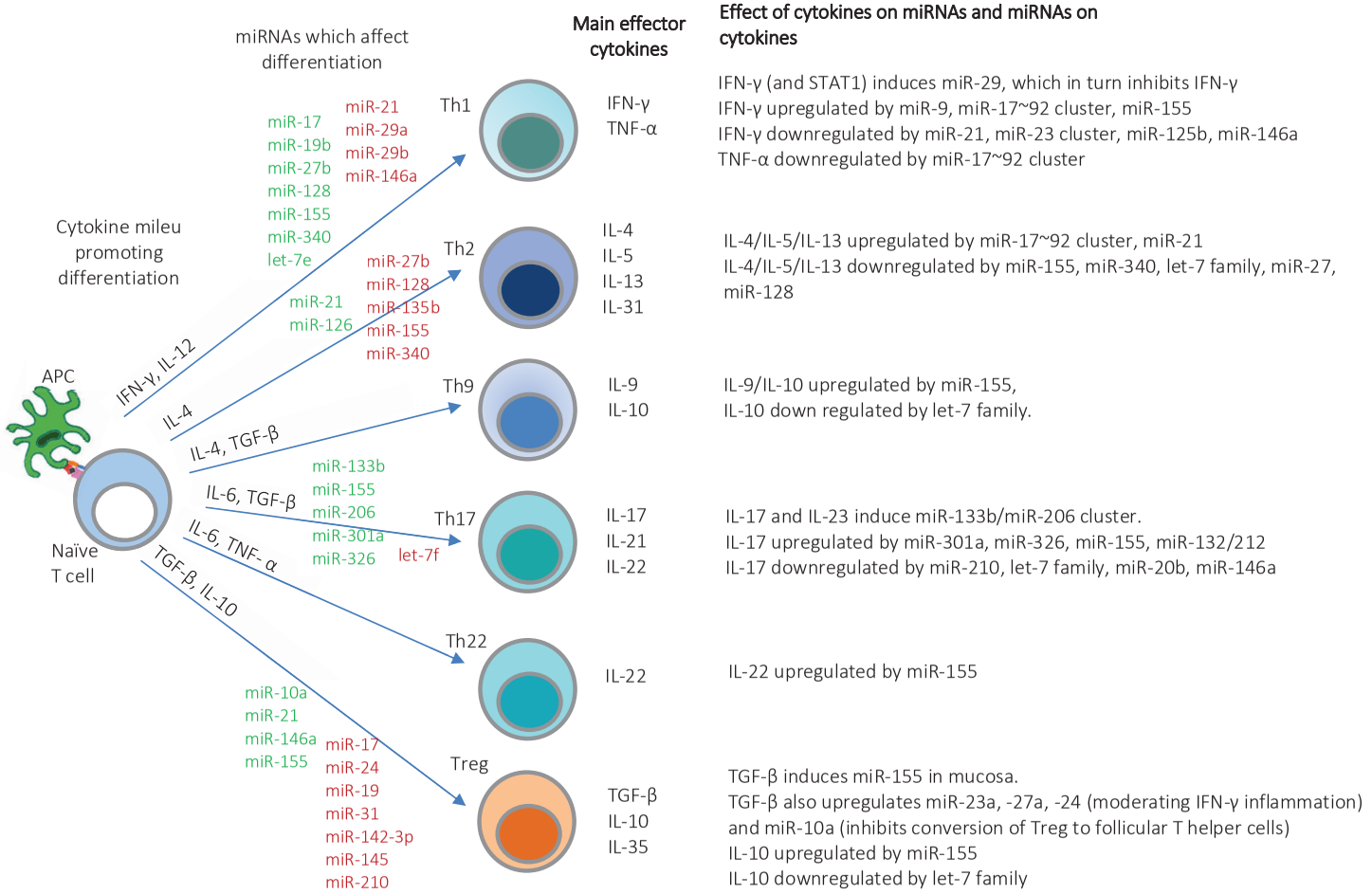


Figure 7: T cell differentiation with miRNAs. MiRNAs, which are reported to affect T cell differentiation, are shown in green or red if they promoted differentiation of that subset, respectively. Figure is based on Figure 3 in Sethi et al (2013)[135] and the text in Amado et al (2015)[140]. APC: antigen presenting cell; IFN: interferon; IL: interleukin; TGF: transforming growth factor; Th: T helper cell; TNF: tumor necrosis factor; Treg: regulatory T cell.

1.6.1 Transfer of administered probiotic bacteria from mother to child

With respect to the effect of the ProPACT regime on the maternal and infant intestinal microbiotas, we found that maternal probiotic supplementation with LGG, La-5 and Bb-12 increased the presence and relative abundance of all three bacteria in the mothers' stools[142]. Similarly, we observed a transient increased presence and relative abundance of LGG in infant stools up to 3 months of age[142]. The ability of LGG to be transferred from mother to child after maternal supplementation has not been consistently observed. Rather than the transfer of LGG, two previous studies have suggested that maternal ingestion of LGG promotes Bifidobacterium colonisation of infants[143,144]. This effect was not apparent in the ProPACT study, nor did we observe a meaningful effect on the presence or relative abundance of La-5 or Bb-12, or overall microbiota diversity in infant stools[142]. When considering the association between the presence of LGG in infant stools samples and the development of atopic dermatitis, it is apparent that the presence of LGG cannot entirely explain the observed preventative effect (Unpublished results). Therefore, we consider it likely that the ProPACT regime prevented atopic dermatitis through a combination of LGG transfer and maternal immunomodulation.

1.6.2 Intestinal epithelial barrier function and inhibition of pathogens

Defective intestinal barrier function and increased intestinal permeability in infancy has been associated with infantile gastrointestinal diseases and a predisposition to inflammatory diseases later in life, including autoimmune diseases and atopic dermatitis[145]. Animal, *ex vivo* and *in vitro* experiments have shown that probiotics improve intestinal barrier function by up-regulating epithelial tight junction and adherence junction genes, increasing mucus section, reducing cytokine-induced epithelial damage and initiating repair after damage[141]. Assessing the effect of probiotics on the human intestinal barrier function *in vivo* is more challenging. One *in vivo* human experiment demonstrated upregulation of tight junction associated proteins, Zonula occludens (ZO)-1 and transmembrane protein occludin, after *Lactobacillus plantarum* treatment[146]. Specific probiotic bacteria have also been shown to inhibit colonisation with pathogenic bacteria though several mechanisms including: occupation of mucosal adhesion sites, production of antimicrobial substances and other metabolites, depletion of essential nutrients, and stimulation of defensin release from epithelial cells.

Defensins are small peptides or proteins which are both anti-microbial and promote local intestinal barrier function[141].

1.6.3 Modulation of the immune system by probiotics

There are several experimental and animal studies which suggest that probiotics may directly modulate the innate and adaptive immune system, and that these effects may be strain specific. *Lactobacilli* species have been found to affect Toll-like receptor (TLR) expression[147], and inhibit T cell activation[148]. Others have demonstrated that combinations of *Lactobacilli* and *Bifidobacteria* can modify the cytokine profiles expressed by dendritic cells and epithelial cells, which in turn can promote or inhibit T helper cell subtypes[149-153]. In particular, there is experimental evidence to suggest that probiotic bacteria can upregulate Treg cells and thus moderate both Th1 and Th2 inflammation[154,155]. Whilst T cell mediated inflammation and control is considered particularly important in the development of atopic dermatitis and other allergy related diseases, there is varying evidence regarding the ability of probiotics to affect T cells in infants from clinical trials. Conflicting evidence exists regarding the effect of probiotics in promoting a "non-allergic" T helper (Th) type 1 cytokine profile[78,156-159]. Our own findings are in agreement with others, showing no significant impact of probiotic supplementation on Th1, Th2 or Treg cells, or the Th1/Th2 ratio[78,159]. However, we found that 3-month-old infants in the probiotic arm of the ProPACT trial had a decreased proportion of Th22 cells and this was related to a decrease in the cumulative incidence of atopic dermatitis at 2 years of age[160]. This was the first study to consider the more recently described T helper cell subtypes (Th9, Th17 and Th22) and will need to be confirmed in future studies.

1.7 Breast milk

Breast milk is first and foremost a source of nutrition and hydration for the new-born infant. However, these roles are perhaps the most readily replaceable functions of breast milk. It is well established that breast milk also provides a direct immune defence against pathogens through maternal immune cells and molecules such as secretory IgA, lysozymes and lactoferrin[161-163]. Correspondingly, breastfed infants have a lower rate of respiratory and gastrointestinal infections when compared to formula fed infants[163]. Breast milk also contains numerous biologically active components and is considered to play an important role in the promotion of intestinal barrier function and immune system development[164-166]. Among these

components are cytokines and miRNAs, which are presented in more detail below. Additionally, breastfeeding is one of the major factors influencing the early establishment of the intestinal microbiota in newborns, both through the immunologically active components and the transfer of microbes during breastfeeding. Breastfeeding and the complex composition of breast milk have been implicated in a number of immune related health outcomes later in life[167,168] suggesting that breastfeeding may have immunological consequences beyond the period which a child is actually breastfed. The remainder of this introduction will focus on breastfeeding and breast milk components within the context of allergy related diseases and probiotic supplementation.

1.7.1 Breastfeeding and allergy related diseases

When I commenced this doctoral work in 2012, there was a degree of uncertainty around the association between breastfeeding and the development of allergy related diseases. A recent review had concluded that methodological differences and weakness in the current research meant that no definitive conclusions could be made regarding the preventative effect of breastfeeding[163]. Specifically for atopic dermatitis, they concluded that "… current evidence does not support a beneficial effect of breastfeeding on development of atopic dermatitis"[163], a finding that was consistent with an earlier meta-analysis of prospective cohort studies[169]. With respect to the other allergy related diseases, they noted that there is an apparent protective effect on asthma symptoms and wheezing in early childhood, possibly due to the protective effect of breastfeeding on respiratory tract infections. Later in life, there was some evidence that breastfeeding may increase the risk of asthma and allergic rhinoconjunctivitis, although there is a significant risk of reverse causality in many studies that have reported allergy related disease outcomes beyond infancy[163].

Over the past 5 years, several additional observational studies have been published and, 2 years ago, Lodge et al published a new meta-analysis concluding that breastfeeding reduces the risk of asthma throughout childhood[170]. Lodge et al also found that breastfeeding appears to have a protective effect on the development of atopic dermatitis before 2 years of age and allergic rhinitis up to 5 years of age, although the evidence for these effects was weaker. There was a suspicion of publication bias for all of these findings. Heterogeneity between studies, and methodological challenges within studies, remain a significant limitation in the interpretation

of results from individual studies and meta-analyses. In particular, studies differ with respect to study design, the definition of breastfeeding exposure, the diagnostic criteria for the allergy related diseases and age of diagnosis. Within observational studies, recall bias, reverse causality and failure to adequately account for confounding factors are substantial risks, and especially in cross-sectional studies, which have been the main study design used in older children[170].

Whilst breastfeeding and breast milk has not been conclusively proven to prevent all allergy related diseases, breastfeeding should be recommended for its other health benefits. Many of these benefits are presumed to come from the multiple biologically active components of breast milk.

1.7.2 Breast milk composition and allergy related diseases

Breast milk contains an impressive list of ingredients that have evolved to support both mother and child[171,172]. In addition to the classic milk related carbohydrate (lactose) and proteins (whey and casein), breast milk contains a multitude of fats and micronutrients, as well as factors considered to be influential in the developing gastrointestinal and immune systems of the neonate. These include a diverse microbiota, inflammatory mediators (cytokines, chemokines), hormones and growth factors, miRNAs, leukocytes, immunoglobulins, human milk oligosaccharides (HMO), soluble receptors and PUFAs[173,174]. Cytokines are the most widely studied breast milk component with respect to maternal probiotic supplementation and allergy related diseases in infants as described below. The breastfeeding-associated microbiota and miRNAs in human milk are also introduced. An introduction to the remainder of the breast milk factors, and their potential relationship to allergy related diseases, is beyond the scope of this thesis, and I refer to recent comprehensive reviews that include these other components[173,175,176]. By and large, there is insufficient evidence to confirm any associations between individual breast milk factors and the development of allergy related diseases. Methods of integrated analysis may provide a more instructive overview of the characteristics of breast milk profiles that are more or less protective against both allergy related diseases and other immune related diseases[173].

1.7.3 Breastfeeding-associated microbiota in human milk

Breastfeeding is one of the major factors affecting the early development of the infant gut microbiota and weaning is associated with a shift in the gut microbiota towards a more adult-like composition [53,177,178]. Indeed, weaning appears to have a greater influence on the intestinal microbiota of infants than the introduction of solid foods[177]. Multiple components of breast milk contribute to these effects, including human milk oligosaccharides (HMOs) which particularly promote bifidobacteria growth, and lysozymes, lactoferrin and antimicrobial peptides which inhibit the growth of other microbes[179]. A diverse range of microbes, found both on the breast surface and within the mammary glands of lactating women, also contribute to shaping the infant intestinal microbiota[180-182]. Both culture-dependent and -independent techniques have demonstrated a dominance of bacteria belonging to the *Staphylococcus, Streptococcus* and *Propionibacterium* genera, as well as the presence of lactic acid bacteria and bifidobacteria in breast milk[180]. Recently, two studies employing bacterial DNA sequencing, demonstrated that bacteria shared between the mothers' milk and areola surface, and infants stool samples account for a significant proportion of the infants gut microbiota[181,182], and appear to do so in a dose dependent manner[182]. These findings are consistent with several earlier studies which investigated milk and stool samples from mother-infant pairs[181]

The origin of these bacteria is thought to be a combination of the microbiotas associated with the mother's skin flora, the infant's oral mucosa and the maternal gut. Recent studies have suggested the possibility of an entero-mammary route with selective trafficking of commensal bacteria from the maternal gut to the mammary glands via dendritic cells and macrophages in humans, mice and cows[183-185]. It is speculated that physiological changes in pregnancy, including increased vascular and intestinal permeability, decreased intestinal motility, and higher gastric and oral pH, promote bacterial translocation in late pregnancy[183]. In humans, strain level associations have been documented between pairwise and collective analysis of maternal and neonatal stool samples and breast milk samples[184].

Among the studies that have demonstrated apparent maternal gut to breast milk transfer of bacteria are four trials of maternal supplementation with probiotics[186-189]. Together they suggest that *L. rhamnosus* LC705 [187], *L. reuteri* [186], *L. paracaei* DSM 24733 [189], *L. plantarum* DSM 24730 [189], *L. fermentum* CECT5716 [188] or *L. salivarius* CECT5713 [188] results in an increased presence of the administered bacteria in the breast milk of some, but not all, women.

Additionally, one of the studies, which trialled supplementation of a commercially available multi-strain preparation (VSL#3, Alfasigma USA, Inc., Covington, LA), found 6 of the 8 strains were not recovered from breast milk samples in the probiotic group, including *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *B. breve*, *B. infantis, B. longum* or *Streptococcus thermophilus*[189]. In Paper I, LGG was of particular interest because it has been the most commonly administered bacteria in atopic dermatitis prevention studies. Furthermore, LGG was observed to be transferred to infants in the ProPACT study and previous studies of LGG supplementation suggest that ongoing ingestion is required to maintain measurable LGG levels in stool samples[190] . Breastfeeding was therefore investigated as a possible ongoing source of LGG for these infants.

For Paper I, we adopted the term "breastfeeding-associated microbiota", suggested by Sakwinska et al [191], to describe a bacterial community that involves both the breast milk and breast surface microbiotas. This term was chosen because the samples were collected without sterilisation of the breast areola. Whilst this means that we are not sampling the breast milk microbiota alone, we assume that our samples provide a more representative analysis of the bacteria ingested by suckling infants.

1.7.4 Cytokines in human milk

Cytokines are one of the major groups of immunological active components described in breast milk. Many of these have been assessed in association with maternal history of allergy related disease and or the development of these diseases in their offspring. In particular, IL-10, soluble CD14 (sCD14) and TGF-βs. The first of these, IL-10, appears to be present in very low quantities in breast milk. Whilst some studies describe the presence of IL-10 in all samples[192,193], it is often reported as below the limits of detection in significant number of women[156,194-203]. Overall, there are no consistently reported associations between IL-10 detection or concentration and either maternal or infant allergy related disease[156,192-196,198,200-202,204-210]. In contrast, sCD14 is actively secreted and present in very high levels in breast milk[173]. Consistent with the epidemiological studies that have shown lower levels of sCD14 in allergy related disease, early studies reported that breast milk sCD14 might be protective[211,212], however this has not been confirmed in other studies[78,156,206,207,209,213].

The analyses in Paper II focused on TSLP and the TGF-β isotypes. TSLP had only been reported once previously in breast milk samples and the biological effects on the mother and child are unknown[214]. Although TSLP is implicated in the establishment and maintenance of allergy related diseases, potential associations between breast milk TLSP and the development of these diseases had not been previously assessed. On the other hand, all three isoforms of TGF-β had been reported in breast milk and several studies have assessed the influence of maternal atopy and probiotics on breast milk TGF- βs and their association with later allergy related disease. Current research suggests that high levels of TGF β_2 in human breast milk may be associated with the development of atopic dermatitis[192,194], sensitisation[196] and asthma symptoms[195], whilst TGF β_1 may be protective[196,209], however these findings are not consistent across all experiments[194,195,204,206,215-219]. Studies investigating the effect of probiotic and or prebiotic supplementation on breast milk TGF-β concentrations have also produced conflicting results (Table 2) [78,156,192,204,207,220,221].

Current research provides insufficient evidence, or conflicting results, regarding the effect of probiotics on a number of other immune related breast milk components, including IL-2, IL-4, IL-6, IL-10, IL-13, tumour necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), total and secretory IgA or soluble CD14 (sCD14)[78,156,173,192,207,222]. Similarly, the associations between mother-infant characteristics and various breast milk cytokines have been investigated, but remain inconclusive due inconsistent findings between studies or lack of confirmatory studies. These mother-infant characteristics include maternal history of allergy related disease[192,193,197,200-202,206,210,212,223-227], cigarette smoking[228], maternal weight[229], physical activity, maternal diet (fish oil, PUFAs, black current seed oil)[198,230-232], parity[215,224,232,233], alcohol[234], presence of mastitis[235,236] and pregnancy complications (preeclampsia and prematurity)[237-239], geography and ethnicity[175,196,233,240].

Table 2: Summary of previous studies investigating effect of probiotic and / or prebiotic supplementation on breast milk TGF-β.

Author	Probiotic and/or Prebiotic	Administration period	Details of analysis methods	Results: clinical outcomes and breast milk TGF-β measurements[a]
Rautava[221], 2002	LGG ($2x10^{10}$ CFU)	36 weeks to 3 months if breastfeeding.	TGF-β_1 and TGF-β_2 at 3 months (n=62). Implied normal distribution, t-test.	Reduced relative risk of AD at 2 years with probiotics (n=62, RR 0.32, 95% CI 0.12 – 0.85). TGF-β_2 higher with probiotics, no change in TGF-β_1 "No direct correlation… between breast milk TGF-β_2 and… atopic disease or sensitisation…"
Huurre[207], 2008	LGG ($1x10^{10}$ CFU) and Bb12 ($1x10^{10}$ CFU)	1st trimester to end of breast-feeding.	TGF-β_2 at 1 day (n=53-58) and 1 month (n=65-68). Pre-treated with sodium taurochlorate (bovine bile acid) Log transformed, t-test.	No significant effect of probiotics on development of AD at 1 year (n=140, RR 0.55, 95% CI 0.23 – 1.32). TGF-β_2 higher with probiotics at day 1, no change at 1 month or in a range of other breast milk cytokines. Testing of association between TGF-β_2 and AD not reported.
Prescott[156], 2008	Two probiotic groups which received either *L. rhamnosus* HN001 ($6x10^9$ CFU) or *B. animalis* subsp. *lactis* HN019 ($9x10^9$ CFU)	~36 weeks to 6months if breastfeeding	TGF-β_1 at 1 week (n = 105), 3 and 6 mths (unreported sample size). Mann-Whitney U-test.	*L. rhamnosus* HN001, but not *B. lactis* HN019, reduced risk of AD at 2 years (n = 310, RR 0.54, 95% CI 0.34 – 0.87)[71] TGF-β_1 higher in both probiotic groups at 1 week postpartum, but not at later time points. No relationship found between breast milk TGF-β1 (or a range of other cytokines) and AD or other allergic outcomes.
Böttcher[204], 2008	*L. reuteri* (1×10^8 CFU)	36 weeks to delivery. Child received postpartum.	TGF-β_1 & TGF-β_2 at 3 days and 1 month (n=109). Activation and neutralisation reported. Mann-Whitney U-test	No significant effect of probiotics on AD at 2 years (n=188).[241] TGF-β_2 lower in probiotic group at day 3, no difference in TGF-β_1 or for either isoform at 1 month. No association between TGF-β_1 or TGF-β_2 and development of AD during first 2 years.
Boyle [78], 2011	LGG (1.8×10^{10})	36 weeks to delivery. Neither mother or child after birth	TGF-β_1 at 7 and 28 days. Mann-Whitney U-test.	No significant effect of probiotics on AD at 1 year. Non-statistically significant lower level of TGF-β_1 at 7 days in probiotic group. No difference in TGF-β_1 at 28 days postpartum.
Kuitunen[192], 2012	LGG $5x10^9$ *L. rhamnosus* LC70 $2x10^8$ *B. breve* Bb99 $2x10^8$ *Propionibacterium freudenreichii* ssp. *Shermanii* JS $2x10^9$	36 weeks to delivery. Child received postpartum with 0.8 g galacto-oligosaccharide.	TGF-β_2 at 0-3 days (n=364) and 3 months (n=245). Log-transformated, 2-way ANOVA with treatment allocation and maternal atopy status.	Reduced risk of AD at 2 years with probiotics (n=925, RR 0.81, 95% CI 0.66 – 0.99).[242] Trend towards lower TGFb2 in colostrum in probiotic group. High TGFb2 levels in mature milk were positively associated with development of allergy and non-IgE associated eczema.

Table 2 continued: Summary of previous studies investigating effect of probiotic and / or prebiotic supplementation on breast milk TGF-β.

Author	Probiotic and/or Prebiotic	Administration period	Details of analysis methods	Results: clinical outcomes and breast milk TGF-β measurements[a]
Baldassarre [243], (2016)	Multi-strain preparation[b] (9×10^{11} bacteria)	36 weeks gestation to 4 weeks postpartum.	TGF-β_1 at 0-3 days and 30 days (n=66) T-test and 2-way ANOVA. Unclear if concentrations were log-transformed prior to statistical testing.	Newborns were followed for 30 days with respect to gastrointestinal symptoms. Lower relative risk of colic and regurgitation in probiotic group. TGF-β_1 higher in probiotic group at 30 days postpartum, but not at 0-3 days.
Nikniaz [244], (2013)	Trial of synbiotics including a multi-strain preparation[c]	From 3 to 4 months postpartum	TGF-β_1 and TGF-β_2 at 3 and 4 months (n=75) Paired t-test. Reported to be normally distributed.	TGF- β_2 was higher at the end of the supplementation period (4 months) in the probiotic group. TGF- β_1 was unchanged.
Kubota [245], (2013)	Trial of prebiotics – fructo-oligosaccharides	26 weeks gestation until 1 month postpartum.	TGF-β_1 in first days after birth (n=64)	No infant outcomes reported. Non-statistically significant lower level of TGF- β_1 in colostrum samples.

All studies used commercial ELISA kits to quantify TGF-β1 and or TGF-β2 ; AD: atopic dermatitis; Bb12: *Bifidobacterium animalis* subsp. *lactis* Bb-12; CFU: colony forming units; LGG: *Lactobacillus rhamnosus* GG ; OR: odds ratio; TGF: transforming growth factor. [a]The summarised results detail the observed effect of probiotics on atopic dermatitis, with risk ratios calculated from the original studies, with the observed effect of probiotics on breast milk TGF-βs and their association to the development of atopic dermatitis. Other conclusions regarding the effect of probiotics or TGF-βs on other allergy related diseases and those based on subgroup analyses are not covered; [b]*L. paracasei* DSM 24733, *L. plantarum* DSM 24730, *L. acidophilus* DMS 24735, *L. delbrueckii* subsp. *Bulgaricus* DSM 24734, *B. longum* DSM 24736, *B. infantis* DMS 24737, *Streptococcus thermophilus* DSM 24731; [c]*L. casei* PXN 37, *L. rhamnosus* PXN 54, *Streptococcus thermophilus* PXN 66, *B. breve* PXN 25, *L. acidophilus* PDN 35, *B. longum* PXN 30, *L. bulgaricus* PXN 39, and fructo-oligosaccharide.

1.7.1 Human milk microRNAs

Breast milk is known to contain high quantities of RNA, including miRNAs, and many of these miRNAs are "immune-related"[174,246-248]. This has led to the hypothesis that breast milk miRNAs are one of the mechanisms that breastfeeding affects the early development of an infant's gastrointestinal and immune systems. In support of the biological plausibility of this hypothesis, *in vitro* studies have demonstrated that breast milk miRNAs are stable under a variety of harsh conditions, including prolonged exposure to acidic solutions simulating the stomach environment [174,248]. Using extracellular vesicles derived from human milk, *in vitro* experiments have also demonstrated that gene expression and protein synthesis is regulated in recipient cells after exposure to the milk extracellular vesicles[246,247]. Furthermore, *in vivo* porcine experiments have shown that the concentration of specific immune-related miRNAs in breast milk is proportional to the concentrations seen in serum samples from suckling piglets[249]. This is most likely evidence of absorption of milk-derived miRNA by the piglets and suggest that breast milk miRNAs may be a means of vertically transferred epigenetic regulation of infant protein synthesis. However, there is ongoing debate as to whether these molecules are functionally active or simply contribute to the nutrient components of breast milk[250]. Paper III presents the only study of human milk miRNAs in relation to maternal probiotic supplementation and with an infant health outcome.

2 Aims

The overall aim of this thesis was to investigate if alterations in certain breast milk components are partially responsible for the prevention of childhood atopic dermatitis observed in the ProPACT study. Papers I, II and III focus on different components of human breast milk, each considering the overriding research questions:

- Did maternal supplementation with the probiotic bacteria, LGG, Bb-12 and La-5, affect the breast milk component in question?

- Did changes in these breast milk components mediate the effect of the probiotic regime on atopic dermatitis in offspring?

Additionally, Papers I and III employed high-throughput sequencing techniques to assess the breastfeeding-associated microbiota and miRNA profiles of breast milk, respectively. At publication, these papers represented two of the largest studies of these components of human breast milk and a significant portion of these papers provide a descriptive presentation of the results.

2.1 Specific breast milk components and aims in Papers I, II and III

Paper I: Breastfeeding-associated microbiota

The aims of Paper I were to: (a) investigate whether maternal probiotic supplementation with LGG, La-5 and Bb-12 affected the presence and relative abundance of these strains among the bacteria transferred during breastfeeding at 10 days and 3 months postpartum, (b) assess their association with the later development of atopic dermatitis, and (c) examine the general microbiota associated with breastfeeding, considering temporal trends, and relationships between the composition of the microbiota transferred during breastfeeding, probiotic supplementation and atopic dermatitis.

Paper II: Cytokines

The aims of this paper were to: (a) determine if perinatal maternal probiotic supplementation alters the concentration of TLSP, TGF-β1, TGF-β2 or TGF-β3 in breast milk at 10 days and 3 months postpartum and (b) investigate if these breast milk cytokines contribute to the preventative effect of maternal probiotic supplementation on the development of atopic dermatitis at 2 years of age through causal mediation analysis.

Paper III: miRNA

The aims of this paper were to: (a) to determine the miRNA profile of human breast milk samples, (b) to examine if this profile is influenced by maternal probiotic supplementation and (c) to assess if any changes in the miRNA profile are associated with the development of atopic dermatitis in offspring.

3 Material and methods

All three papers presented in this thesis used breast milk samples collected during the ProPACT study. The following sections detail the design of the ProPACT study, the collection and laboratory analysis of breast milk samples, and the statistical analyses methods.

3.1 Design and participants

The ProPACT study is a double-blind randomised controlled trial. Pregnant women were recruited from the larger community based study which was being conducted by our research group at the time – the PACT study. All pregnant women who had not reached 36 weeks gestation were eligible for inclusion if they understood Norwegian, planned to breastfeed during the first three months after birth, and they liked and tolerated fermented milk. Women were ineligible if they had been taking probiotic supplements during the last 4 weeks, if they were planning to move away from Trondheim within 25 months of randomisation, or were at risk of developing pregnancy related complications.

After inclusion, the women were randomised to receive 250 ml per day of either a probiotic or placebo fermented milk from 4 weeks before their expected due date until 3 months after birth. The probiotic milk was a commercially available fermented milk product (Biola™, Tine AS, Norway) that contains 5 x 10^{10} colony-forming units (CFUs) *Lactobacillus rhamnosus GG* (LGG), 5 x 10^{10} CFUs of *Bifidobacterium animalis* subsp. lactis Bb-12 (Bb-12) and 5 x 10^{9} CFU *L. acidophilus* La-5 (La-5) per 250mL. The placebo milk contained no probiotic bacteria strains and had been heat treated (75 °C for 4 seconds) after fermentation. The probiotic and placebo milks were neutrally packaged and had an equivalent taste. The producer, Tine AS, was responsible for the distribution of the study milk throughout the trial period and conducted regular culture testing to ensure consistent bacterial concentrations in the probiotic milk and effective sterility of the placebo milk.

During the period of supplementation, women were requested to complete a diary detailing their daily consumption of the study milk. After birth, they also recorded how frequently the child was breastfed, the consistency, colour and frequency of the infant's bowel motions, and symptoms of infant colic. Women were considered to be compliant with the study protocol if they consumed at least 250 mL of study milk on at least 50 % of days during the intended intervention period, they did not consume other products containing probiotics and the child

was at least partially breastfed until 3 months of age. The consumption of study milk was only recorded in the diaries, and thus women who did not submit a completed diary were considered to be non-compliant.

3.1.1 Lifestyle and child health questionnaires

Participating families completed lifestyle questionnaires at baseline (around 30-36 weeks gestation), 6 weeks, 1 year and 2 years postpartum. These lifestyle questionnaires detailed maternal and family history of allergy related disease, parental smoking habits, housing conditions and indoor environment, household pets, and semi-quantified food frequency information for the mother during pregnancy and whilst breastfeeding. Questionnaires completed postnatally included additional information regarding the child's sex, birthweight, gestational age and prematurity, breastfeeding status and duration, and semi-quantified food frequency information for the child. Child health questionnaires were completed at 2 and 6 years of age. These were adapted from the ISAAC questionnaires and focused on signs and symptoms of allergy related diseases, common childhood infections and medication use. Both the lifestyle and child health questionnaire are available in Norwegian in the appendices of Dr. Torbjørn Øien's doctoral thesis (available at NTNU, 2010:47)[251]. English translations of the questions relevant to this thesis are presented in Appendix I. Data regarding mode of delivery was collected after publication of Paper II and Paper III, and is therefore only included as a baseline variable in Paper I.

3.1.2 Clinical examination

The predefined primary outcomes were atopic dermatitis, allergic rhinoconjunctivitis and asthma during the first 2 years of life and atopic sensitisation was a secondary outcome. At 2 years of age, all children were invited to attend a structured clinical interview and examination conducted by a paediatrician. Additionally, families were encouraged to attend a clinical examination by a trained nurse at the Department of Dermatology, St Olavs Hospital (Trondheim, Norway), if the child developed an itchy rash lasting more than 4 weeks at any point before 2 years of age. Atopic dermatitis was diagnosed according to an age appropriate modified UK Working Party's diagnostic criteria[252] at these clinical examinations (APPENDIX II). A child was deemed to have current asthma if they had experienced at least three episodes of wheezing during the past 12 months and had been treated with inhaled glucocorticoids or had

signs of suspected airway hyper-reactivity (e.g. cough or wheeze with exertion or disturbed sleep because of cough or wheeze) in the absence of upper respiratory tract infection. Allergic rhinoconjunctivitis was diagnosed based on the paediatrician's clinical assessment. The secondary outcome of atopic sensitisation was defined as a positive skin prick test (SPT, wheal ≥ 3mm) or an elevated specific IgE (sIgE, ≥ 0.35 kU/L). In both instances, children were tested for sensitivity to 7 aeroallergens and 5 food allergens. Aeroallergens included mite (*Dermatophagoides pteronyssinus*), mould (*Cladosporium herbarum*), cat and dog dander, birch, timothy and mugwort pollen. Food allergens included egg white, codfish, hazelnut, peanut and cow's milk. SPT were conducted by experienced research assistants using the ISAASC II protocol[253] and standardised extracts from Soluprick® allergens (ALK Abelló, Hørsholm, Denmark) with the exception of cow's milk where fresh skimmed milk was used. Specific IgE was measured in sera from venous blood samples with the Immulite® 2000 Allergen-specific IgE system (Siemens Medical Solutions Diagnostics, Deerfield, IL, USA).

Attendance at the 2-year clinical examination was an inclusion criterion for all of the breast milk sample analyses presented in this thesis. Participating children have subsequently been invited to clinical follow-up at 6 years of age, although the outcomes of this assessment were not used in the conjunction with the analysis of breast milk.

3.2 Breast milk samples and analysis

3.2.1 Collection and storage of breast milk samples

The participating women were provided with sterile containers and requested to express up to 30 mL breast milk at 10 days and 3 months postpartum. They did not receive explicit instructions regarding the timing or method of collection and this information was not consistently recorded. We therefore have insufficient information regarding potentially influential variables such as the time of day of collection, whether fore- or hindmilk was collected, how long it had been since that breast had been used for either breastfeeding or expressing, or if the breast areola was cleaned prior to expressing the milk samples. Participants stored the breast milk samples in their home freezer until they could be delivered to the laboratory, where they were stored at -80°C until analysis. Styrofoam boxes were used during transportation to the laboratory to prevent thawing. Breast milk samples were thawed

and aliquoted and used immediately for breast milk cytokine analysis. The remaining aliquots were re-frozen and were subsequently analysed for the microbial content and miRNA profile.

A total of 503 samples from 259 mother-infant pairs were available for analysis. However, not all samples had sufficient volume or technical quality to be included in all analyses (Figure 8). The number of breast milk samples used in each analysis varies for differing reasons as described in the following sections.

3.2.2 Microbial analysis

The microbial content of breast milk samples was analysed in collaboration with Prof. Knut Rudi at the Norwegian University of Life Sciences, Ås, Norway. Members of Prof. Rudi's laboratory conducted the sample preparation, DNA extraction, and quantitative real-time PCR (qPCR) analysis of the total and administered bacteria. The 16S rRNA gene sequencing, used in the investigation of the general breastfeeding-associated microbiota, was conducted at The Centre for Intergrative Genetics (CIGENE, Ås, Norway). Each of these steps is described briefly below and in detail in Paper I.

Subgroup included in microbial analyses. Chronologically, breast milk samples were analysed for cytokine concentrations first. As a result, only 472 of the original 503 samples had sufficient volume to be included in the subsequent microbial content analyses. DNA was extracted from all of these 472 samples and used in the analysis of the administered bacteria. However, only 142 samples from 125 women could be used in the analysis of the general breastfeeding-associated microbiota due to low quantity and quality of bacterial DNA.

Sample preparation. After thawing, 2 mL of breast milk was centrifuged at 21 500 *g* for 30 mins and the fat layer and supernatant were discarded. The resulting pellet was resuspended in 100 µL of stool transport and recovery buffer (S.T.A.R) to inactivate infectious organisms, minimise nucleic acid degradation and enhance the binding of nucleic acids to magnetic beads during the subsequent DNA extraction steps[254].

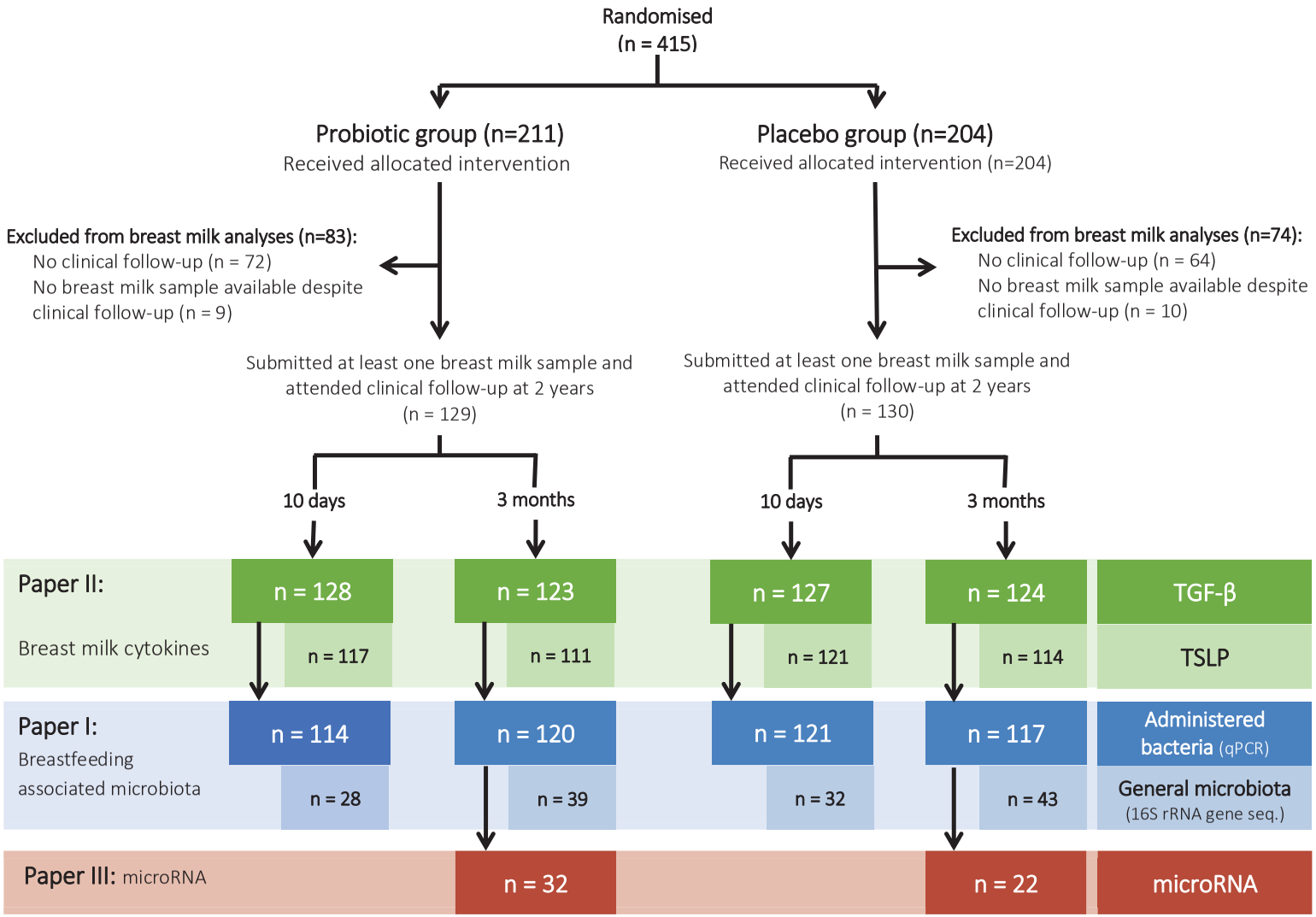


Figure 8: Participant and sample flow diagram for Papers I, II and III.

DNA extraction. DNA was extracted from the resuspended pellet using LGC Mag™ DNA extraction kit (LGC Genomics, UK) on a robotic KingFisher Flex magnetic particle processor (ThermoScientific, MA, USA) according to the manufacturers' instructions. The DNA was extracted once from the breast milk samples and used in the analysis of total and administered bacteria (qPCR) and the general breastfeeding-associated microbiota (16S rRNA gene sequencing).

Analysis of total and administered bacteria (qPCR). Due to the availability of appropriate primers and probes, the qPCR analysis was conducted using two different assay types. For LGG and La-5, samples were analysed with a double stranded DNA binding dyes EvaGreen® assay. This assay involved identification of a threshold cycle (C_t) and melting point determination. Samples were considered positive if they had both a C_t value ≤ 35 and a melting point of 86°C for LGG or 80°C for La-5. A probe-based TaqMan assay was used for the analysis of Bb-12 and total bacteria, and samples were considered positive if they had a C_t value ≤ 35. Primers and probes used for each assay are presented in Table 1 of Paper I.

Analysis of general microbiota (16S rRNA gene sequencing). Following DNA extraction, the V3 – V4 regions of the 16S rRNA gene was amplified using PRK341F/PRK806R primers adapted for Illumina sequencing. Paired end reads (300-bp) were obtained on the Illumina MiSeq platform (Illumina Inc., CA, USA) using v3 sequencing chemistry. The resulting reads were processed and assigned Operational Taxonomic Units (at 97 % similarity) with the Quantitative Insights Into Microbial Ecology (QIIME) pipeline and the Ribosomal Database Project classifier. The number of reads was standardised by rarefying each sample to 1000 reads before descriptive and comparative analysis of the breastfeeding-associated microbiota. Samples with less than 1000 reads were not included in these analyses. An OTU, or taxonomic unit, was considered "present" for a given sample if it accounted for at least 1% of reads (10 reads) in the rarefied data set.

3.2.3 Cytokine analysis

The concentrations of selected cytokines were analysed at the Department of Cancer Research and Molecular Medicine, NTNU. The assays were conducted by research assistants, Guri Helmensen and Liv Ryan. In addition to the published results, quantification of soluble CD14 (sCD14) and a panel of cytokines was attempted, as described below. Due to technical

difficulties, only the TSLP and TGF-β results were suitable for publication (Paper II) and these results are summarised in Chapter 4.

Subgroup included in cytokine analyses. Analyses were performed on all 503 available samples for TGF-β and sCD14 quantification. However, only 463 samples could be analysed for TSLP because of insufficient quantity of reagents. A multiplex analysis of 27 cytokines was trialled in a pilot study of 6 samples from 3 individuals, and a panel of 5 cytokines was constructed and attempted in all available samples.

Sample preparation. Breast milk samples (1.5 mL) were centrifuged at 16 100 *g* for 10 minutes at 4°C. The aqueous portion was extracted from beneath the fat layer, without disturbing the pellet, and was used in the subsequent quantification of cytokines.

Quantification of TSLP. TSLP concentrations were measured using a human TLSP DuoSet Enzyme Linked Immunosorbent Assay kit (ELISA kit; R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. The range of detection of the assay was from 31.25 to 2000 pg/mL. The samples were analysed over seven ELISA plates with samples from the probiotic and placebo group randomly distributed between and upon the plates. Samples collected at 10 days postpartum were primarily analysed on plates 1 – 3 (~90 % of samples), whilst samples collected at 3 months postpartum were analysed on plates 4 – 7, exclusively. The standards and an internal control breast milk sample were conducted in triplicate on each plate. Based on the calculated concentrations of the internal control (average 266.6 pg/mL, SD 61.1 pg/mL), the intra-assay coefficient of variation (CV) was <13.5 % and inter-assay CV was 23 %. Breast milk samples were analysed as singlets.

Nearly half of the samples were found to contain TSLP concentrations either above (22 %) or below (23 %) the limits of detection for this assay. We therefore categorised the TSLP concentrations into four categories for analysis: below detection, low detectable, high detectable and above detection. For samples within the range of detection, they were defined as low or high based on their calculated concentration relative to the internal control samples. The choice of the internal control sample as a cut-off maximised the inter-assay comparability and its calculated concentration was marginally higher than the sample median for those in the range of detection (183 pg/mL, IQR: 91 – 576 pg/mL).

Quantification of TGF-β isotypes. The concentrations of all three human isotopes of TGF-β (TGF-β_1, TGF-β_2 and TGF-β_3) were measured using a multiplex assay (Bio-Plex Pro TGF-β assay, Bio-Rad Laboratories, Oslo, Norway) following the manufacturer's instructions. Extracellular TGF-β is primarily found in a latent, or inactive, form. Therefore, an activation step is recommended prior to measurement. We used the acid activation and neutralisation protocol suggested by the assay manufacturers. Specifically, 20 µL of 1 N HCl was added to 100 µL of the aqueous portion of breast milk and left at room temperature for 10 minutes. The sample was then neutralised using 20 µL of 1 N NaOH with 0.5 M HEPES. As for the TSLP analysis, breast milk samples were analysed for TGF-βs over seven plates with the same distribution of probiotic, placebo, 10-day and 3-month samples as before. The standard and two internal control samples, one high and one low internal control, were conducted in duplicate. For some plates, external standard curves were utilised to maximise the inter-assay comparability. All concentrations were multiplied by 1.4 due to the dilution in the activation-neutralisation process. Based on the calculated concentrations of the high and low internal control, the intra-assay CVs were <14.6 % and the inter-assay CVs were <14.3 %. The working range of the assay was 1.7 – 27 616 pg/mL for TGF-β_1, 14.7 – 30 080 pg/mL for TGF-β_2 and 2.8 – 15 031 pg/mL for TGF-β_3.

Quantification of sCD14. For sCD14 quantification, breast milk samples were diluted 1:10000 in PBS and reagent diluent prior to analysis with a human sCD14 DuoSet ELISA kit (R&D Systems, Minneapolis, USA). The range of detection for this kit was 62.5 to 4000 pg/mL. Samples were analysed over seven plates as for TSLP and TGF-β, and standards and an internal control sample were analysed in triplicate. The calculated concentration of the internal control samples varied widely, both between and within each plate. After dilution of samples 1:10 000, the estimated concentration of sCD14 was within the assays limits of detection for all but one sample. However, based on the calculated concentration of the internal control, the intra-assay CV ranged from 8 – 34 % and the inter-assay CV was 77 %. The unacceptably high inter-assay CV meant that the calculated concentrations could not be considered reliable and, with a relatively high intra-assay CV on most plates, it was not possible to dichotomise the results based on the internal control sample. Therefore, these results were deemed unsuitable for publication. It appears that the high level of uncertainty was introduced during the dilution

process as the standard curves, which were also conducted in triplicate, showed much less variation in their optical densities and estimated concentrations.

Multiplex quantification of cytokine panel. It was our intention to investigate a number of other cytokines involved in the Th1-Th2 spectrum. Initially, 6 samples from 3 individuals were analysed using a 27-cytokine multiplex kit (Bio-Rad Laboratories, Oslo, Norway) according to the manufacturer's instructions. The 27-plex included analyses of PDGF-BB, IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, FGF2, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1a, MIP-1b, RANTES, TNF-α and VEGF. Based on these results, and their previously documented relationship with allergy related diseases, IL-4, IL-10, IL-13, IL-17 and IFN-γ were selected for further analysis of all samples using a customised 5-plex assay (also manufactured by Bio-Rad Laboratories, Oslo, Norway). All 5 of the cytokines selected for multiplex analysis had been detected in the 27-plex assay, yet the 5-plex assay failed to detect these cytokines. Given we were unable to identify which of these multiplexes was more accurate, and observed widely varying results in the published articles, we were reluctant to continue this line of enquiry. The manufacturer was unable to explain the varying results when initially contacted, and we have not chased this up. Due to these technical difficulties, these results remain unpublished.

3.2.4 miRNA analysis

The analysis of miRNA was conducted in collaboration with Dr. Gaute Brede of the Department of Cancer Research and Molecular Medicine at NTNU. The preparation of samples, and isolation of extracellular vesicles and RNA were conducted by me, and the small RNA sequencing was performed by Ocean Ridge Biosciences, Florida, USA. The sequenced data was analysed with assistance from BioCore, the Bioinformatics Core Facility at NTNU.

The ProPACT study recruited pregnant women during 2002 to 2004, at a time that miRNAs were only recently identified and were yet to be described in breast milk. Even at the start of my project, in 2012, there was limited understanding of the impact of methodological choices such as collection and storage, extracellular vesicle isolation or enrichment, and the small RNA sequencing protocol. Through four pilot / validation experiments, we have investigated aspects of the methods that were used in Paper III (Figure 9). For the purposes of discussion,

I have included a brief description of these experiments at the end of this section and the results are presented in Chapter 5.

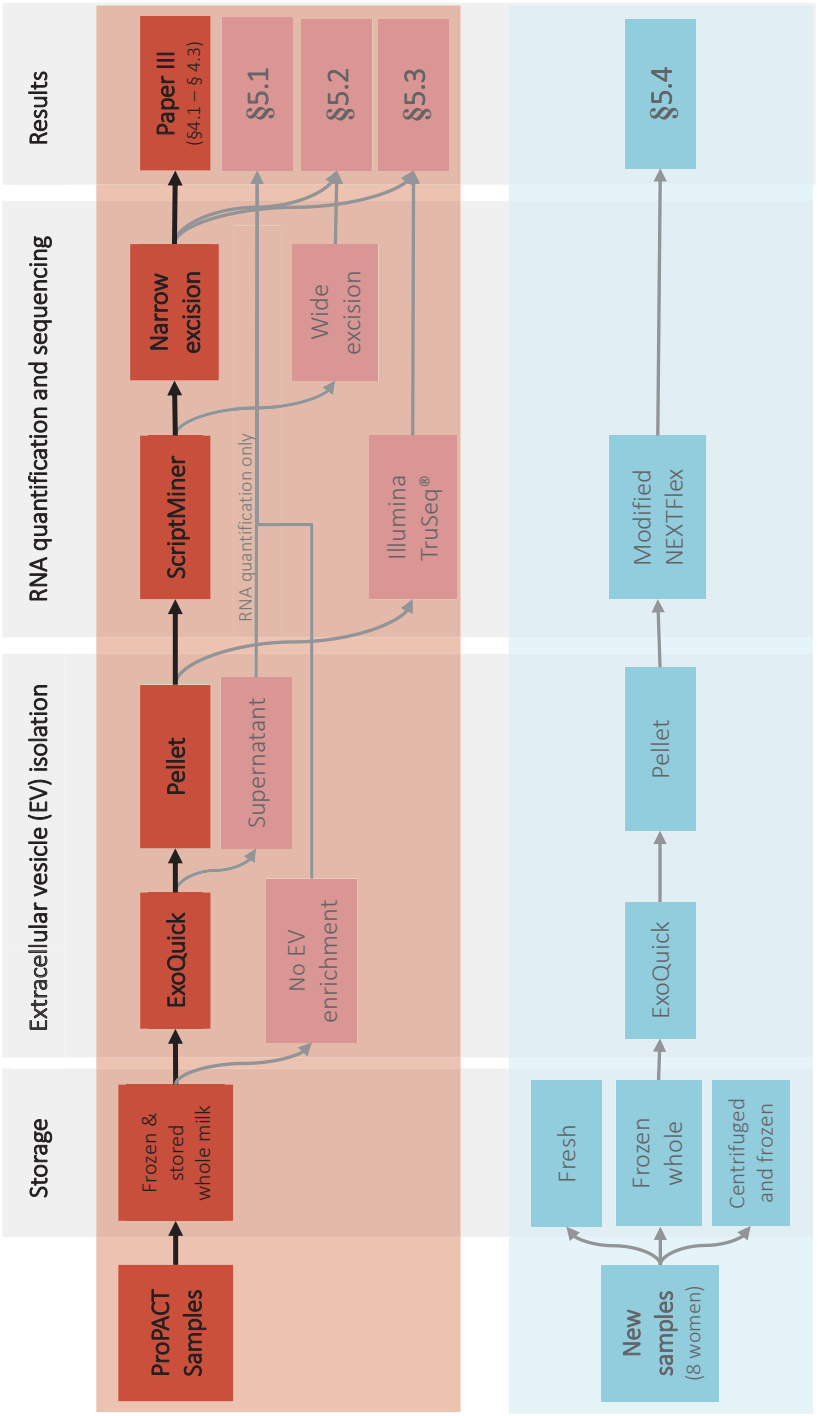


Figure 9: Investigation of different processing steps on the influence of the miRNA profile of human breast milk. The upper dark red path represents the laboratory analysis flow for the samples presented in the published paper (Paper III). Additionally, we investigate various alternative methods of sample preparation before RNA isolation and sequencing protocols in small pilot / validation experiments (red boxes). Later we recruited 8 new women to investigate the effect of freezing and pre-freezing centrifugation on the miRNA profile (blue boxes).

3.2.4.1 Published miRNA analysis (Paper III)

Subgroup included in miRNA analysis. Due to economic constraints, a semi-random selection of 3-month samples were analysed for miRNA (n = 54). In addition to the requirement that participants had attended the 2-year clinical examination, inclusion to the miRNA analysis was restricted to mother-infant pairs who had also supplied a blood sample from the infant and stool samples from both mother and child when the child was 3 months old. A total of 124 women had provided breast milk and met these inclusion criteria. The 54 samples were chosen based on the following semi-random process: first, 10 samples were randomly selected from each of the probiotic and placebo groups, independent of atopic dermatitis status at 2 years; second, among those who had developed atopic dermatitis, 10 and 12 samples were randomly selected from the probiotic and placebo groups, respectively; finally, 12 samples randomly selected from those without atopic dermatitis in probiotic group (Figure 1. in Paper III).

Sample preparation. The preparation of samples for miRNA analysis was developed with the intention of profiling extracellular miRNA, and miRNA in extracellular vesicles in particular. As such, the samples were centrifuged three times to ensure complete removal of the fat portion, cells and debris. After thawing on ice, 1.5 mL of breast milk were spun at 2000 *g* for 15 minutes, then 16 000 *g* for 40 minutes and again at 16 000 *g* for a further 60 minutes. Each centrifuge step was conducted at 4 °C.

Extracellular vesicle isolation. 250 µL of ExoQuick Exosome Precipitation Solution™ (System Biosciences, CA, USA) was added to 500 µL of the cell- and debris-free, defatted breast milk and refrigerated for 12-14 hours at 4 °C. The precipitate was pelleted at 1500 *g* for 30 minutes at 4 °C, resuspended in 100 µL of RNase free water and used immediately in the RNA isolation process.

RNA isolation and quantification. The extracellular vesicle solution was lysed in 500 µL QIAzol solution (QIAGEN, Hilden, Germany) for 5 minutes at room temperature. RNA was isolated using the QIAGEN miRNAeasy mini kit according to the manufacturer's instructions, without the optional Buffer RWT and second Buffer RPE washing steps. To ensure maximum recovery of RNA, the samples were eluted, twice, using 50 µL RNase-free water each time in the final steps of the isolation procedure. Total RNA concentration was measured using a NanoDrop 1000 (Thermo Scientific, Wilmington, USA) and selected samples were analysed with Agilent

Bioanalyzer 2100 using the Agilent RNA 6000 Nano Kit for total RNA and Small RNA kit for a focused review of the small RNA.

Sequencing. At Ocean Ridge Biosciences, samples were treated with RNase-free DNase I and repurified on RNeasy MiniElute columns (QIAGEN) prior to subsequent sequencing. Sequencing libraries were constructed from the re-purified RNA samples using ScriptMiner™ Small RNA-Seq Library Preparation Kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's instructions with minor modifications. After ligation of 3'-Adapter Oligo, samples were treated with 1 µL Tobacco Acid Pyrophosphatase (TAP) and incubated at 30 mins at 37 °C. This is an optional step in the ScriptMiner™ protocol to include 5'-capped and 5'-triphosphyorylated RNAs, as well as 5' monophosphate RNAs. Additionally, the protocol was modified to include an RNA fractionation step between RNA linker ligation and cDNA synthesis. Fractionation occurred on a 10% acrylamide-urea Gel and RNA fragments of 65-82 nt were excised. This corresponded to reads of 11-28 nt in length and ensured that reads of 17-23 nt were recovered at a minimum. Purified libraries were electrophoresed using 8% native polyacrylamide gel and fragments of approximately 122-144 nt were recovered, purified, quality controlled, quantified and pooled at equimolar concentrations.

The samples were sequenced using 50-bp single-end reads using the TruSeq SBS Kit version 3 – HS (Illumina Inc.) on the HiSeq 2000 sequencing system (Illumina Inc.). Demultiplexing and production of FASTQ files was conducted using CASAVA software version 1.8. Sequencing was completed within 3.5 months from breast milk extracellular vesicle isolation and RNA isolation.

Bioinformatics pipeline. Raw reads were processed with cutadapt[255]. Low-quality bases (<20) were excluded, adapter sequences were removed and the final reads were required to have a length of at least 17 bp for inclusion in downstream analyses. Mapping of reads to the human genome was done with STAR version 2.4.0[256], requiring a perfect match alignment. featureCounts version 1.4.0[257] was then used to count every hit of miRNAs in miRBase version 20, and finally a count matrix was produced using local scripting. Subsequent statistical analysis and functional prediction methods are described below (§3.4.3).

3.2.4.2 Assessing extracellular vesicle enrichment and RNA recovery

Four breast milk samples from the ProPACT study were randomly selected to evaluate the influence of extracellular vesicle enrichment on the quantity of RNA recovered, and investigate if there was residual RNA in the supernatant after extracellular vesicle precipitation. The possibility of residual RNA was investigated because it had been suggested that up to 90 % of extracellular miRNA in serum was also extravesicular yet stable due to binding with large RNA-binding proteins[130].

After thawing, samples were aliquoted and processed using the following steps:

1. The first aliquot of 500 µL was combined as whole breast milk with 250 µL of ExoQuick™ and refrigerated overnight. The sample was then pelleted (1,500 g, 30 mins, 4 °C), resuspended in 100 µL RNase free water and used immediately for RNA isolation.
2. The second aliquot of 1.5 mL was processed with the same triple centrifugation and extracellular vesicle isolation described above (§3.2.4.1).
3. The supernatant, after ExoQuick™ treatment and pelleting, was ultracentrifuged at 100,000 g for 90 mins. The ultracentrifuged pellet was resuspended in 100 µL RNase free water and used in the RNA isolation procedure.

RNA was isolated using the miRNeasy kit as described above for all samples. The quantity of RNA was assessed using NanoDrop 1000 and on a Small RNA chip with the Agilent Bioanalyzer 2100.

The results of this experiment are presented in §5.1

3.2.4.3 Assessing library preparation methods

Numerous different sequencing platforms and protocols are available for RNA sequencing, some specifically aimed at sequencing small RNAs and miRNAs. In terms of the influence on the end results, one of the major steps differentiating these kits is “library preparation”. The small RNA sequencing protocol used by Ocean Ridge Biosciences in the analysis presented in Paper III was relatively non-specific for miRNAs and resulted in a large proportion of reads aligned to other non-coding RNAs, particularly tRNAs and rRNAs. This was observed in a pilot study and prompted a change in the protocol. The final protocol used in Paper III included a physical selection of shorter RNAs in order to increase the relative proportion of miRNAs. The

chemistry used in the ScriptMiner™ kit also resulted in less miRNA specific sequencing, particularly because of the TAP step. We therefore also reviewed the effect of kit (chemistry) choice on the miRNA profile in a validation experiment conducted in conjunction with the local Genomics Core Facility.

In both the assessment of physical and chemical selection of miRNA sequences, only the sequencing protocol differed. The samples were otherwise processed identically to Paper III with respect to extracellular vesicle enrichment, small RNA isolation and bioinformatic analysis of the data.

Physical selection. As described above, the library preparation protocol used in Paper III used the ScriptMiner™ Small RNA-Seq Library Preparation Kit and involved fractionation of the samples after RNA linker ligation and before cDNA synthesis, and again after library construction. In the initial pilot sequencing project, wider excisions were made at both of these fractionation steps. Specifically, between RNA linker ligation and cDNA synthesis, fragments of 65-95 nt (instead of 65-82 nt used in Paper III) were excised from the 10% acrylamide-urea gel. This corresponded to reads of 11-41 nt and ensured recovery of reads of 17- 35 nt. In the second fractionation step, fragments of ~125-155 nt were recovered and purified, rather than the narrower excision of 122-144 nt used in Paper III.

The results from 12 samples sequenced with both the wide and narrow excision protocols are briefly presented in §5.2. In particular, the proportion of reads aligning to miRNAs, tRNA and rRNA are described, the overall miRNA profile is assessed using principle component analysis (PCA) and a simple assessment of expression levels of individual miRNAs was performed using paired t-tests.

Chemical selection. After the publication of Paper III we decided to expand our investigation of miRNAs in human breast milk and include samples that were collected at 10 days post-partum. By this time, the local Genomics Core Facility at our own university had purchased an Illumina next generation sequencing platform. We therefore opted to have the 10-day samples sequenced locally using their standard small RNA protocol using Illumina's TruSeq SmallRNA Library Preparation Kit as per the manufacturer's instructions and sequencing with a NextSeq HighOutput flow cell on a NextSeq 500 instrument (Illumina Inc.). To investigate the influence of the different sequencing protocols on the general miRNA profile, 12 of the 54 samples used in the analysis presented in Paper III were re-sequenced using the TruSeq SmallRNA library

preparation protocol (including 9 of the samples also sequenced after the wide excision ScriptMiner™ protocol).

The results of this validation experiment are briefly presented in §5.3 using PCA and a scatter plot of relative abundance for each miRNA in paired samples. The results from the 10 days samples are incompletely analysed, and are beyond the scope of this thesis.

3.2.4.4 Assessing the impact of sample storage

As described, the ProPACT samples were frozen after collection, initially at -20 °C and shortly afterwards at -80 °C. At the time of sequencing, the samples were between 7 and 9 years old. It was not possible to evaluate the effect of storage on these samples. In particularly, we hypothesised that the process of freezing samples may have influenced the small RNA and miRNA profiles of breast milk, and had perhaps contributed to a relatively high proportion of tRNA derived fragments. We have subsequently conducted a validation study of 8 women, comparing the miRNA profile of samples that were processed fresh, frozen after centrifugation (i.e. frozen as de-fatted, cell- and debris-free milk) or frozen whole milk.

Breast milk samples were collected from 8 women at 8-11 days after an uncomplicated, term birth. Prior to expressing the breast milk samples, participating women were contacted to arrange a time that I could pick up the sample and they were encouraged to minimise the time between expressing the sample and the arranged time point. When expressing, they were asked to wash the breast surface, express 5-10 mL milk, and store the sample in their home refrigerator until collection. Women also answered questions regarding maternal-infant characteristics and sample collection, including the time of day and time elapsed since previous breastfeeding or expression of milk from that breast.

Upon transfer to the laboratory, the samples were aliquoted into 1.5 mL volumes and the first three aliquots were processed using the following procedures:

1. The first aliquot was processed immediately using the same protocol for extracellular vesicle and RNA isolation as described above. That is to say, without being frozen, these aliquots were centrifuged three times at 2,000 *g*, 16,000 *g* and 16,000 *g* for 15, 40 and 60 minutes, respectively. Each time the aqueous portion below the fat layer was recovered and ultimately 500 µL of cell- and debris-free, defatted breast milk was combined with 250 µL ExoQuick™. After refrigeration for 13 – 16 hours, the sample

was pelleted at 1,500 *g* for 30 minutes and the pellet was resuspended in 100 µL RNase free water and used immediately in the miRNeasy mini RNA isolation procedure described in the methods section for the published paper.

2. The second aliquot was also centrifuged 3 times immediately using the same settings as above. Five hundred microliters of the cell- and debris-free, defatted breast milk was frozen at -80 °C for at least 1 week. Upon thawing, these aliquots were mixed with 250 µL ExoQuick™ and refrigerated for 13-16 hours. As before, samples were pelleted at 1,500 *g* for 30 minutes, resuspended in 100 µL RNase free water, and used immediately in the miRNeasy RNA isolation procedure.
3. The third aliquot was frozen as whole milk over night at -20 °C, without prior centrifugation, and transferred to a - 80 °C for at least 1 week before being thawed and processed as per the protocol used in Paper III.

The isolated RNA was sequenced locally at the Genomics Core facility (NTNU) using the NEXTflex™ Small RNA-Seq Kit v3 for Illumina® Platforms (Bioo Scientific, Austin, USA) protocol with incorporated steps designed to mimic the chemistry used in the now discontinued ScriptMiner™ library preparation kit used by Ocean Ridge Biosciences. Specifically, modification of the 3'-ends was performed by incubating 9.7 µl of RNA, 1.1 µl T4 10X Reaction Buffer and 0.4 µl T4 PKN (T4 Polynucleotide Kinase(3' phosphatase minus)) (New England BioLabs, Ipswich, USA) at 37 ˚C for 40 min. The T4 PKN was then heat-inactivated at 65 ˚C for 20 min. This step freed the 3' ends of OH groups, allowing ligation of 3' end with 3' 4N Adenylated Adapters at ½ dilution as per the NEXTflex™ manual (v16.06). After removal of excess 3' adaptor, 5'-capped and 5' triphosphate ends were modified to generate 5'-monophophate ends by combing 11.5 µl of the eluted samples with 2.0 µl 10X Cap-Clip Acid Pyrophosphatase Reaction Buffer and 0.5 µl Cap-Clip Acid Pyrophosphatase (CELLSCRIPT™, Madison, USA) and incubation at 37 ˚C for 60 min. Excess adapter inactivation, 5' adapter ligation, reverse transcription, bead clean-up and PCR amplification were conducted as per the manufacturer's instructions.

Libraries were pooled at equal amounts, purified using QIAquick PCR Purification Kit (QIAGEN) and eluted in 30 µl of EB Buffer. 15 µl of the purified library pool were subjected to size selection on a DNA 300 Chip (PerkinElmer, Inc., Waltham, USA) and run on a PerkinElmer LabChip XT instrument (PerkinElmer). The size selected was fragments between 138 bp to 206

bp, which should correspond to fragment insert size between ~18 bases to ~86 bases. Subsequently, Libraries were denatured as described in NextSeq "*System Denature and Dilute Libraries Guide"* [258] and sequenced on the Illumina NextSeq 500 system with the use of a High OutPut Flow Cell 50 Cycles (Illumina Inc.).

The results of this experiment are yet unpublished and preliminary analyses have been summarised in §5.4 for the purpose of discussion.

3.3 Trial registration and ethical approval

The Regional Committee for Medical Research Ethics for Central Norway granted ethical approval for the ProPACT study (Ref. 097-03 and 2012/2123/REK midt). This approval encompasses the initial ProPACT study, as well as later revisions made to the breast milk samples analysis section. These revisions were made to include the use of modern analysis methods and a validation study of storage effects on the small non-coding RNA profile of breast milk. The study was granted a license by the Norwegian Data Inspectorate to process personal health data (Ref. 2003/953-3 KBE/), and one of the parents signed a written informed consent form. The ProPACT trial was registered in Clinical Trials.Gov (identifier NCT00159523).

3.4 Statistical analysis

The types of data produced by the laboratory analyses for each Paper also required different statistical methods, as described in the coming sections (§4.4.1 – §4.4.3). Before presenting the details of these methods, I will first explain the theoretical framework that was used to create each analysis plan.

The overall aim of this thesis was to determine if alterations in certain breast milk components are partially responsible for the prevention of childhood atopic dermatitis observed in the ProPACT study. To address this aim, we had two main research questions, which required different conceptual and statistical approaches in order to draw causal inferences (Figure 10).

In the question of whether maternal supplementation with LGG, Bb-12 and La-5 led to changes in specific breast milk components, treatment allocation is the "exposure" variable. The "outcome variables" are the breast milk components, each considered individually. The underlying randomised controlled study design allows an unbiased assessment of the effect of probiotic supplementation in Papers I and II. The semi-random selection of samples for

analysis of breast milk miRNAs (Paper III) was based on treatment allocation and the development of atopic dermatitis. Therefore, the statistical model includes both of these factors as described below (§4.4.4).

The second overriding research question in this thesis was "Did changes in selected breast milk components mediate the effect of the probiotic regime on atopic dermatitis in offspring?" Once again, treatment allocation is the "exposure" variable, but the "outcome" variable is now the development of atopic dermatitis. The breast milk components are now potential "mediators", or intermediate variables, on the hypothetical causal pathway from maternal ingestion of LGG, Bb-12 and La-5 to the observed reduction of atopic dermatitis among infants in the probiotic group. In order to answer this question, we adopted a pragmatic approach assuming that any given breast milk component was only a potential mediator if we could demonstrate a statistically and practically significant effect of maternal probiotics on that component. That is to say, if maternal probiotics had no certain meaningful effect on the breast milk component, then we concluded that it could not help explain why the ProPACT regime had a beneficial effect on the development of atopic dermatitis. On the other hand, when a breast milk component appeared to be influenced by maternal supplementation, we conducted causal mediation analysis to quantify the magnitude of the preventative effect that was due the impact on the breast milk component.

Ultimately, causal medication analysis was only conducted for breast milk TLSP at 10 days postpartum and these methods are therefore presented in more detail under the section on statistical analysis in Paper II (§3.4.2)

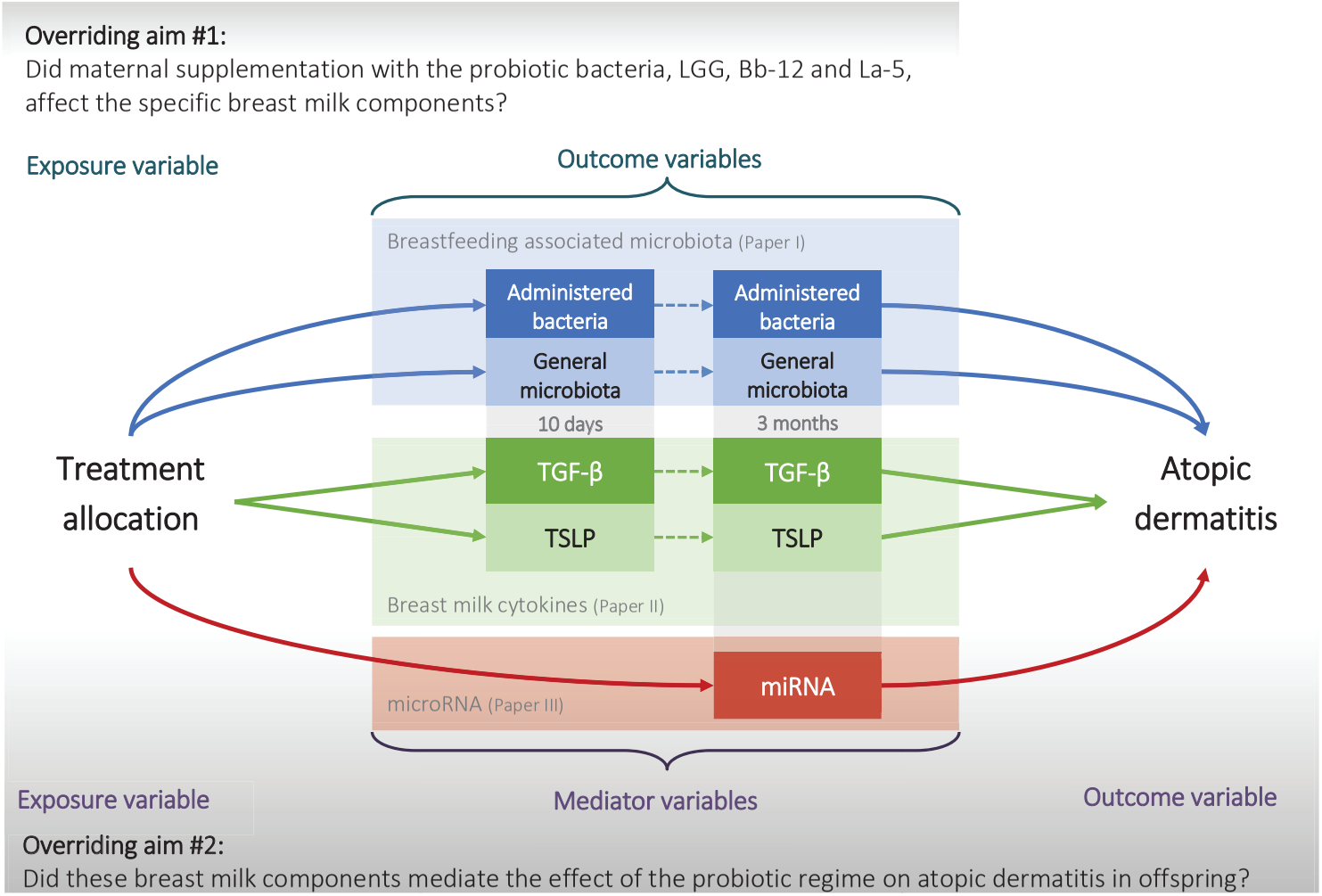


Figure 10: Conceptual framework for the statistical analyses for each of the overriding aims.

3.4.1 Breastfeeding-associated microbiota in human milk (Paper I)

Statistical analyses and graphics presented in Paper I were conducted using Stata IC 13.1 (StataCorp, College Station, Texas). Additionally, α- and β-diversity calculations were conducted in MATLAB 2016b (MathWorks, Natick, Massachusetts).

Probiotics and the administered bacteria (qPCR results). The qPCR data was used to estimate the relative risk (RR) of the presence of the administered bacteria in breast milk in the probiotic group compared to the placebo group. The RR estimate is presented with a 95 % confidence interval (CI) and p-value from a Fisher exact test.

Probiotics and the general microbiota (16s rRNA gene seq.). The effect of probiotic supplementation on the presence of individual genera was assessed using a χ^2 test (or a Fisher exact test, where indicated). Due to a high degree of individual variability, the distribution of relative abundances for most OTUs and genera were heavily skewed to the right. Therefore, the effect of probiotic supplementation on the relative abundance was assessed using the non-parametric Wilcoxon rank-sum test. In assessing both the presence and relative abundance of individual genera at 10 days and 3 months, multiple comparisons were required. Under the assumption that less common or very low abundance OTUs are of minimal clinical interest, we limited the assessment of the effect of probiotic supplementation to common OTUs and genera that were present in at least 10 % of samples (i.e. ≥ 10 reads in ≥ 6 samples from 10 days or ≥ 9 samples from 3 months). Additionally, we calculated False Discovery Rates (FDRs) using the Benjamini-Hochberg[259] method in the `multiproc` Stata command for each set of comparisons. A raw p-value of ≤0.05 was considered of potential interest, and an FDR < 0.1 considered acceptable.

The effect of probiotic supplementation on the diversity of the breastfeeding-associated microbiota in human milk was assessed using measures of α- and β-diversity. Measures of α-diversity reflect the number and or relative distribution of species within a local "habitat" – in this case, within each breast milk sample. We compared the species richness (number of observed species), phylogenetic diversity, and the Shannon and Simpson diversity indices between the probiotic and placebo group at each time point. Both the Shannon and Simpson diversity indices incorporate species richness and evenness (relative abundance of species) to produce an overall measure of diversity. Changes in species richness tend to have a greater

influence on Shannon's index, whilst Simpson's index is more sensitive to species evenness[260]. The β-diversity reflects the differentiation between "habitats", i.e. between breast milk samples. We present principle coordinates analysis (PCoA) plots from three methods of calculating β-diversity: unweigthed and weighted UniFrac distances, and Bray Curtis dissimilarity. Essentially, each of these methods produces a matrix with pairwise distances (or dissimilarities) between all samples. Unweighted UniFrac distance matrices incorporate phylogenetic information with qualitative (presence/absence) data for the bacterial species in each sample. Weighted UniFrac distance matrices weights branches of the phylogenetic tree based on the relative abundance of the species[261]. The incorporation of phylogenetic information in both UniFrac distance matrices means that these distances also reflect the relative relatedness of the bacterial species in the samples. We also present results from the Bray Curtis dissimilarity matrix, another common β-diversity metric that incorporates relative abundance, but not phylogenetic, information. The PCoA plots, otherwise known as multidimensional scaling (MDS) plots, are used to visualise the main axes producing separation of samples. If probiotic supplementation had a significant effect on the β-diversity, we would expect that the breastfeeding-associated microbiota in milk from women in the probiotic group would be more similar to each other than to samples from women in placebo group. If this was the case, we would see a clear divide between samples from women in the probiotic and placebo group on the PCoA plot(s).

General microbiota and atopic dermatitis. As an exploratory analysis, we investigated possible associations between common genera in the general breastfeeding-associated microbiota and the development of atopic dermatitis using the χ^2 and Wilcoxon rank-sum tests. Since this association is not randomised it may be confounded by other mother-infant characteristics. We therefore performed alternate multivariable logistic regression models assessing the presence of common genera and atopic dermatitis and included treatment allocation, presence of older siblings, maternal atopy and sex of the infant as additional covariates. The Wilcoxon rank-sum test was used for investigating association between relative abundance of common genera and atopic dermatitis because of the skewed distribution of reads for most genera, however this test cannot accommodate multivariable analyses.

General microbiota and mother-infant characteristics. To further investigate what factors might shape the general breastfeeding-associated microbiota in human milk, and thereby gain an

impression of the likelihood of confounding, we also investigated potential associations between common genera and mother-infant characteristics. Specifically, the 16S rRNA gene sequencing data was also used to assess associations between the general breastfeeding-associated microbiota and stage of lactation, sex of the infant, presence of older siblings and maternal atopy. Once again, χ^2 and Wilcoxon rank-sum tests were used and FDRs were calculated for each set of comparisons. The PCoA plots derived from the unweighted and weighted UniFrac distance matrices and Bray Curtis dissimilarity matrix were assessed to determine if any of these characteristics defined the overall composition of the breastfeeding-associated microbiota in human milk.

Stage of lactation and temporal variation was specifically considered among 17 women who had samples with at least 1000 reads at both time points. A further 99 women had samples from both time points sequenced, but either the 10-day sample (n = 18), 3 month sample (n = 44) or both samples (n = 37) returned less than 1000 reads. For those with adequate reads at both time points, an OTU was considered "persistent" if it accounted for at least 1% of the reads (10 reads) at both time points. The contribution of these persistent OTUs to the total microbiota at 10 days and 3 months was assessed and used to comment on the stability of the milk microbiota among women who had and had not received the probiotic supplementation.

3.4.2 Breast milk TSLP and TGF-β (Paper II)

All statistical analyses presented in Paper II were performed using Stata IC 13.1 (StataCorp, College Station, Texas). As described, TSLP concentrations were categorised into four groups, and the effect of probiotics was assessed using ordinal logistic regression. Since the distributions of TGF-βs were right-skewed, effect of probiotic supplementation on TGF-β concentrations was assessed using linear regression on log-transformed concentrations. The concentrations are reported as medians and interquartile ranges.

Due to the randomised, placebo controlled design of the ProPACT study, the effect of probiotic supplementation on cytokine concentration should not be confounded by mother-infant characteristics. All the same, alternate regression models that included maternal atopy, maternal smoking and the presence of older siblings were assessed. It was considered particularly relevant to assess these alternate models because the covariates were later included in the mediation analysis described below.

The effect of stage of lactation on the concentrations of TSLP and TGF-βs was assessed by comparing cytokine concentrations measured at 10 day and 3 months postpartum. Ordinal logistic regression was again used to estimate the association between stage of lactation and TLSP category, this time with individuals consider as clusters when deriving the variance-covariance matrix. Wilcoxon matched-pairs signed-rank test was used to compare the concentration at 10 days and 3 months for the 243 women with TGF-β isoforms measured at both time points.

3.4.2.1 Causal mediation analysis

It is intuitively appealing to consider how an intervention, or more generally an "exposure", causes an observed outcome by investigating intermediate steps along the causal pathway. Understanding the intermediate steps, which ultimately contribute to how an intervention works, has the potential to further our understanding of the biological cause of diseases and improve interventions[262,263]. In the case of this thesis, we have been interested in identifying factors in breast milk that may have been influenced by maternal probiotic supplementation, which in turn reduced the likelihood of developing atopic dermatitis for the infants (Figure 11a). The breast milk components in this situation are considered "mediators", or intermediate variables. Any given mediator tends to be only partially responsible for the overall effect of an exposure on an outcome, such that the total effect can be partitioned into a so-called "indirect effect", i.e. the effect that occurs via a mediator, and a "direct effect", i.e. the effect that occurs via other mechanisms (Figure 11a). Sometimes, the indirect and direct effects are referred to as the mediated and unmediated effects, respectively[263]. "Mediation analysis" refers to the process of estimating the size of the direct and indirect effects of an exposure on an outcome. The term "causal mediation analysis" has been adopted to describe modern, regression based techniques that apply a counterfactual framework and certain assumptions to allow causal interpretation of the indirect and direct effects.

The counterfactual, or potential outcomes, framework is the most widely used conceptual way of defining causal effects. If we first consider a situation where we want to find the true total effect of an exposure, we could select a relevant group of people who are all "exposed" (e.g. to a medication or risk factor) and follow them with respect to the outcome of interest. To find the unequivocal true total effect of that exposure, we would need to simultaneously follow exactly the same group, this time without the exposure, and measure the outcome. The

comparison of these two hypothetical groups will give us the true causal effect of the exposure on the outcome. This is an ideal, yet impossible, scenario. Individually, people are either exposed or unexposed. One of these scenarios is observable and the other scenario is described as "counter-to-the-fact" or counterfactual. The great advantage of a well-designed and well conducted randomised controlled trial (RCT) is that it mimics this counterfactual ideal. However, when we extend the counterfactual thinking to include mediation and direct and indirect effects, the randomised controlled design does not guarantee that we will obtain an unbiased estimate of the direct and indirect effects without certain assumptions. Extending the counterfactual framework to the investigation of mediators, we are hypothetically following three groups: (i) an unexposed group who have their "natural" level of the mediator (akin to the placebo group in an RCT), (ii) an exposed group whose mediator maintains its "natural" level as if the treatment had not effected mediator (an unobservable group, even in an RCT), and (iii) an exposed group whose mediator level is affected by the exposure (akin to the treatment group in an RCT). Comparisons between these groups will give us the natural indirect effect (NIE), the natural direct effect (NDE) and the total effect (TE) (Figure 11a). Although group (ii) is unobservable, we can still estimate the NIE and NDE under certain assumptions about potential confounders and lack of unmeasured confounding, as described below.

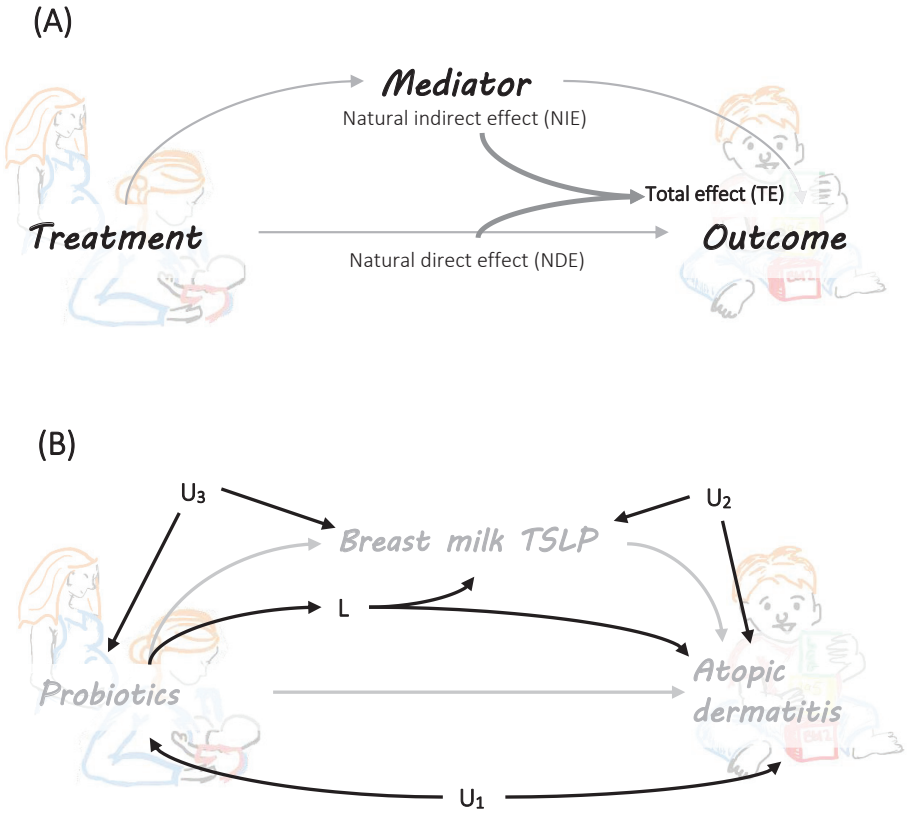


Figure 11: Schematic diagram of the mediation (A) and the required assumptions about confounding (B). In the upper panel (A), the total effect (TE) is partitioned into the natural indirect effect (NIE) and the natural direct effect (NDE). In the current study, causal inference of the estimated NIE and NDE require the following 4 assumptions to hold (B): there is no unmeasured confounding between probiotics and atopic dermatitis (U_1), breast milk TSLP and atopic dermatitis (U_2) and probiotics and breast milk TSLP (U_3), and there is no factor which is affected by probiotics and confounds the relationship between breast milk TSLP and atopic dermatitis (L). See text on following page for a further discussion of these assumptions.

In Paper II, we performed causal mediation analysis using the user written command, *paramed.* The NIE and NDE were estimated using a log-binomial regression model for atopic dermatitis, since atopic dermatitis is not a rare outcome, and the resulting estimates are interpreted as risk ratios [263-265]. The *paramed* command does not currently support models with ordinal mediator variables and TSLP concentrations were therefore dichotomised for mediation analysis. Maternal atopy, maternal smoking during the first year of life and the presence of older siblings were included in the mediation analysis as potential confounders of the relationship between TSLP level and atopic dermatitis. The model also allowed for the possibility of an interaction between probiotic supplementation and TSLP level on the development of atopic dermatitis[263].

In this study, the NIE represents the effect of the probiotic regime on the development of atopic dermatitis that can be attributed to its effect on the breast milk TSLP. The NDE represents an estimation of what the risk ratio of developing atopic dermatitis after maternal probiotic supplementation would have been, had there been no effect on the concentration of the breast milk TSLP. The estimated NIE and NDE are unbiased estimates of the causal pathways under the following assumptions (Figure 11b):

1. There is no unmeasured confounding of the relationship between the intervention (probiotic supplementation) and the outcome (atopic dermatitis), U_1.
2. There is no unmeasured confounding of the relationship between the mediator (breast milk TSLP) and the outcome, U_2
3. There is no unmeasured confounding of the relationship between the intervention and the mediator, U_3, and
4. There is no confounder of the mediator-outcome relationship that is affected by the exposure, L.

Whilst the randomised design ensures that the effect of probiotics on both breast milk cytokines and atopic dermatitis can be estimated without confounding, the relationship between cytokine concentration in breast milk and the development of atopic dermatitis is not randomised. That is to say, the first and third assumptions are automatically satisfied due to the study design. However, the second and fourth assumptions are not necessarily satisfied by randomisation. The second assumption will not hold if there are factors that affect both the mediator and outcome which have not been included in the regression models used to

estimate the NIE and NDE. Hence the inclusion of maternal atopy, the presence of older siblings and maternal smoking as potential confounders. The fourth assumption may be violated if probiotic supplementation affected some other factor that in turn affected both the level of TSLP and the development of atopic dermatitis. This assumption is noted to be a particularly strong assumption in most situations. Inclusions of such factors in the mediation analysis model requires a more advanced modelling strategy and program.

3.4.3 Breast milk miRNAs (Paper III)

All statistical analyses presented in Paper III were performed using R version 3.03[266] and functions available in the limma package[267,268]. Due to the varying proportions of miRNA in each sample, the mature miRNA reads were normalised to the total number of mature miRNA reads to create a count per million (cpm) value for each miRNA. The count data was processed using the voom function[269] and a linear model was fitted using treatment allocation and atopic dermatitis as independent variables. Subsequently, comparisons were made to assess the marginal effect of probiotic treatment on the expression levels for each miRNA and their association with the development of atopic dermatitis. Comparisons were limited to miRNAs which had an expression level of ≥500 cpm in ≥4 samples (n=125 miRNAs) in order to increase the likelihood of identifying biologically significant differences. Since the selection of samples for miRNA analysis disrupts the original randomised design, the marginal effects of treatment allocation and atopic dermatitis diagnosis were also assessed in an alternate model, which included maternal atopy and the presence of older siblings as covariates. A raw p-value of ≤0.05 was considered of potential interest and false discovery rate (FDR) was controlled for using the Benjamini-Hochberg method[259] with a FDR of ≤0.05 being considered acceptable.

Prediction of target genes and functional consequence. Potential target genes were predicted for the 20 highly expressed miRNAs and for the lists of differentially expressed miRNAs using a locally executed TargetScan version 7.0 algorithm[270-273] with an upper threshold for the context score at -0.2. TargetScan is an internet-based resource that predicts miRNA targets by identifying perfect and imperfect complimentary matches between the seed region of miRNAs and conserved sites in the 3' UTR of mRNAs. The lists of unique target genes were subsequently uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7[274,275] to gain insight into potential functional consequences of these miRNAs.

DAVID is also an internet-based resource and aims to provide a biologically meaningful interpretation of a large gene lists by identifying functional clusters and biological pathway enrichments. DAVID defined defaults for annotation categories were used. When few target genes were identified, they were assessed individually using the DAVID annotation table for evidence of involvement in atopic dermatitis and other allergy related disease. After publication of Paper III, I have also compared the list of target genes of differentially expressed miRNAs with genes identified in candidate gene association studies and GWAS.

The intestinal epithelium is presumably one of the first sites of action for the miRNAs ingested by suckling infants. To investigate the potential functional consequence of common breast milk miRNAs in the intestinal epithelium, we reviewed functional annotation clusters associated with genes target by the 20 most highly expressed miRNAs that were also identified as up-regulated in epithelial tissue in the "UP_TISSUE" chart under the tissue expression category in DAVID.

4 Main results

4.1 The effect of maternal probiotic supplementation on specific breast milk components

Overall, we found that a four-month period of supplementation with LGG, Bb-12 and La-5 had minimal effect on the composition of human milk with respect to the presence of these bacteria, concentrations of TSLP and TGF-β isotypes, and the relative expression of miRNAs.

In Paper I we found that breastfeeding is unlikely to be a substantial source of the administered bacteria for infants in the probiotic arm of the trial. Whilst the majority of women had measurable quantities of bacterial DNA in breast milk samples from both time points (392 of 472 samples), only 10 samples from 9 women had detectable levels of any of the administered bacteria at either time point. These 9 women included 8 from the probiotic group and 1 from the placebo group. Considering the breastfeeding-associated microbiota of human milk more generally, probiotics had no statistically significant effect on the presence or relative abundance of any of the common genera at either time point (Suppl. Table S4 and Figure 2 in Paper I). Nor did supplementation appear to influence the α or β diversity of the breast milk microbiota at either time point (Suppl. Figure S2 and Figure S3 in Paper I).

In Paper II, we found that probiotic supplementation might increase breast milk TSLP levels at 10 days, but does not appear to influence TGF-β isotype concentrations. Ordered logistic regression revealed a borderline statistically significant effect of probiotics associated with an increase in TSLP levels at 10 days postpartum (OR 1.55 95% CI 0.98 – 2.45, p = 0.062) (Figure 2 in Paper II). This effect was not sustained at 3 months, but was marginally enhanced when considering only women compliant with the study protocol (n = 209, OR 1.74, 95% CI 1.06 – 2.84, p = 0.028). The lack of effect of probiotics on TGF-βs was not altered when considering the subgroup of compliant women (n=224 and n=220 at 10 days and 3 months, respectively, data not shown). None of the effect estimates were affected when adjusting individually or in combination for maternal atopy, maternal smoking or the presence of older siblings (data not shown).

In Paper III, we found that probiotics did not convincingly alter the miRNA profile of breast milk samples collected at 3 months postpartum from a subgroup of 54 women. Maternal probiotic supplementation was associated with differential expression of 5 miRNAs, none of which had

an acceptable FDR (Table 2 in Paper III). All 5 miRNAs had a low level of expression, accounting for an average of 0.03 to 0.2 % of mature miRNA reads. These results were unaltered when maternal atopy and presence of siblings were included as covariates (data not shown).

4.2 Breast milk components as mediators of the preventative effect of probiotics on atopic dermatitis

We considered a breast milk component as a potential mediator of the preventative effect of the ProPACT regime only when it was affected by probiotic supplementation.

Supplementation with LGG, Bb-12 and La-5 appears to lead to the presence of these bacteria, primarily Bb-12, in the breastfeeding-associated microbiota for a small subgroup of women. However, with so few positive samples, we concluded that breastfeeding was not a meaningful source of these bacteria, and their transfer during breastfeeding cannot be a major contributor to the preventative effect seen on atopic dermatitis at 2 years of age. Formal statistical assessment of the presence or relative abundance of the administered bacteria as mediators was not feasible.

In terms of the cytokines and miRNAs investigated, only TSLP concentrations at 10 days postpartum were found to be potentially influenced by probiotic supplementation. Causal mediation analysis was performed to estimate to what extent the increased levels of TSLP may have contributed to the preventative effect of probiotics. For this analysis, TSLP concentrations at 10 days were dichotomised. Consistent with the ordinal logistic regression analysis, standard logistic regression on the dichotomised TSLP values also suggested that perinatal probiotic supplementation increased the odds of a high TSLP concentration at 10 days (OR 1.72, 95% CI 1.03 – 2.87, p = 0.039). However, mediation analysis suggested that the effect of probiotics on TSLP does not appear to contribute to the prevention of atopic dermatitis seen at 2 years of age (Figure 4 in Paper II). The RR^{NIE} was 1.04 (95 % CI 0.94 – 1.15, p = 0.45), and can be interpreted as the relative risk of developing atopic dermatitis following probiotic supplementation and its subsequent effects on TSLP. In contrast, the RR^{NDE} was 0.63 (95 % CI 0.42 – 0.95, p = 0.03). This can be interpreted as the expected risk of developing atopic dermatitis following probiotic supplementation had it had no influence on TSLP levels at 10 days postpartum. Together these indicate that the primary preventative effect of probiotics appears to operate via pathways other than effects on breast milk TSLP.

Breast milk TSLP at 3 months post-partum, TGF-β isotypes at both 10 days and 3 months, and miRNAs at 3 months were not demonstrably affected by probiotic supplementation. As such, changes in these breast milk cytokines and individual miRNAs are unlikely to be responsible for the reduced risk of developing atopic dermatitis observed after maternal probiotic supplementation and no estimation of NIE and NDE was conducted for these.

4.3 Breast milk components and the development of atopic dermatitis

The data presented in Papers I, II and III were also used to explore possible association between the composition of breast milk at 10 days and 3 months and the later development of atopic dermatitis, independent of treatment allocation. Whilst we found that individual genera and miRNAs were potentially associated with the development of atopic dermatitis based on raw p-values, none of the comparisons had an acceptable FDR. Furthermore, there was no clear biologically meaningful explanation for the association between the highlighted miRNAs and atopic dermatitis.

In Paper I, the presence and relative abundance of *Staphylococcus* and the presence of *Veillonella* in breast milk samples at 3 months tended to be associated with a greater risk of developing atopic dermatitis by two years, although without an acceptable FDR. In Paper II, neither TGF-βs nor TSLP, including TSLP at 10 days, were convincingly associated with the development of atopic dermatitis when assessed independently of treatment allocation or after adjusting for treatment allocation. The one possible exception to this observation is TGF-β_3, which may have a protective effect on the development of atopic dermatitis. The median (IQR) concentration of TGF- β_3 in milk received by infants that developed atopic dermatitis was 23.4 (13.3 – 32.8) pg/mL, compared to 27.9 (18.2 – 45.9) pg/mL for infants who did not develop atopic dermatitis (adjusted p = 0.023). In comparison to TGF- β_1 and TGF-β_2, TGF-β_3 has a very low level of expression and it is uncertain if this small difference in concentration is of biological importance.

In Paper III, the development of atopic dermatitis was associated with the differential expression of 13 breast milk miRNA: 5 upregulated and 8 downregulated. Five of the differentially expressed miRNAs were relatively highly expressed including miR-146b-5p, miR-21-5p, miR-22-3p, miR-375 and let-7f-5p (Table 2 in Paper III). Once again, none of these miRNAs had an acceptable FDR. In total, these 13 miRNA had 2269 predicted target genes,

and function predictions indicated that these genes were enriched in a diverse range of functional clusters. There was no clear clustering of target genes with respect to allergy related disease pathways in DAVID. On comparison of gene lists identified by candidate gene association studies and GWAS, 13 of the 2269 predicted target genes have been previously identified in association with atopic dermatitis (including BNDF, CD207, CYP24A1, CYSLTR1, GPSM3, IL10, IL13, IL31, ILBRA1, OVOL1, SOCS3, STAT3, ZBTB10).

4.4 Insights into human breast milk composition

Through these analyses, we have also gained greater insight into the breastfeeding-associated microbiota and miRNA profile of human milk, as well as temporal changes and the potential influence of mother-infant characteristics on breast milk composition.

4.4.1 Breastfeeding-associated microbiota in human milk

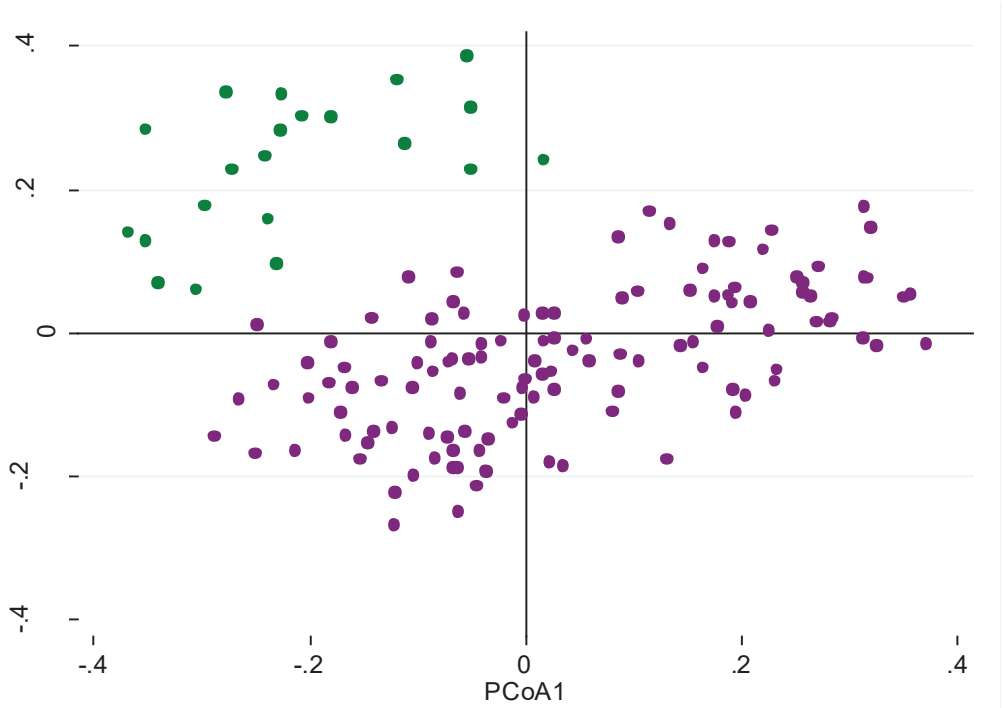
Due to low quantity and quality of bacterial DNA in the breast milk samples, the 16S rRNA gene sequencing produced sufficient reads for 142 of the 472 samples. These reads were attributed to a total of 307 OTUs belonging to 147 genera. However, only 45 and 69 genera were present with ≥10 reads in at least one sample from 10 days and 3 months, respectively, and only 11 genera were present in at least 10% of samples at either time point (Table 3 and Table S3 in Supplementary material for OTU level summary in Paper I). No genus was present in all samples. Even at the phyla level, there was no phylum which was present in all breast milk samples, although Firmicutes bacteria were present in all but one 3-month sample.

The general breastfeeding-associated microbiota at 10 days and 3 months postpartum was dominated by *Streptococcus* and *Staphylococcus* genera in most women (Suppl. Figure S1 in Paper I). A subgroup of women had a milk microbiota dominated by other genera, such as *Acinetobacter* and genera from the *Bacillaceae* family (Suppl. Figure S1 in Paper I) or uncommon genera, such as *Klebsiella, Lactobacillus zeae, Alkanindeges, Stenotrophononas* and genera from the *Caulobacteraceae* family (Data not shown, these genera fall into the category of "other" in Figure S1 in Paper I). Over time, it appears that the prevalence and relative abundance of *Staphylococcus* genus decreases, with a concurrent increase in the number of observed species, phylogenetic diversity and Shannon's diversity index. Despite temporal changes in individual genera, the PCoA plots did not indicate a clear separation of the

breastfeeding-associated microbiota based on stage of lactation. However, 10-day samples were more likely to be associated with divergent microbiotas in the unweighted UniFrac and Bray Curtis analyses (Figures 13 and 14).

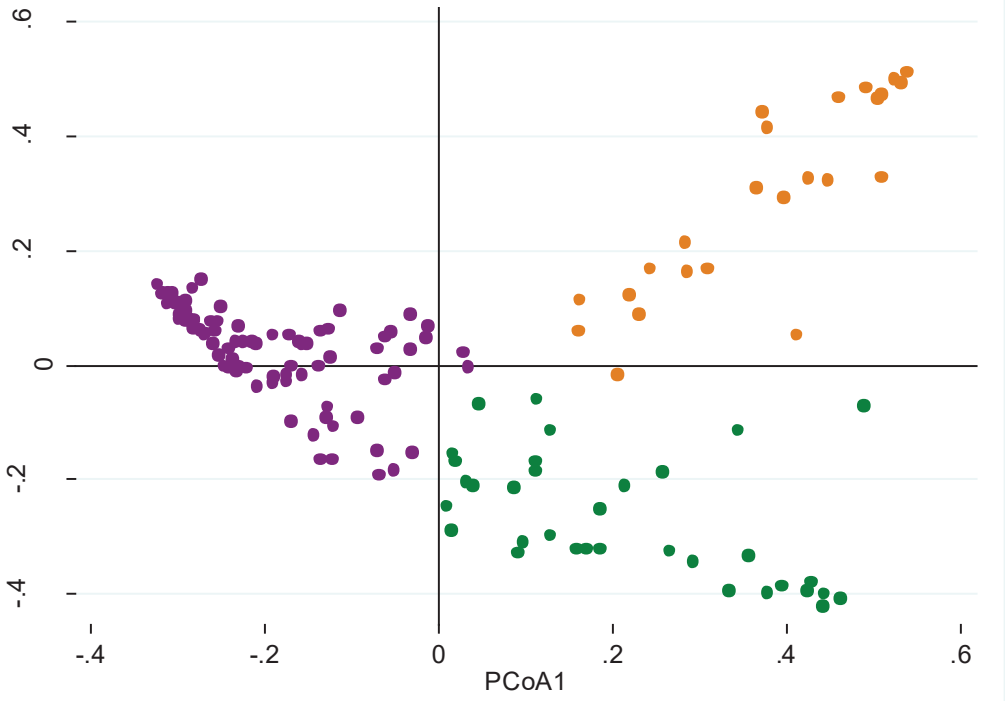
Due to the high degree of individual variability, we also assessed temporal changes in the breastfeeding-associated microbiota for the subgroup of 17 women with adequate reads at both 10 days and 3 months postpartum. Most of these women had a breastfeeding-associated microbiota dominated by Streptococcus and Staphylococcus at both 10 days and 3 months postpartum (Figure S4 in Paper I). For some women Streptococcus genera was responsible for more than 50% of the reads at both time points, whilst for other women there was a shift from a Staphylococcus to Streptococcus dominance. The remaining women had more variable composition of their milk microbiota. At the OTU level, the breastfeeding associated microbiota was relatively stable for most women, since OTUs shared between both time points accounted for 70 – 80 % of reads at each time point. Probiotic supplementation may have a positive influence on the stability of the breastfeeding-associated microbiota (RR of stable breastfeeding-associated microbiota after probiotic supplementation: 2.37, 95% CI 0.94 – 5.97, Fisher's exact p = 0.050).

Maternal atopy and infant sex were potentially associated with the presence or relative abundance of common genera at individual time points. Specifically, the *Gemellaceae* family was more common in 10 day samples from mothers with a personal history of allergy. In contrast the *Rothia* genus was more commonly observed from mothers with no allergic history at 10 days postpartum, and mothers of female infants at 3 months postpartum. No other associations were found between individual genera and these mother-infant characteristics, nor do they appear to define the overall composition of the breastfeeding-associated microbiota in milk on review of the PCoA plots (Figure 12 and 13, and Suppl. Figure S3 – S6 in Paper I).



	Main group (purple)	Divergent group (green)	p-value
	% (95% CI)	% (95% CI)	Chi² test
Probiotic treatment	48.8 (39.9 – 57.7)	38.1 (19.8 – 60.5)	0.366
Age of child, 10 days	**38.8 (30.5 – 47.9)**	**61.9 (39.5 – 80.2)**	**0.048**
Sex. males	46.3 (37.5 – 55.3)	42.3 (23.5 – 64.7)	0.771
Presence of older siblings	43.0 (34.4 – 52.0)	42.9 (23.5 – 65.0)	0.992
Maternal atopy	48.8 (39.9 – 57.7)	47.6 (27.3 – 68.8)	0.923
Atopic dermatitis	34.7 (26.7 – 43.7)	33.3 (16.4 – 56.1)	0.902
	Mean (95% CI)	Mean (95% CI)	t-test
Maternal age	34.7 (26.7 – 43.7)	33.3 (16.4 – 56.1)	0.902

Figure 12: Unweighted UniFrac PCoA plot of axis 1 against axis 2. The accompanying table provides a comparison of baseline characteristics, treatment allocation and development of atopic dermatitis in the "main group" (purple) and the group of individuals which might be considered to have a divergent breastfeeding associated microbiota (green). The "divergent" group has a substantially higher proportion of 10-day samples, however this was not the case in the weighted UniFrac derived PCoA plot which considers also the relative abundance.



	Main group (purple)	Upper divergent group (orange)	Lower divergent group (green)	p-value
	% (95% CI)	% (95% CI)	% (95% CI)	Chi^2 test
Probiotic treatment	46.4 (36.9 – 57.2)	66.7 (45.5 – 82.7)	58.8 (41.5 – 74.2)	0.156
Age of child, 10 days	**34.5 (25.0 – 45.4)**	**29.2 (14.3 – 50.5)**	**70.6 (53.0 – 83.6)**	**0.001**
Sex. males	46.4 (35.9 – 57.2)	45.8 (27.0 – 65.9)	44.1 (28.3 – 61.2)	0.976
Presence of older siblings	47.6 (37.1 – 58.4)	41.7 (23.6 – 62.2)	32.4 (18.6 – 50.0)	0.303
Maternal atopy	52.4 (41.6 – 62.9)	54.2 (34.1 – 73.0)	35.3 (21.0 – 52.8)	0.203
Atopic dermatitis	34.5 (25.0 – 45.4)	37.5 (20.4 – 58.4)	32.4 (18.6 – 50.0)	0.939
	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)	ANOVA
Maternal age	30.8 (29.9 – 31.7)	31.6 (30.0 – 33.3)	30.6 (29.2 – 32.0)	0.598

Figure 13: PCoA plot from Bray-Curtis dissimilarity matrix (axis 1 against axis 2). The accompanying table provides a comparison of baseline characteristics, treatment allocation and development of atopic dermatitis in the "main group" (purple) and two groups of individuals which might be considered to have a divergent breastfeeding associated microbiota (orange and green). The "green" group has a substantially higher proportion of 10-day samples.

4.4.2 miRNAs and other small RNAs

Between 12.4 and 247.5 ng/µL was isolated from the 54 breast milk samples. These isolates consisted of RNA up to 1000 nt in length (Figure 2b in Paper III). After sequencing, the majority of reads were mapped to tRNA and rRNA fragments which on average (SD) accounted for 35.5 (15.5) and 40.3 (15.3) % of reads, respectively, and mature miRNAs accounted for an average of 9.1 % (SD 3.8 %, range 2.8 – 20.2 %) (Figure 2c in Paper III). More than half of these mature miRNA reads were mapped to 5 miRNA that were consistently highly expressed across all samples: miR-148a-3p, miR-22-3p, miR-30d-5p, let-7b-5p and miR-200a-3p.

The 20 most abundant miRNAs were predicted to have 3498 unique gene targets and functional predictions indicated that the breast milk miRNAs may have a wide range of biological effects. Regulation of transcription and gene expression, embryonic development and metabolic or biosynthetic processes, were among the identified annotation clusters. Using the list predicted target genes, we also considered which tissue types might be most affected by the group of breast milk miRNAs and what effects they might have on epithelial cells. Consistent with the hypothesis that the intestinal epithelium may be one of the main tissues influenced by breast milk miRNAs, “epithelium” was identified as having an upregulation of genes that are predicted targets of these miRNAs. However, it was difficult to glean the overall consequence of breast milk miRNAs on epithelium based on the broad range of annotation clusters. Other tissues with upregulation of target genes included brain, female reproductive, foetal, and haemopoetically involved tissues.

5 Results from preliminary and validation experiments

5.1 Extracellular vesicle enrichment and RNA recovery

The extracellular vesicle precipitation solution ExoQuick™ resulted in a substantially higher yield of RNA from breast milk samples compared to when they were processed without any form of extracellular vesicle (EV) enrichment (Table 3). The supernatant remaining after pelleting the ExoQuick was essentially devoid of RNA, with concentrations of ≤ 5 ng/ µL (Table 3).

Table 3: Concentration of RNA in samples after before and after extracellular enrichment, and in the resulting supernatant.

Sample	RNA concentration (ng/µL)		
	No EV enrichment	ExoQuick	Ultracentrifuged supernatant
A	53	90	.
B	15	41	5
C	90	204	3
D	5	15	3

5.2 Library preparation with physical selection of miRNA sized sequences

The proportion of reads mapped to tRNAs was significantly higher using the original "wide excision" protocol (Table 4). Conversely, the modified "narrow excision" protocol increased the proportion of miRNAs from an average (SD) of 2.6 (0.9) % to 9.1 (3.8) %.

Table 4: Proportion of reads mapped to miRNA, tRNA and rRNA in the wide and narrow excision protocols.

	Wide excision		Narrow excision	
	Mean (SD)	Range	Mean (SD)	Range
miRNA	2.6 (0.9)	1.5 – 4.7	9.1 (3.8)	2.7 – 20.1
tRNA	78.5 (7.9)	58.3 – 91.6	35.5 (15.5)	8.6 – 74.0
rRNA	13.3 (5.4)	4.1 – 26.4	40.3 (15.3)	12.5 – 76.9

Principle component analysis of samples processed with the narrow and wide library preparation protocols indicates that the size of excision influences the overall miRNA profile (Figure 14). However, there was some overlap between the two miRNA profiles. Using paired t-tests, the relative abundance of 41 miRNAs were statistically significantly different between the two protocols (data not shown). The top 5 miRNA identified in Paper III were also highly expressed in the wide excision protocol, although they tended to have a lower relative abundance (Figure 15).

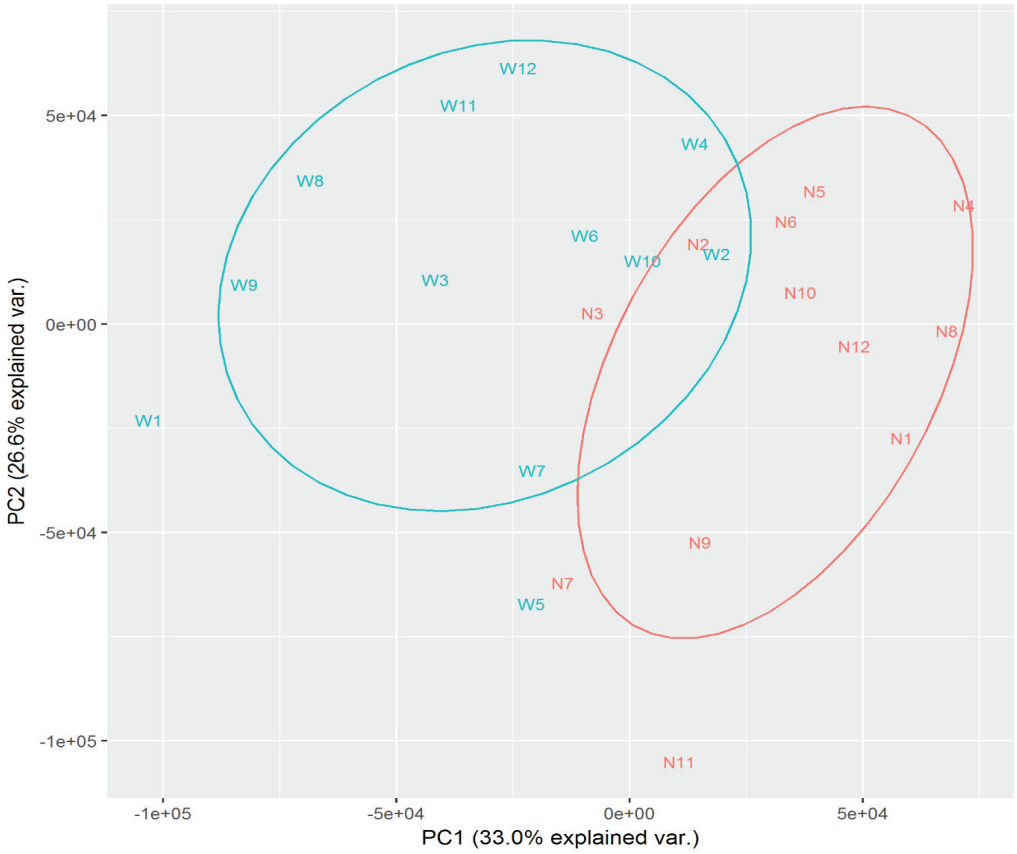


Figure 14: Principle component analysis of miRNA expression profile in paired samples processed using the narrow (N) and wide (W) ScriptMiner™ library preparation protocols.

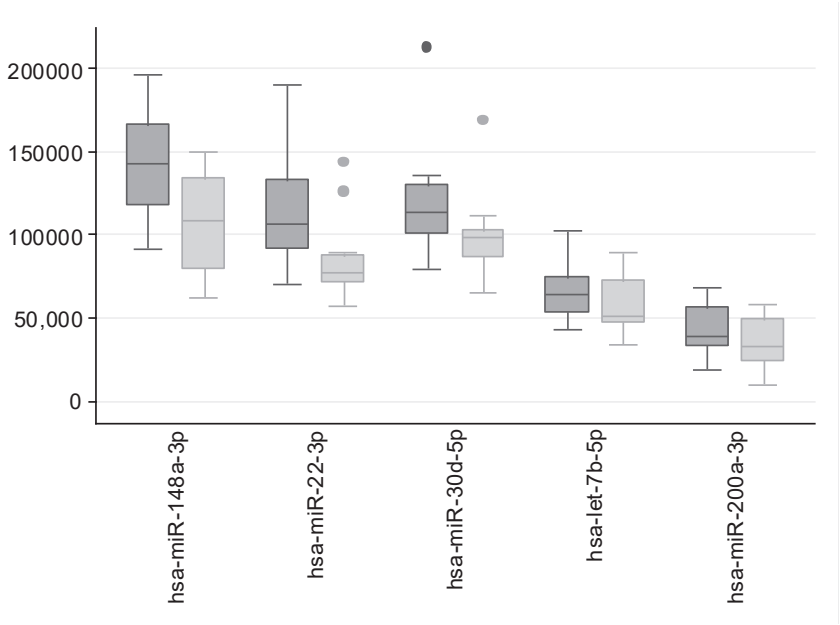


Figure 15: Box plot of counts per million (CPM) of the top 5 miRNA. Samples processed using the narrow (dark grey) and wide (light grey) ScriptMiner™ library preparation protocol. P-values from paired t-tests were miR-148a-3p: 0.031; miR-22-3p: 0.078; miR-30d-5p: 0.083; let-7b-5p: 0.021; miR-200a-3p: 0.371.

5.3 Library preparation with chemical enrichment of miRNA sequences

The miRNA profile for paired samples processed with the narrow excision ScriptMiner™ and standard TruSeq protocol differed substantially. In these samples, the library preparation protocols appear to account for nearly 70 % of the observed variance (Figure 16).

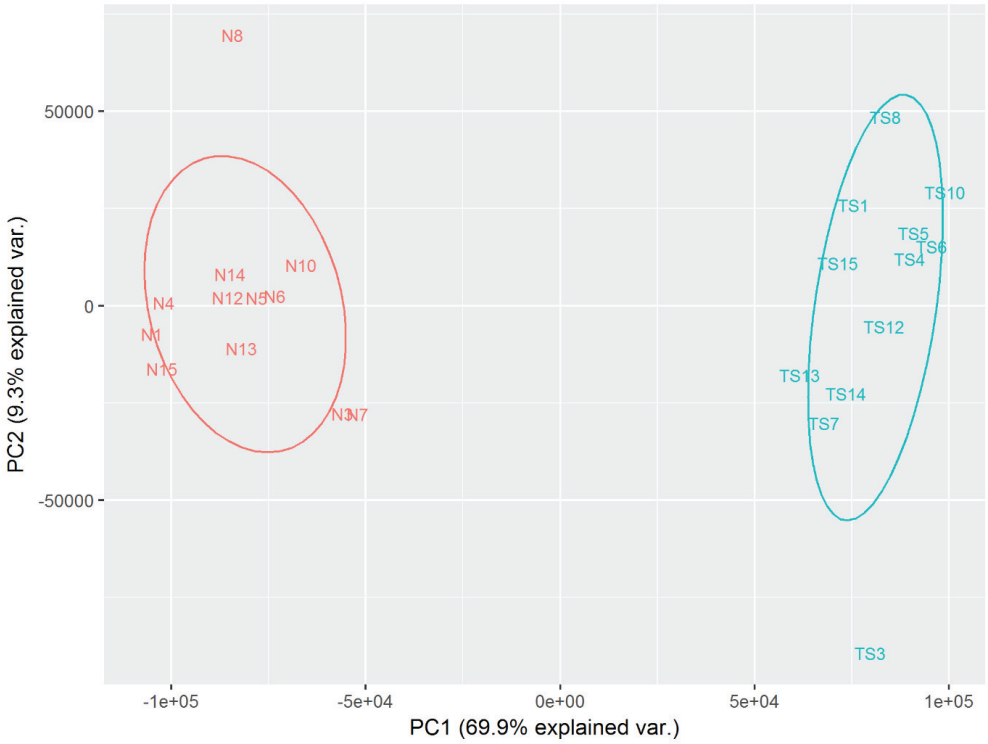


Figure 16: Principle component analysis of miRNA expression profile in paired samples processed using the narrow ScriptMiner™ (N) and TruSeq (TS) library preparation protocols.

Broadly, miR-148a-3p, miR-22-3p, miR-30d-5p and miR-200a-3p (4 of the top 5 from Paper III) were highly expressed after both library preparation methods, although they tended to be more highly expressed after the narrow excision ScriptMiner™ protocol (data not shown). The last of the top 5 miRNA identified in Paper III, let-7b-5p, had a particularly low relative abundance after TruSeq library preparation (Figure 17). On the other hand, a number of miRNAs were observed to have a high relative abundance after the TruSeq library despite having low levels of expression after the ScriptMiner™ protocol.

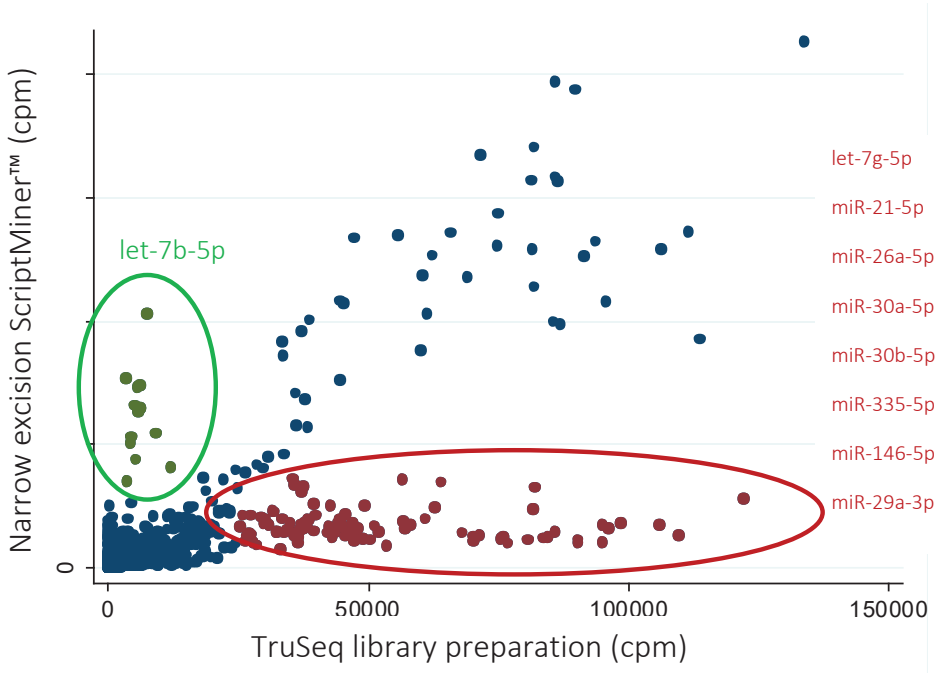


Figure 17: Pairwise comparison of the relative abundance of miRNAs in samples sequenced using the narrow excision ScriptMiner™ and Illumina's TruSeq library preparation protocols. Visually, two groups were identified which particularly distinguish the miRNAs from the two protocols. These are circled in green and red. In all 12 samples, let-7-5p was comparatively highly expressed using the ScriptMiner™ kit. The miRNAs listed in red represent miRNAs that fell into the red group in at least 8 of 12 samples. cpm: counts per million.

5.4 Fresh vs frozen skimmed or whole milk

The characteristics of the 8 mother-infant pairs included in this validation experiment are provided in Table 5. Samples collected by the women between 08:30 and 11:25 AM. At time of collection, a median of 2 ½ hours had passed since the newborn had drunk from that breast, or milk had been expressed from that breast (range: 5 minutes to 4 ½ hours). Seven of the 8 samples underwent the first centrifugation step within 2 hours after they were expressed, and the 8th sample was centrifuged 4hrs 20 minutes after expression (median time to laboratory was 1:27, range – 0:30 to 4:20).

Table 5: Characteristics of mothers and infants included in validation experiment of fresh and frozen breast milk samples (n = 8 mother-infant pairs).

Characteristic	Value
Maternal characteristics	
Maternal age, years, mean (SD)	29.7 (3.9)
Primiparious, n (%)	5 (63)
Previous breastfeeding, n (%)	3 (38)
Maternal atopy, n (%)	2 (25)
Infant characteristics	
Sex of infant, male, n (%)	6 (75)
Gestational age, days, mean (SD)	288 (5.8)
Birth weight, gm, mean (SD)	3439 (460)
Presence of siblings, n (%)	4 (50)

The library preparation protocol used in this validation experiment results in a high proportion of reads from rRNA and tRNA fragments in all samples (approximately 80 % of reads).

Overall, there was 106 differentially expressed miRNAs between the fresh and frozen whole milk samples (as defined as an FDR < 0.1, Table S1 in Appendix III), and a clear separation of the miRNA profiles following these two storage methods on principle component analysis (Figure 18). Among the differentially expressed miRNAs were 4 of the top 5 miRNAs identified in Paper III. Specifically, miR-148a-3p and miR-22-3p were significantly more abundant in

frozen samples and miR-30d-5p and let-7b-5p was more abundant in samples processed fresh. Another 11 differentially expressed miRNAs belonged to the top 20 highly expressed miRNAs described in Paper III.

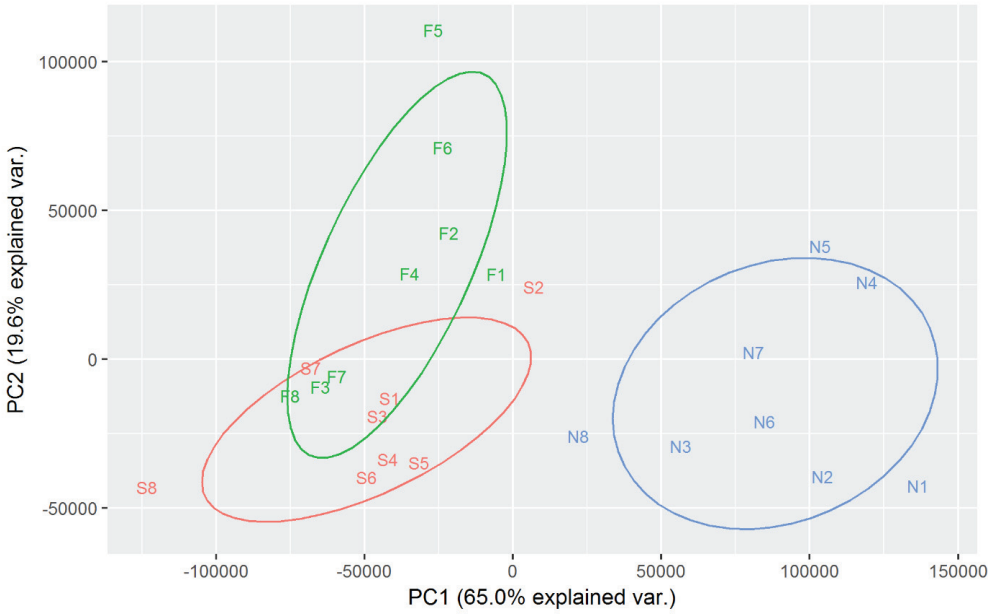


Figure 18: Principle component analysis of miRNA expression profile in paired samples processed fresh (F), or frozen as defatted, cell and debris free (S) and whole milk (N).

There was also 81 differentially expressed miRNA in the comparison between fresh samples and those centrifuged prior to freezing (Table S2 in Appendix IV). However, these tended to be less markedly changed and the resulting miRNA profile was overlapping (Figure 18). Consistent with the PCA analysis, the average expression levels for abundant miRNAs were more similar between the fresh samples and those centrifuged prior to freezing than to the samples frozen as whole milk (Figure 19).

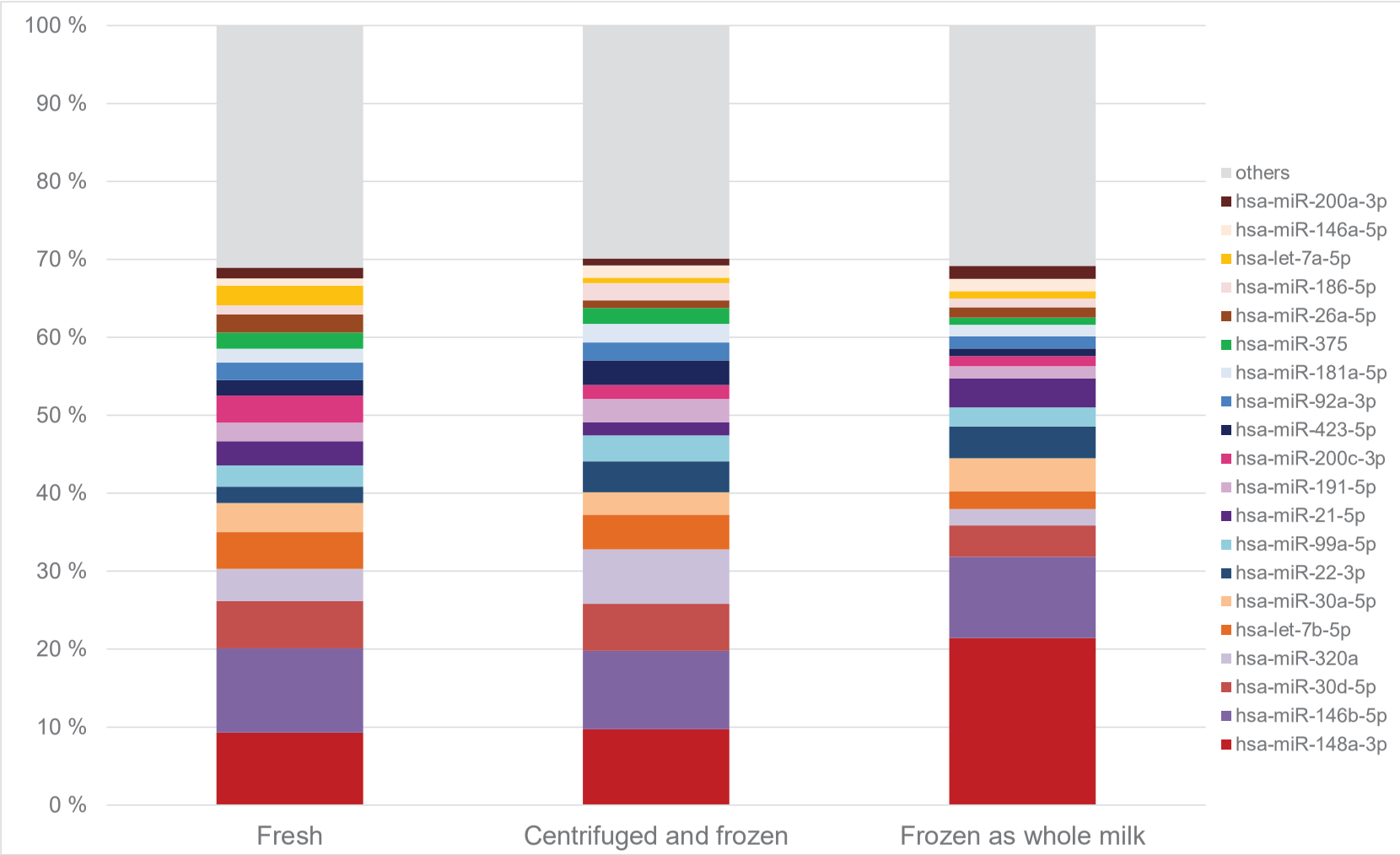


Figure 19: Relative abundance of highly expressed miRNAs in samples after processing fresh or processed after freezing with and without prior centrifugation. The 20 most highly expressed miRNAs are presented here based on the average expression in these samples (not those in Paper III)

6 Discussion

6.1 Summary of main findings

Overall, we found that pre- and postnatal maternal probiotic supplementation with LGG, La-5 and Bb-12 led to minimal changes in the breast milk composition with respect to the breastfeeding-associated microbiota, TSLP, TGF-βs, and miRNAs. Furthermore, our data would suggest that none of these breast milk components significantly contributed to the preventative effect of maternal probiotic supplementation against the development of atopic dermatitis in infants.

Women in the probiotic arm of the trial were statistically more likely to have Bb-12 in their breast milk at either 10 days or 3 months postpartum. However, with only 9 of 252 women found to have any administered bacteria at either time point, breast milk is unlikely to be a substantial source of LGG, La-5 or Bb-12 for the infants in the ProPACT study. Turning our attention to the immune active breast milk components that were investigated, we found that probiotic supplementation may increase breast milk TSLP concentration at 10 days postpartum, but does not appear to significantly alter the concentration of TGF-β isotypes or the relative abundance of individual miRNAs. Causal mediation analysis suggested that the increase in TSLP at 10 days does not contribute to the prevention of atopic dermatitis in infants following maternal probiotic supplementation. With no demonstrated effect of probiotics on breast milk TGF-β or miRNAs, these components are unlikely to be mediators of the preventative effect of probiotics on the development of atopic dermatitis.

Through these analyses, we have also gained greater insight into the composition of breast milk. In particularly, papers I and III remain two of the larger studies published on the breastfeeding-associated microbiota and human milk miRNA profile, respectively.

6.2 Methodological considerations

When interpreting the findings presented in this thesis, we must consider the precision of the estimates, the risk of systematic bias, and to whom the results are applicable. In the coming sections I address these considerations through a discussion of random error, internal validity and external validity, respectively.

6.2.1 Random error

The term random error is used to describe the variability in the data that is unexplainable after considering systematic errors that may have been introduced through selection bias, confounding or measurement errors[276]. A study with a low degree of random error will have a high degree of precision. The primary strategy to decrease the amount of random error, and thus increase the precision of the estimates, is to increase the study sample size.

Prior to commencement of the ProPACT study, a sample size calculation was performed for a study with 80 % power and a two tailed 5% significance level based the following expectations: A cumulative incidence of atopic dermatitis of 40 % in the placebo group, a relative reduction of 40 % in the probiotic group, and a dropout rate of 30 % in both groups[6]. Two-hundred and eight mother-infant pairs were to be randomised to each trial arm. Implicit in this calculation is the fact that the size of the ProPACT study was solely determined based on the expected incidence and prevention of atopic dermatitis, and not the other allergy related diseases or secondary outcomes such as modifications in breast milk components. As such, the studies presented in this thesis may be underpowered to detect small differences in cytokines or miRNA in breast milk following probiotic supplementation. The investigation of probiotic supplementation on the presence of the administered bacteria was sufficiently powered to detect relatively small increases in these bacteria, assuming a naturally low presence of these bacteria as seen in the placebo group. Also, considering the fact the probiotic regime resulted in a substantial increase in presence and relative abundance of LGG in children, Paper I has a large enough samples size to establish if LGG transferred during breastfeeding was a significant source in these children.

The measurement of the breast milk components may be both imprecise because of inherent variability in the analysis methods or inaccurate if the methods have led to a systematic bias in the results. The likelihood of measurement errors, both random and systematic, are discussed together below (§6.2.2.3), as they could both lead to a biased estimation of the association between probiotic supplementation and the specific breast milk components. However, any random measurement error would have reduced the precision of the estimates in the descriptive aspects of Papers I and III.

6.2.2 Internal validity

A study with a high degree of internal validity is one that has minimised the extent of systematic error through appropriate study design, addressing the potential influence of confounders, and employing precise and accurate measurement techniques. Internal validity implies that the conclusions from the study are valid for the members of the source population[276].

The first of the overriding aims of this thesis was to assess if maternal probiotic supplementation affected the breastfeeding-associated microbiota, selected cytokines or miRNAs in breast milk. The randomised, placebo controlled design of the ProPACT study and the internal validity of this study is integral to the ability to draw unbiased conclusions about the effects of the probiotic regime (§6.2.2.1). The second overriding aim of this thesis was to investigate if any changes in the selected breast milk components may be mediators in the preventative effect of probiotics. The randomised controlled design is still an advantage in addressing this question. However, we needed to employ a more nuanced statistical model to quantify the degree of mediation since this estimation is not immune to more classical epidemiological sources of bias, such as confounding (§6.2.2.2). Precise and accurate measurement of the specific breast milk components are integral to both aims and probably represent the greatest challenge in the analysis of breast milk components (§6.2.2.3).

6.2.2.1 Study design

As detailed in the *Cochrane Handbook for Systematic Reviews of Interventions*, the internal validity of RCTs is primarily reliant on adequate sequence generation, allocation concealment during recruitment, blinding of participants and assessors, and appropriate handling of incomplete outcome data[277]. Selective reporting of (usually positive) outcomes and the resulting "reporting bias" is also included when assessing risk of bias in RCTs.

Selection and attrition bias. Randomisation was conducted using a computer-generated sequence at the Department for Applied Clinical Research at the Norwegian University of Science and Technology, NTNU. The group allocation was sent directly to the producers of the study milk who were also responsible for its distribution. The doctors and midwives recruiting patients did not know, and were unable to predict, which treatment a given woman would receive. As such, there should have been no risk of selection bias at recruitment.

The original ProPACT cohort included 415 women, yet 162 of these are not included in any of the present studies due to lack of breast milk sample(s) and or clinical follow-up at 2 years. There were similar attrition rates between the probiotic and placebo groups, and the remaining 259 mother-infant pairs were representative of the original study population with respect to baseline characteristics and allergy related disease outcomes at 2 years (Suppl. Table S1 in Paper I). During the follow-up at 6 years of age, it was apparent that children who had developed atopic dermatitis by 2 years of age were more likely to attend the 6-year follow-up[86]. It is reasonable to assume that there may be a degree self-selection present also at 2 years. Otherwise, there is no indication that there was a differential cause of attrition (or retention) between the two study groups, and it seems unlikely that this has biased the results. The analysis of administered bacteria (qPCR) and cytokines was conducted for all available samples, although slightly fewer are included in the analysis of administered bacteria and TLSP as described previously. Due to the low quantity of bacterial DNA in breast milk, only 142 samples from 125 women had adequate reads and were included in the investigation of the overall composition of the bacteria transferred during breastfeeding. Again, the baseline characteristics of these women were representative of the original population.

The subgroup included in the analysis of breast milk miRNA (Paper III) included a semi-random selection of 54 samples collected at 3 months postpartum. The investigation of miRNAs was an exploratory study and samples were selected in such a way that increased statistical power to assess differences between the breast milk miRNA profile associated with the following two categories: (1) the mother received probiotics and the infant did not develop atopic dermatitis, and (2) the mother received the placebo and the infant did develop atopic dermatitis. Selection was influenced by both treatment allocation and the outcome of atopic dermatitis. Particularly in the placebo group, the selection criteria favoured the inclusion of mother-infant pairs where the infant had developed atopic dermatitis and there was a maternal or familial history of allergy related disease. The statistical analyses employed in Paper III include both treatment allocation and atopic dermatitis such that the results reflect the marginal effect (association) of each of these individually. We further investigated the impact of the selection procedure by including maternal atopy and siblings as covariates in an alternate statistical model without significant changes in the results.

Performance and detection bias. The participants, the paediatrician assessing the children, and the investigators conducting the data analyses were all blinded to group allocation until publication of the 2-year follow-up results. Therefore, the risk of performance and detection bias was also minimised. Whilst information on group allocation was available before starting the laboratory analyses presented in this thesis, the analysis process was not influenced by group allocation. Importantly, when there was a need to conduct laboratory analyses in batches, group allocation did not influence the distribution of samples between batches.

Baseline imbalance. Imbalances in baseline characteristics may bias the results of an RCT if the unevenly distributed factors are also strongly related to the outcome. The probiotic and placebo groups of the original ProPACT study had comparable baseline and clinical characteristics, with the exception of a slightly higher proportion of male infants and children with older siblings in the probiotic group compared to the placebo group. In terms of estimating the effect of probiotics on specific breast milk components, these imbalances may introduce bias if they are strongly related to individual breast milk components. There is insufficient evidence to confirm associations between sex or birth order to the breastfeeding-associated microbiota (Urbaniak et al[278] and Paper I), cytokines levels[175,279], or miRNAs[280]. We do not believe these small imbalances have substantially affected the results presented in Papers I, II or III.

Non-compliance. Approximately 90% of participants in both treatment groups adhered to the study protocol. The influences of compliance on the results in Paper I and Paper III are considered to be minimal. In Paper I, all of the women who had detectable levels of the administered bacteria in breast milk samples were compliant with the study protocol, with the exception of 2 women who only drank the study milk after birth. Similarly, in Paper III, two women of the 54 women consumed the study milk only after birth, but otherwise all women in this study were compliant. In Paper II, we present a per-protocol analysis including only women compliant both before and after birth and find that the observed effect of probiotic supplementation on breast milk TSLP levels at 10 days postpartum is mildly increased.

6.2.2.2 Causation, mediation and confounding

This thesis aimed to investigate if maternal probiotic supplementation *caused* changes in selected breast milk components, and if any changes could be *mediators* of the effect of probiotics on the development of atopic dermatitis in infants.

As discussed in the section on causal mediation analysis (§3.4.2.1), the randomised controlled design of the ProPACT study is a significant strength in the assessment of the effect of probiotic supplementation on breast milk components. Randomisation distributes both known and unknown confounders and allows for an unbiased assessment of the effect of probiotics. In each paper, we have also assessed associations between each breast milk component and the development of atopic dermatitis. Since this relationship is not randomised, we have attempted to investigate the likely influence of confounding by either inclusion of potentially influential factors or assessing for associations between these factors and each breast milk component. Specifically, the presence of older siblings, mode of delivery, maternal atopy and infant sex were assessed as potential confounders of the associations between individual genera and atopic dermatitis (Paper I). Presence of older siblings, maternal atopy and maternal smoking were included as confounders in the mediation analysis presented in Paper II, and only the first two were included in Paper III due to the smaller sample size.

Modern regression based techniques were used to formally assess breast milk TSLP as a mediator of the effect of probiotics on atopic dermatitis. The strength of these analyses is that we can estimate if and to what extent TSLP contributes to the protective effect whilst accounting for potential confounders and interaction between probiotics and TSLP level. This analysis showed that the vast majority of the effect of probiotics occurs due to effects other than the influence on breast milk TLSP. As described (§3.4.2.1), the first two assumptions required for drawing causal inferences from the NIE and NDE estimates are satisfied by the RCT design. To address the third assumption, we included baseline characteristics that were potentially associated with both TSLP level and the development of atopic dermatitis (i.e. maternal atopy, older siblings and maternal smoking). It is not unlikely that other factors might affect both breast milk TSLP and atopic dermatitis, however we do not think there are any strong unmeasured confounders. The fourth assumption, that probiotic supplementation does not affect any (post baseline) confounder(s) of the relationship between breast milk TSLP and atopic dermatitis, is more difficult. Conceivably, probiotic supplementation might affect

the maternal immune system, resulting in increased levels of TSLP in breast milk and influencing the infants risk of atopic dermatitis via mechanisms other than breast milk TSLP. Indeed, prenatal supplementation appears to strengthen the preventative effect of probiotics[5,281]. This would suggest that the interaction between the immune systems of a mother and her foetus may be modified by probiotics and contribute to their preventative effect on atopic dermatitis. Cord blood samples from one of the probiotic trials showed higher levels of INF-γ after supplementation with *L. rhamnosus* HN001, but not *B. lactis* HN019[156]. Other cord blood cytokines (IL-5, IL-6, IL-10, IL-13 and TGF-β_1) were not observed to be affected by probiotic supplementation[156]. In other studies, stimulated and unstimulated cord blood mononucleocytes (CBMC) do not appear to be influenced by maternal probiotic supplementation with respect to: expression of FoxP3[282], transcription factors (GATA-3, T-bet)[283], cytokine receptors (IL-12R, IL-4) [283] or cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IFN-γ, TNF-α, TGF-β_1)[282-284]; dendritic cell phenotype or CD4+ T cell proliferation[282]. Although analysis of cord blood samples has not provided convincing evidence of a probiotic effect on foetal immune development, it seems likely that there are complex and cumulative effects of pre- and postnatal supplementation that prevent later atopic dermatitis. Returning to the fourth assumption for causal mediation analysis, the question is: "does probiotic supplementation significantly effect a strong confounder of the relationship between breast milk TLSP and atopic dermatitis, such that it masked a mediating relationship?". This possibility cannot be excluded, however we do not think that any single post-randomisation factors is a strong confounder in this situation.

6.2.2.3 *Measurement error and misclassification*

Accurate and precise measurements of all study variables are integral for the correct interpretation of results from any research. There are multiple steps in the processing of breast milk sample that could have introduced measurement errors, including aspects of sample collection, the storage of samples, samples preparation and the laboratory analyses. To limit the possibility of systematic batch-related measurement errors, samples were processed and analysed in the laboratories over a short period of time and without regarding treatment allocation or atopic dermatitis. As such, any measurement error should be non-differential[276]. That is to say, samples from the probiotic and placebo groups are presumably equally affected. Similarly, the samples from mothers of children who did and did not developed atopic

dermatitis would also have been equally affected. Non-differential measurement errors generally result in underestimation of the any associations[276]. In the coming sections, I will discuss the method of collecting, storing, processing and analysing the breast milk samples. For each of these steps, we believe that the any non-differential measurement error has not significantly altered our conclusions, although have likely reduced our ability to identify small differences between groups.

Sample collection. As described in the methods, women were not given any specific instructions regarding the timing or sterility of samples collection. The composition of breast milk has been reported to change during feeds (fore- and hindmilk) and throughout the day. The nutritional components in breast milk have been extensively studied with respect to these factors. In particular, the fat content of breast milk is greatest in the mornings and afternoons, and increases during each feeding[285]. In comparison, the lactose and protein concentration in the defatted portion remains relatively constant during feeds[286]. It is uncertain if cytokine concentrations are similarly unchanged. On the other hand, a small study found that there may be diurnal variations of the cytokines IFNγ, IL-2, IL-4, IL-5 and IL-10[287]. No studies were found that assess changes in TSLP or TGF-β isotype concentrations during feeding or during the day.

Similarly, none of the studies which used sequencing technologies to investigate the breast milk or breastfeeding-associated microbiotas have systematically assessed these sources of potential variation. Intuitively, one might expect a greater contamination from the infant oral microbiota in hindmilk samples and there may be a degree if this in our data. One culture based study assessing the recovery of *Staphylococcus aureus* in dairy cows with mastitis found a higher number of CFUs in pre-milking samples (foremilk), suggesting that there may be bacterial growth in the mammary gland between feeds[288]. The effect of non-sterile collection is a major limitation of our assessment of the breastfeeding-associated microbiota. These results cannot be interpreted as indicative of the breast milk microbiota (alone), since non-sterile collection would have resulted in a degree of contamination from the areola and breast surface microbiota and possibly the infants' oral microbiota, as described above. We therefore adopted the term "breastfeeding-associated microbiota". However, we have not specifically sampled the areola and breast surface microbiota, and we cannot know if our samples contain a representative balance between the skin and milk microbiotas that is ingested by the suckling

infant. It seems plausible that infants would consume an even more skin "contaminated" milk sample that what we have analysed. Regardless, it is unlikely that the sample collection method has masked a significant transfer of the administered bacteria.

With respect to miRNA, a recent publication shows that the fore- and hindmilk miRNA profiles is overlapping in both the lipid and defatted fractions of breast milk[289]. This is reassuring for our study where we do not have this information. Another study, which investigated selected miRNA using PCR, demonstrated that there may be a diurnal fluctuation in the level of miR-16-5p[290]. It will be interesting to see in future studies if there is a diurnal shift in the whole miRNA profile or just individual miRNAs.

Storage of samples. Another limitation is that the breast milk was stored at -80 °C for between 6 and 11 years prior to analysis. There was no difference in the average length of storage between the treatment groups, but it is unclear what effect this would have had on the bacterial DNA, cytokine levels or miRNA profile.

To my knowledge, no studies have directly assessed the effect of long term storage of breast milk on the culture-independent analyses of the microbiota. Culture based techniques have demonstrated viability of bacteria in human milk stored for up to 2 weeks at -20 °C, including for LGG and Bb-12[291], but a marked reduction in the number of viable bacteria when samples were stored at -80 °C for up to 9 months[292]. One would expect culture-independent techniques to be more stable. Nonetheless, culture-independent analyses of stool[293,294] and nasopharangeal[295] samples suggest that short-term storage (7 days to 6 months) of samples at -80 °C has a minimal to modest effect on the microbiota. Perhaps the most pertinent question here is: can the apparent absence of LGG, La-5 and Bb-12 be due to bacterial DNA degradation during storage? Analysis of stool samples suggests that bacterial DNA appears to retain sufficient quantity and integrity for PCR amplification after storage at -80 °C for up to 14 years[296]. Breast milk has comparatively low quantities of bacterial DNA such that the results may be more susceptible to small degrees of DNA degradation. Reassuringly, the majority of breast milk samples from the ProPACT study had detectable quantities of total bacterial DNA. It is possible that the administered bacteria would have been recovered in more of the samples if they had been analysed fresh, however we believe the absence of these bacteria is primarily because they are not transferred via breast milk.

The effect of temperature and duration of storage on the concentration of IgA, EGF, TGF-β_1, TGF-β_2, TNF-α, TNF-RI, IL-6 and IL-8 was investigated by Ramirez Santana et al[297]. Specifically, samples were stores for up to 48 hours at 4°C or for 6 and 12 months at -20 °C and -80°C. Cytokine concentrations were unaffected by both refrigeration and freezing up to 6 months, with the exception of IL-10 which showed a slight reduction after 48 hours of refrigeration. After 12 months of frozen storage at both -20 °C and -80 °C, IL-8 and TGF-β_1 concentrations were reduced and IL-10 was reduced after storage at -20 °C only. The other cytokines were unaffected by 12 months frozen storage. Other studies have reported that freezing, or centrifugation before freezing, had no effect on the concentration of IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-8, IFN-γ, TGF-β_1, TGF-β_2, TNF-α, eotaxin, RANTES and sCD14, however results of these experiments and the details of length of storage are not provided[199,218,223].

Based on our own analysis of fresh and frozen miRNAs, the process of freezing has probably distorted the miRNA profile. This is presumably because of cell lysis and disruption of lipid particles and subsequent "contamination" with miRNAs from the cellular and lipid fractions of breast milk. Cell lysis would lead to release of both intracellular miRNAs as well as intracellular stores of extracellular vesicles which has previously been demonstrated after freezing milk samples[298]. This would imply that our data does not represent the miRNA profile of the skimmed milk (defatted, cell and debris free) portion alone. Centrifugation prior to freezing may be a pragmatic approach when the processing of fresh samples is not feasible and the skimmed milk fraction is under particular investigation.

Sample preparation. Breast milk samples were prepared with a slightly different set of centrifugation steps for each presented paper. Bacterial DNA was isolated from the pellet and cytokines from the skimmed milk portion after a single centrifugation. The duration and speed of centrifugation varies between studies that have assessed these breast milk components, although it is unclear if these, relatively minor, variations have a significant effect on the results. We have assumed that they are not major sources of variability. On the other hand, the processing of breast milk samples for miRNA analysis may have greater implications as miRNAs are present in both the lipid, skimmed milk, and cellular fractions. Alsaweed and co-workers have thoroughly investigated these different fractions, also using different RNA isolations kits. They found the highest concentration of total RNA and miRNA in the cell and lipid fractions of breast milk, with the lipid fraction containing 8 – 10 times the concentration

of miRNA compared to the skimmed milk fraction[299]. Later, the same research group confirmed that these milk fractions appear to have different miRNA profiles with multiple differentially expressed miRNAs[300,301]. It remains uncertain if the miRNAs in one milk fraction is more biologically relevant than the other fractions. Also, even though the concentration of miRNAs in the skimmed milk may be lower, infants consume a far greater volume of this fraction than either the lipid or cellular fractions. Unfortunately, I have been unable to find a clear description of the proportions of these fractions by volume in breast milk samples. Mature milk has been reported to contain an average of ~50 g/L of fats throughout the day[302]; however, this does not reflect the volume of the lipid fraction after centrifugation. From my own processing of samples, it was rare to observe a fat layer that was more than the upper 3 – 4 mm of a 1.5 mL Eppendorf tube and the pellet was discernible, but not substantial.

Another aspect of sample preparation which may have influenced the miRNA profile is the enrichment of EVs using the precipitation solution, ExoQuick™. Our own results (§5.1) and others[303] suggest that this step has maximised the recovery of RNA and has likely resulted in precipitation of RNA associated with larger protein structures in addition to the extracellular vesicle. Other methods exist for exosome, or EV, isolation including ultracentrifugation with or without density gradient solutions, immunoaffinity isolation using antiboties to surface proteins, or size exclusion using a physical filter[304]. The recovery of EVs is generally lower using these methods, although the pool of isolated EVs are more pure and better suited to functional analysis than those treated with ExoQuick™. Since we were primarily interested in characterising the miRNA profile in breast milk, we believe that the use ExoQuick™ is pragmatic and efficient in this setting.

Microbial analysis. The analysis of the administered bacteria with qPCR has a high sensitivity and specificity for the administered bacteria. We therefore consider the risk of measurement error to be minimal. The 16S rRNA gene sequencing has the advantage of being able to provide an overview of the entire bacterial community. However, these results are limited since only 30% of samples had enough reads to be included in further analysis and only 17 individuals had adequate reads at both time points. The multitude of choices for DNA isolation, primer selection, library preparation and sequencing platform are likely to influence the final microbiota[305]. In a comprehensive comparison of various sequencing protocols, D'Amore and co-workers concluded by recommending that small pilot projects using multiple protocols

should be undertaken for each experiment, although they consider the Illumina MiSeq platform to be a pragmatic choice for most situations. To make matters more complex, the choices in data processing after sequencing also introduces a source of variation in microbiota analyses[306,307]. The QIIME pipeline used in Paper I is one of the most commonly used methods for analysing 16S rRNA gene sequencing data. Other methods appear to provide better fine-scale resolution, which may allow for a greater degree of strain-level assessment of Illumina data in future studies[307].

Cytokine analysis. Analysis of breast milk cytokines, both within the context of allergy related diseases, probiotic supplementation, or more generally, has produced widely varying concentrations. For TGF-β_1 average concentrations ranged from 18 pg/mL[199] to 1500 pg/mL[297], and from 4.3 pg/mL[199] to 5600 pg/mL[297] for TGF-β_2. Even assuming that these two studies have produce unusually extreme values, other studies still report a 10 to 20 fold difference in TGF-β concentrations[192,193,196,201,205,219,233,237]. Some of this variation may be due to mother-infant characteristics, such as diurnal and fore-/hindmilk variations, ethnicity, maternal atopy status and stage of lactation. Although, the few studies which have specifically investigated maternal atopy and ethnicity have, at most, found a 3-fold difference in the relative concentrations of a range of cytokines [175,196,205,233,308]. Differences in sample storage, preparation, analysis methods and between laboratory practices are therefore likely to play a major role in the measured concentrations. As such, it is difficult to compare results between studies beyond a rough comparison of the relative relationships between study groups.

Activation of samples prior to TGF- β analysis is inconsistently reported between studies. Manufacturers of the most commonly used ELISA and multiplex assay kits recommend activation, often stating that this is necessary. It is therefore likely that all studies have undertaken activation without necessarily reporting on this step. However, there was an intriguing pattern where the three studies reporting higher TGF-β_1 or TGF-β_2 after probiotic supplementation did not mention this activation step in their methods. One of these studies, however, used an alternate activation procedure which is thought to additionally release lipid bound TGF-β[207,309]. All other studies have removed the lipid fraction prior to cytokine analysis.

miRNA. Similar to the sequencing analysis of the breastfeeding-associated microbiota, multiple steps in the miRNA sequencing protocol can introduce variability. In addition to the previously discussed freezing, centrifugation and use of ExoQuick™ precipitation, different RNA isolations

kits, library preparation and sequencing methods affect the overall miRNA profile. When comparing the lipid, skim milk and cellular fraction of breast milk, Alsaweed and co-workers also investigated the total RNA and miRNA content recovered using 8 commercially available isolation kits. The miRNeasy mini kit used in Paper III performed comparative very well in terms of RNA purity and total miRNA quantities, and had a reasonable recovery of total RNA and miRNA to small RNA ratio[299]. As demonstrated in our pilot and validation experiments, library preparation and sequencing protocol play a major role in what types of RNA are observed and, to a lesser degree, the overall miRNA profile. The ScriptMiner™ library preparation appears to capture the entire small-RNA transcriptome [310], whilst the TruSeq protocol is more miRNA specific because only 5'-monophosphate sequences are captured. Once again, this raises the question: what is most biologically relevant? MicroRNAs have the most well defined functional role, however there is some evidence that other non-coding RNAs, such as the abundant tRNA fragments, also contribute to epigenetic regulation[311,312].

Atopic dermatitis diagnosis. The main potential measurement error lies in the analyses of the breast milk components. For completeness sake, the diagnosis of atopic dermatitis is also subject to the risk of misclassification. The UKWP diagnostic criteria used at the clinical follow-up is one of the most well studied and validated criteria for diagnosing atopic dermatitis. The participants and pediatrician were blinded to treatment allocation to ensure no detection bias, attrition bias, or reporting bias. We therefore assume that any misclassification was non-differential.

6.2.3 External validity and clinical utility

The external validity of a study refers to the extent to which the results can be generalised to other populations, and is inherently reliant on adequate internal validity. The ProPACT population was broadly similar to the participants of the larger community based intervention study (PACT study). Whilst there appears to be a degree of self-selection throughout the study period, with high risk families being more likely to attend the clinical follow-ups, there is still a significant proportion of the families with no history of allergy related disease. We therefore consider the ProPACT study to be applicable to a general population. Additionally, the analyses presented in this thesis address some of the potential biological mechanisms of probiotic supplementation acting via breast milk. It seems reasonable that these biological mechanisms

are common to other populations. However, these are all explorative analyses and currently have little clinical utility.

6.3 Appraisal of main findings

The main findings are discussed within their respective papers. I will use these last sections to discuss the results of the three papers collectively and consider publications that have arisen or been identified since their submission.

6.3.1 Breast milk, maternal probiotic supplementation and atopic dermatitis

This thesis has only just scratched the surface of the complexity of breast milk. The bacterial content is only one group of microbes found in breast milk[313], and is only partially responsible for the effect of breastfeeding on the infant intestinal microbiota. TSLP, TGF-β_1, TGF-β_2 and TGF-β_3 are only 4 of more than 250 immunologically active proteins[173] and, although abundant in mammalian milk, miRNAs represent only a fraction of the potentially active non-coding RNAs present in human milk. Not to mention the maternal cells, HMOs, antibacterial peptides and range of fats, which appear to contribute to the development of the microbiota, intestinal tract and immune system of the newborn infant. Considering this complex and evolutionary driven composition, it is perhaps unsurprising that the investigation of individual breast milk factors after maternal supplementation produce inconclusive and inconsistent results. Indeed, when an infant exclusively receives formula milk there is a total absence of most of these factors and yet there remains some uncertainty regarding the extent of the protective benefits of breastfeeding for allergy related diseases. Thus, the balance between breast milk components may be particularly relevant to understand their role in later allergy related disease. Nonetheless, the papers presented in this thesis provide some important observations within the context of maternal probiotic supplementation and the development of atopic dermatitis.

In Paper I, we found no evidence that breastfeeding is a significant source of LGG, La-5 or Bb-12 after maternal supplementation. In particular, breastfeeding does not appear to be an ongoing source of LGG, which was more often present and abundant in stool samples from infants up to 3 months of age in the probiotic arm of the trial[142]. Broadly, the increased presence of administered bacteria in a small subset of women is consistent with other studies

of probiotic supplementation[186-189,314]. The increased recovery of administered bacteria supports the idea of an entero-mammary route, although contamination from the oral mucosa of infants is a possible explanation rather than internal trafficking of microbes. Even if we assume that these results indicate a true entero-mammary route, these studies do not suggest that breastfeeding is a substantial route of transfer of most probiotics. Interestingly, *L. fermentum* and *L. salivaris,* which were originally isolated from human breast milk, are reported to be present in as many as 54% of women after supplementation with these strains [188]. This would suggest that some strains have a natural affinity for breast milk. However, it is unclear if this is because they are more readily trafficked to the mammary glands or because they are more suited to the nutritional and immunological environment of the mammary glands. In this sense, other components of human milk, such as cytokines and antibacterial peptides, may play an important role in shaping the breast milk microbiota and determine which probiotic supplementation regimes can result in transfer via breast milk.

In Paper II, we provide the first report of increased breast milk TLSP levels at 10 days postpartum after probiotic supplementation. However, there was only a marginal increase in the proportion of samples with high and above detectable levels of TSLP in the probiotic group and comparison of the treatment groups showed a borderline statistically non-significant result. These observations will need to be confirmed in further studies. Our mediation analysis suggests that increases in breast milk TSLP does not contribute to the preventative effect of probiotics on atopic dermatitis, nor does it appear to be associated with the development of atopic dermatitis. Indeed, increased TSLP following probiotic supplementation was contrary to what we initially expected, since TSLP has primarily been associated with promotion of a Th2 and allergic type inflammation, particularly in the lung and skin[101,315]. On the other hand, research into TSLP in the context of inflammatory bowel disease suggests that it may regulate intestinal inflammatory responses, particularly in response to commensal bacteria[315-318]. This is partially due to the Th2 promoting capacity of TSLP, which counteracts the Th1 inflammation typical of inflammatory bowel disease, but also through promotion of tolerogenic dendritic cells and macrophages and expansion of colonic Treg cells[316,317]. Once again, this highlights the complexity of investigating individual breast milk cytokines and a need for analysis methods that incorporate multiple components.

Few studies have attempted to investigate the combined effects of multiple breast milk cytokines. Using principle component analyses of 19 breast milk cytokines, Jepsen et al[216] found that breast milk with a combination of higher levels of IL-1β, IL-17A, and CCL17 and lower levels of CXCL1 and TSLP was protective against the development of atopic dermatitis. Multivariate analysis methods are an important tool for understanding the impact of the relative balance between breast milk components, however, I think the results presented by Jepsen et al should be interpreted cautiously for a number of reasons. The identified profile is based on the characterisation of samples along the third principle component, which described only 9 % of the total variation. There was no association between the development of atopic dermatitis and the first or second principle components, which accounted for 41 % and 10 % of the variation, respectively. Graphically, there is substantial overlap of the cytokine profile in breast milk samples from the "atopic dermatitis" and "no atopic dermatitis" groups, also along the third principle component. This overlap would suggest that the composition of breast milk in terms of the measured breast cytokines is broadly the same for mothers whose infants do or do not develop atopic dermatitis. Dr Daniel Munblit presented a similar PCA analysis with 11 cytokines in his doctoral thesis[319], also showing a substantial overlap in the cytokine profile in colostrum samples for infants with or with any immunological outcome (eczematous rash, cough or wheeze or allergic sensitisation) at 1 year of age. There was a tendency towards an increased risk of any immunological outcome when the breast milk profile had higher levels of hepatocyte growth factor (HGF) and the three TGF-β isotypes. This was at least partially consistent with individual analysis of cytokines, which suggested that TGF-β_2 was positively correlated with parental reports of eczematous rash[194,319]. Our own data in Paper II does not lend itself to informative multivariate analyses since only 4 cytokines were analysed. However, we intend to incorporate the results from breast milk analyses, together with analyses of other samples, to investigate the potential mechanistic pathways of probiotic supplementation collectively.

The role of breast milk TGF-βs in the prevention of atopic dermatitis after maternal probiotic supplementation and, more generally, in the risk of developing atopic dermatitis remains uncertain. It was with a sense of familiarity that I came across the comment that "… these data add to a confusing picture from studies of breast milk immune constituents in relation to maternal probiotic supplementation or allergy outcomes"[320]. This statement is still relevant

after more than 5 years and the addition of 3 publications investigating the effect of probiotics on breast milk TGF-βs[243,244,321] and 6 publications assessing relationships between breast milk cytokines and later allergy related disease[194,195,215-217,322]. Among the recent studies assessing the effect of probiotics on breast milk TGF- βs are Paper II and a subsequent publication by Baldassarre et al. Overall, changes in TGF-βs are more frequently reported in colostrum or transitional milk samples compared to mature milk, although not consistently in the same direction (Table 6). Perhaps unsurprisingly, none of the studies where the mother only received supplementation prenatally demonstrated changes in breast milk TGF-βs in mature milk. Thus, the apparent greater effect on colostrum or transitional milk may be due to the supplementation regime rather than the underlying biology. In the studies by Jepsen et al[216] and Munblit et al[194], mentioned in the previous paragraph, TGF-βs were among the cytokines assessed in association with allergy related disease outcomes. Considering TGF-β_1 individually, Jepsen et al found no significant association between TGF-β_1 and eczema or recurrent wheeze[216]. Munblit et al provides some support for a positive correlation between TGF-β_2 levels in mature milk and the development of an eczematous rash in the first 6 months of life. This association was not statistically significant. Crude analysis would also suggest that high TGF-β_2 in mature milk tended to be associated with later cough/wheeze, although this was apparently not noteworthy in the subsequent regression modelling[194]. Based on these recent studies, and previous studies, no certain conclusions can be made about the influence of breast milk TGF-βs on allergy related diseases.

In Paper III, a number of miRNAs were differentially expressed with respect to treatment allocation and the development of atopic dermatitis. No individual miRNA had an acceptable FDR and it was difficult to glean any functional significance of these differentially expressed miRNAs based on lists of predicted gene targets. Breast milk miRNAs were first described in 2010[126,174]. Since these first reports, most publications have focused on characterisation of the miRNA profile in human milk and their possible biological functions, as discussed below. To my knowledge, Paper III remains the only publication to investigate human milk miRNAs in conjunction with any infant health outcome, although it adds little definitive understanding of the role of miRNAs in the longer-term health of infants. A major challenge in understanding the functional consequences of miRNAs is that current methods do not estimate their collective impact on gene targets or within the context of different “target tissues”.

Together, Papers I, II and III do not provide definitive knowledge regarding breast milk composition in allergy related disease, beyond confirming that breastfeeding is not a substantial source of LGG, La-5 or Bb-12 for infants after maternal supplementation. This may partially be due the fact that the breast milk components under investigation have been assessed individually, as discussed above. However, it is worth noting that not all researchers in the field of allergy are convinced that breastfeeding or breast milk composition is protective. In 2006, Prof. Agnes Wold pointed out that the composition of breast milk evolved during a period when allergies were essentially nonexistent and passive protection from infection was a more pressing requirement[323]. The implication is that, with adequate microbial exposure in infancy, the neonatal immune system was stimulated such that allergy related inflammatory processes did not occur. The immunological capacity of breast milk would have primarily develop to protect against infection in this microbe-rich environment. The anti-microbial properties of breast milk may even be detrimental in the relatively microbe-poor urbanised, westernised societies of today. Perhaps it is unreasonable, as Wold suggests, "… to ask for protection from this modern disease by breast milk, the composition of which has evolved during millions of years."[323]. That said, there has be a co-evolution of the human microbiota, immune system and breast milk. They are likely to all contribute to human health.

Table 6: Changes in breast milk TGF-β_1 and TGF-β_2 after maternal probiotic supplementation with probiotics with or without prebiotics.

Author	Probiotic strain(s)	Administration period	Colostrum / Transitional milk	Mature milk
TGF-β1				
Rautava[221], 2002	LGG	Pre and postnatal	.	Weak trend ↓
Prescott[156], 2008	*L. rhamnosus* HN001	Pre and postnatal	↑	No change
	B. animalis subsp. *lactis* HN019		↑	No change
Böttcher[204], 2008	*L. reuteri*	Prenatal only	Weak trend ↓	No change
Boyle[78], 2011	LGG	Prenatal only	Weak trend ↓	No change
Nikniaz[244], 2013	Synbiotics and a multi-strain preparation[a]	Postnatal only	.	No change
Baldassarre[243], 2016	Multi-strain preparation[b]	Pre and postnatal	No change	↑
Simpson[c321], 2016	LGG, La-5 and Bb-12	Pre and postnatal	No change	No change
TGF-β2				
Rautava[221], 2002	LGG	Pre and postnatal	.	↑
Huurre[207], 2008	LGG and Bb12	Pre and postnatal	Trend ↑	No change
Böttcher[204], 2008	*L. reuteri*	Prenatal only	↓	No change
Kuitunen[192], 2012	LGG, *L. rhamnosus* LC70, *B. breve* Bb99, *Propionibacterium freudenreichii* ssp. *Shermanii* JS	Prenatal only	Trend ↓	No change
Nikniaz[244] , 2013	Synbiotics and a multi-strain preparation[a]	Postnatal only	.	↑
Simpson[c321], 2016	LGG, La-5 and Bb-12	Pre and postnatal	No change	No change

↑ and ↓ indicate a statistically significant increase and decrease, respectively. Trend and Weak trend indicates an observed difference without statistical significance. [a]*L. casei* PXN 37, *L. rhamnosus* PXN 54, *Streptococcus thermophilus* PXN 66, *B. breve* PXN 25, *L. acidophilus* PDN 35, *B. longum* PXN 30, *L. bulgaricus* PXN 39, and fructo-oligosaccharide; [b]*L. paracasei* DSM 24733, *L. plantarum* DSM 24730, *L. acidophilus* DMS 24735, *L. delbrueckii* subsp. *Bulgaricus* DSM 24734, *B. longum* DSM 24736, *B. infantis* DMS 24737, *Streptococcus thermophilus* DSM 24731; [c]Paper II

There certainly appears to be overlapping consequences of a "healthy" intestinal microbiota and breastfeeding, particularly with respect to intestinal barrier function. *In vitro* and *in vivo* animal studies have shown breastfeeding to be associated with reduced permeability[145,164], earlier morphological maturation[166,324] and altered gene expression[108,325] in neonates. These are considered to be some of the beneficial effects of commensal bacteria. Epidemiological evidence also provides indirect support for the idea that a combination of a microbe-rich environment and breastfeeding may be protective against allergy related diseases. The preventative effect of breastfeeding on asthma throughout childhood, and on atopic dermatitis up to 2 years of age, was stronger in low- and middle-income countries in the recent meta-analysis by Lodge et al[170]. However, income-specific factors were not found to be a statistically significant source of variation between the studies. Overall, there remains stronger evidence that a microbe-rich environment is substantially more protective against allergy related diseases than breastfeeding when they are assessed individually[323].

6.3.2 Insights into breast milk composition

In this final section of the discussion, I would like to dwell briefly on two general questions regarding the composition of human breast milk. Firstly, what components of human milk are biologically relevant for the infant? In particular, I will consider what components are biologically active in the maturation of the gastrointestinal and immune systems. There is good evidence that breast milk has short and probably long term immunological consequences for breastfed infants, however it is not given that everything in breast milk significantly contributes to these effects. Breast milk has evolved for the benefit of mammalian infants and their mothers. Some factors in breast milk may therefore be present primarily because of their involvement in regulation of mammary tissue development, or regulation and elimination of the mammary microbiota[171]. The second question is very related to the first one: how do these components support the transition from foetal to neonatal life? Papers I, II and III, cannot directly answer either of these questions. However, I consider them important questions in our general understanding of the composition of breast milk, and particularly with respect to breast milk miRNAs.

When investigating the health effects of breast milk components, there is a need to identify which ones are biological relevant for the infant and whether these can be accurately measured. Breast milk reportedly contains $10^3 – 10^5$ CFU/mL of viable bacteria[326] and, together

with bacteria found on the breast areola, these appear to be a source of some of the early colonising microbes in breastfed infants[181,182]. The breastfeeding-associated microbiota can therefore be considered to be biologically relevant. Culture-independent techniques, such as the 16S rRNA gene sequencing used in Paper I, allow us to characterise the general microbiota and include microbes which are difficult to culture. However, it is also worth remembering that culture-independent techniques will also measure non-viable bacterial, or DNA remnants, which are presumably less biologically active. There is evidence that certain heat- or protease-treated probiotic strains retain some of their immunomodulatory potential[327], but it is unclear if this could also be the case for non-viable bacteria in breast milk. Breast milk TGF-β also appears to be biologically relevant for the infants. Mice experiments and an *in vitro* study using a foetal intestinal epithelial cell line demonstrated that orally administered TGF-β survives the gastrointestinal tract, enhances oral tolerance and can limit the gastrointestinal inflammation in necrotising enterocolitis[328,329]. To my knowledge, oral administration of TSLP has not been studied. For accurate measurement of breast milk cytokines, there is a need for standardisation of sample collection, preparation and analysis.

The biological role of miRNAs in human milk is less certain. Many researchers presume that they are active after ingestion because breast milk contains a high quantity of miRNA compared to other body fluids[126], they are stable under conditions mimicking the gastrointestinal tract, and because *in vitro, ex vivo* and animal studies provide indirect evidence of their ability to be taken up and influence gene expression. On the other hand, some authors have suggested that miRNAs contribute only the nutritional content of breast milk. This hypothesis is based on results from three animal models that fail to demonstrate significant uptake of milk miRNAs in breastfed pups[330,331]. However, the physiological relevance of these animal models has been described as inherently problematic because the mutations used in developing the models may have also impaired normal extracellular vesicle processing and the inclusion of miRNAs into these vesicles[332]. This highlights another uncertainty, it is unknown if miRNAs enclosed within extracellular vesicles are more biologically active than miRNAs bound to proteins or lipoproteins. As discussed previously, the skimmed milk, lipid and cellular fractions of breast milk contains miRNAs[299]. A recent *in vitro* study demonstrated uptake of milk miRNAs from the both the skimmed milk and lipid fraction in human intestinal epithelial cell lines[333]. Extracellular vesicles are found primarily in the skimmed milk fraction, and were specifically

targeted in Paper III, although the infant consumes whole milk and miRNAs in all fractions are potentially biologically relevant. In Paper III, the use of frozen samples and ExoQuick precipitation solution resulted in a miRNA profile that may partially reflects all three milk fractions, as well as capturing protein bound miRNA. There are also substantial differences in the miRNA profile that arise from the choice of library preparation kit. The TruSeq protocol is more miRNA specific and this provides a greater number of miRNA reads in the dataset. However, protocols that use 5' end modification steps to include the entire small RNA transcriptome offer different insights into other non-coding RNA. Our own results suggest that other non-coding RNAs are also present in high quantities in fresh milk samples. Some very important unresolved questions are: Are any breast milk miRNAs functionally active after ingestion? Are miRNAs associated with maternal cells, lipids or in skimmed milk more or less biologically active? Are other non-coding small RNAs biologically active? And how should breast milk be processed and sequenced to investigate the breast milk miRNA profile that is ingested by breastfeeding infants.

In addition to the apparent short and long-term effects of breastfeeding, there are a number of other reasons to believe that breast milk supports the transition from foetal to neonatal life. Firstly, the proportion of most cytokines and growth factors appear to be higher in colostrum than mature milk[285]. MiRNA are also reported to exist in higher quantities in colostrum. Furthermore, the composition of breast milk may be affected by gestational age. At least for miRNAs, the overall composition of the miRNA profile of samples collected 3-4 weeks after preterm births appears to lie somewhere between colostrum and mature milk samples after full term births[334]. This would suggest that breast milk composition may be gestational-age appropriate. Secondly, breastfeeding supports earlier maturation of the intestinal epithelium with a more rapid decline in intestinal permeability in the neonatal period[145,164,166,324]. Thirdly, breast milk plays a pivotal role in the development of other mammals, particularly marsupials and monotremes. Mammals can be broadly divided into eutherians (often referred to as "placental"), marsupials (metatherian) and monotremes (prototherian)[335,336]. The latter two have a relatively short gestation and give birth to very underdeveloped offspring that are entirely reliant on milk directly after birth. The breast milk in these mammals undergoes a more dramatic compositional change as the offspring develops and is generally divided into three lactation-phases based on the suckling pattern and nutritional complexity of the milk[336].

Although the young of marsupials and monotremes are comparatively tiny, there is a remarkably consistent relationship between the average body mass of young at weaning compared to the adult body mass across all mammals[335]. One gets the sense that there is a continuum of maternal-infant interaction, which switches from an intrauterine to a breast milk based interaction at different gestational ages in the different mammalian groups. Understanding which components support ongoing development and which components support the transition from foetal to neonatal life may be particularly beneficial for preterm and low birth weight infants.

6.4 Conclusions

In conclusion, we find that maternal supplementation with LGG, La5 and Bb-12 does not results in substantial transfer of these bacteria via breastfeeding. Neither the transfer of LGG, La-5 or Bb-12 during breastfeeding or changes in breast milk TSLP, TGF-βs and miRNAs significantly to contribute to the preventative effect of maternal supplementation on the development of atopic dermatitis in offspring. However, probiotic supplementation may lead to a transient increase in TSLP levels in breast milk.

More generally, the results of these studies provide insights into the human breast milk components, particularly with respect to the breastfeeding-associated microbiota and human milk miRNA profile. The breastfeeding-associated microbiota is largely dominated by *Streptococcus* and *Staphylococcus* genera at both 10 days and 3 months postpartum and is relatively stable between these two time points. Breast milk also appears to contain a stable group of core breast milk miRNAs, which are at least partially conserved across a number of mammalian species. The biological functions of this, presumably evolutionarily-driven, collection of miRNAs is uncertain.

7 Future research

Throughout the past five years, I have found myself mulling over many questions and ideas for futures projects. Some of these questions have stemmed directly from the ProPACT study and the analyses of breast milk, whilst other questions are more inspired by these results and lie on the periphery of this thesis. Even focussing on the former category, I find that this research has raised more questions than it has answered.

The question remains, how is the beneficial effect of LGG, La-5 and Bb-12 supplementation transferred from mother to child? Is the transfer of LGG after birth the key to preventing atopic dermatitis? Most recently, we have found that the vaginal canal does not appear to be a source of LGG at the time of birth[337] and we are in the process of analysing oral mucosal swabs from mother and child. Alternatively, it would interesting to investigate prenatal changes occurring in the maternal immune system and the importance of the intrauterine environment and exposures and in future studies.

More generally, further clinical trials into the use of probiotics in the primary prevention of atopic dermatitis and allergy related diseases are needed. It would be beneficial to conduct more head-to-head trials of probiotic strain(s) and administration regimes, i.e. recipient (mother and or child), timing (pre- or postnatal, or both), and duration. New, non-invasive methods could also be employed to assess mRNA expression from intestinal epithelial cells in stool samples and other measures of intestinal permeability to investigate the effect of probiotics on the intestinal epithelial barrier[325,338]. These methods might provide some interesting insights into the interplay between the intestinal microbiota and the intestinal epithelium.

Finally, breast milk is a complex and intriguing fluid. A better understanding of breast milk composition, and its biological effects on the infant immune and gastrointestinal systems, will be beneficial for understanding its relationship to health and disease, and particularly for infants who cannot receive breast milk. The high quantity of short RNA in breast milk, compared to other bodily fluids, is highly suggestive that breast milk was been selectively enriched with RNA. Why, is unclear. We found a particularly high proportion of tRNA fragments, which are not previously described in breast milk and may be biologically active. These molecules will be further assess in the fresh and frozen samples.

8 Corrections

Paper II:

In Figure 1, after randomisation, the heading in the upper right box should read “Allocated to the placebo group (n=204)” not “Allocated to the probiotic group (n = 204)”. The journal has been contacted.

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10 Papers I – III and appendicies

Paper I

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Breastfeeding-associated microbiota in human milk following supplementation with *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* La-5, and *Bifidobacterium animalis* ssp. *lactis* Bb-12

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ABSTRACT

Breastfeeding is one of the major factors affecting the early development of the infant gut microbiota, and weaning is associated with a shift in the gut microbiota toward a more adult composition. Through breastfeeding, infants receive bioactive components that shape their microbiota while also being exposed to the breast milk and breast surface microbial communities. Recent studies have suggested the possibility of an entero-mammary route of microbial transfer, opening the possibility of infant gut microbiota modulation through maternal probiotic supplementation. In this study, we have analyzed breast milk samples collected at 10 d and 3 mo postpartum from women participating in the Probiotics in the Prevention of Allergy among Children in Trondheim placebo controlled trial. Women who were randomized to the probiotic arm of the Probiotics in the Prevention of Allergy among Children in Trondheim trial received a fermented milk supplemented with *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* La-5, and *Bifidobacterium animalis* ssp. *lactis* Bb-12, consuming this daily from 4 wk before their expected due date until 3 mo after birth. In total, 472 breast milk samples were assessed for the administered bacteria using quantitative real-time PCR and the microbiota transferred during breastfeeding was analyzed using 16S ribosomal RNA gene sequencing of 142 samples. We found that breastfeeding is unlikely to be a significant source of *L. rhamnosus* GG, *L. acidophilus* La-5, and *B. animalis* ssp. *lactis* Bb-12 for infants in the probiotic arm of the trial. Furthermore, maternal supplementation did not significantly affect the overall composition of the breast milk microbiota transferred during breastfeeding. We also present a descriptive analysis of this microbiota, which was largely dominated by *Streptococcus* and *Staphylococcus* genera at both 10 d and 3 mo postpartum. Samples collected at 3 mo postpartum had a statistically significant lower presence and relative abundance of the *Staphylococcus* genus. These samples also had a greater number of observed species and diversity, including more operational taxonomic units from the *Rothia*, *Veillonella*, *Granulicatella*, and *Methylbacterium* genera.

Key words: human milk, probiotics, atopic dermatitis, microbiota

INTRODUCTION

Breastfeeding is one of the major factors affecting the early development of the infant gut microbiota and weaning is associated with a shift in the gut microbiota toward a more adult-like composition (Wopereis et al., 2014; Bäckhed et al., 2015; Rodríguez et al., 2015). Multiple components of breast milk contribute to these effects, including human milk oligosaccharides, which promote the growth of some microbes, and lysozymes, lactoferrin, and antimicrobial peptides, which inhibit the growth of others (Cacho and Lawrence, 2017). Additionally, breastfeeding is a source of a diverse range of microbes that are found both on the breast surface and within the mammary glands of lactating women (Fitzstevens et al., 2017). Culture-dependent and -independent techniques have demonstrated a dominance of bacteria belonging to the *Staphylococcus*, *Streptococcus*, and *Propionibacterium* genera, as well as the presence of lactic acid bacteria and bifidobacteria in breast milk (Fitzstevens et al., 2017). The origin of these bacteria is thought to be a combination of the microbiotas associated with the mother's skin flora, the infant's oral mucosa, and the maternal gut. Recent studies have suggested the possibility of an entero-mammary route with selective trafficking of commensal bacteria from the maternal gut to the mammary glands via dendritic cells and macrophages (Rodríguez, 2014; Treven et al., 2015).

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In the Probiotics in the Prevention of Allergy among Children in Trondheim (**ProPACT**) placebo controlled trial, we found that maternal ingestion of 3 probiotic strains, while pregnant and breastfeeding, resulted in an almost 40% reduction in the cumulative incidence of atopic dermatitis among offspring at 2 yr of age (Dotterud et al., 2010). Women in the probiotic arm of the trial consumed fermented milk supplemented with *Lactobacillus rhamnosus* GG (**LGG**), *Lactobacillus acidophilus* La-5 (**La-5**), and *Bifidobacterium animalis* ssp. *lactis* Bb-12 (**Bb-12**), whereas women in the placebo group consumed heat-treated fermented milk without supplemented bacteria. Analysis of stool samples from the ProPACT study revealed that women in the probiotic arm had a higher prevalence and relative abundance of all 3 probiotic bacteria strains at 3 mo postpartum in their stool samples. A similar result was observed for the children of these women at 10 d and 3 mo of age, although only for the LGG (Dotterud et al., 2015). Breastfeeding may therefore be an ongoing source of LGG for these infants. Previous studies suggest that maternal supplementation with *L. rhamnosus* LC705 (Nasiraii et al., 2011), *Lactobacillus reuteri* (Abrahamsson et al., 2009), and *Lactobacillus fermentum* CECT5716 and *Lactobacillus salivarius* CECT5713 (Arroyo et al., 2010) may result in an increased presence of the administered bacteria in the breast milk of some, but not all, women. To our knowledge, no studies have investigated the transfer of LGG, *L. acidophilus,* or *Bifidobacterium* species through breastfeeding after maternal supplementation. *Lactobacillus rhamnosus* GG is of particular interest because it has been the most commonly administered bacteria in atopic dermatitis prevention studies and was observed to be transferred to infants in the ProPACT study.

In the present study, we investigated the bacteria transferred through breastfeeding using breast milk samples taken at 10 d and 3 mo postpartum from women participating in the ProPACT trial. The samples were collected without sterilization of the breast areola and are considered to give a more representative analysis of the bacteria ingested by suckling infants. We have therefore adopted the term "breastfeeding-associated microbiota" suggested by Sakwinska et al. (2016) to describe this bacterial community, which involves the breast milk and breast surface microbiotas in human milk ingested by the suckling infant. Our aim was to investigate whether maternal probiotic supplementation with LGG, La-5, and Bb-12 affected the presence of these strains among the bacteria transferred during breastfeeding at 10 d and 3 mo postpartum, and their association with the later development of atopic dermatitis. We also assessed the general microbiota associated with breastfeeding, considered temporal trends, and the relationships between the composition of the microbiota transferred during breastfeeding, probiotic supplementation, and atopic dermatitis.

MATERIALS AND METHODS

Participant Recruitment and Sample Collection

This study analyzed 472 breast milk samples collected from 252 women participating in the ProPACT trial. The design and clinical results from this randomized, placebo controlled trial have been described in detail elsewhere (Dotterud et al., 2010; Simpson et al., 2015). Briefly, 415 women, who intended to breastfeed, were randomized to receive a commercially available fermented milk (Biola, Tine AS, Oslo, Norway) containing 5×10^{10} cfu of LGG and Bb-12 and 5×10^{9} cfu of La-5 per 250 mL or a placebo fermented milk that contained no probiotic bacteria and was heat treated after fermentation. Participating women were to consume 250 mL per day of their allocated study milk from 36 wk gestation until 3 mo postpartum. Their infants did not receive any probiotic supplementation. The children were assessed for signs and symptoms of allergy-related diseases through questionnaires and clinical examination at 2 and 6 yr of age. Atopic dermatitis was diagnosed according to the UK Working Party diagnostic criteria (Williams et al., 1994) at the clinical examinations.

Participating women were provided with sterile sample tubes and were requested to collect breast milk at 10 d and 3 mo postpartum. The timing, with respect to time of day or whether fore- or hindmilk was collected, was not standardized. The women did not receive explicit instructions regarding washing or sterilization of the breast surface before sample collection. Samples were frozen in their home freezer until transportation to the laboratory where they were subsequently stored at −80°C. All available breast milk samples were included in the current study, provided that the child attended the 2-yr clinical follow-up (Figure 1).

Analysis of Microbiota

Breast milk samples (2 mL) were centrifuged at 21,500 × *g* for 30 min. The resulting pellet was resuspended in 100 μL of stool transport and recovery buffer and DNA was isolated using LGC Mag DNA extraction kit (LGC Genomics, Middlesex, UK) on a KingFisher FLEX magnetic particle processor (Thermo Fisher Scientific, Waltham, MA) according to the manufacturers' instructions. Samples were analyzed for total bacteria

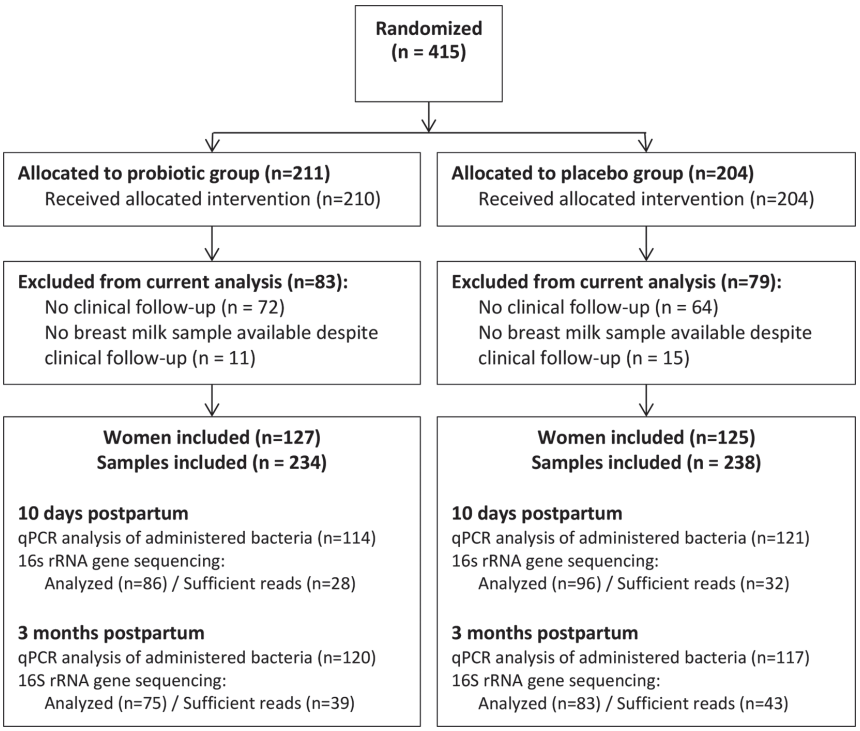

Figure 1. Flow diagram of participants in the Probiotics in the Prevention of Allergy among Children in Trondheim study and analysis of the bacterial content of breast milk samples is given. The number of eligible women who had provided at least one breast milk sample. The precise number of samples analyzed at each time point with each method varies as indicated. Samples were considered to have a sufficient number of reads if the 16S rRNA gene sequencing analysis returned ≥1,000 reads. qPCR = quantitative PCR.

and the 3 administered bacteria using quantitative real-time PCR (**qPCR**) and using 16S rRNA gene sequencing for the general microbial composition.

The qPCR was performed using a double-stranded DNA binding dye-based EvaGreen assay (Biotium Inc., Fremont, CA) for assessing LGG and La5, and a probe-based TaqMan assay (Thermo Fisher Scientific) for Bb12 and total bacteria. The primers and probes for these assays are detailed in Table 1. For the EvaGreen assays, the reaction volumes of 20 μL contained 1 × HOT FIREpol EvaGreen (Solis BioDyne, Tartu, Estonia), forward and reverse primers with a final concentration of 0.2 μ*M* and 1 μL of template DNA. A LightCycler-480 machine (Roche, Basel, Switzerland) was used for the qPCR reaction using the following settings: heating to 95°C for 15 min, followed by 40 cycles of denaturing at 95°C for 15 s, annealing and extension at 60°C for 1 min. Melting point curves were obtained by heating the samples from 60 to 95°C at 0.1°C per second and measuring fluorescence at each 0.2°C temperature increment. For the TaqMan assay, the reaction volume of 20 μL contained 4 μL of 1 × HOT FIREpol, 0.4 μL of forward and reverse primers with a final concentration of 0.2 μ*M*, the probe with a final concentration of 250 n*M* and 1 μL of template DNA. The LightCycler-480 settings for this assay were heating to 95°C for 12 min, followed by 50 cycles of denaturing at 95°C for 30 s, annealing and extension at 60°C for 1 min. For both the EvaGreen and TaqMan assays, the negative control was 1 μL of nuclease-free water and the positive control was DNA isolated from Biola or Bb12 cultures supplied by Tine AS (the producer of Biola) for the measurement of the administered bacteria and 1 μL of *Escherichia coli* DNA for the analysis of total bacteria.

The general microbial composition of the samples was assessed using 16S rRNA gene sequencing of 300-bp paired end reads on the Illumina MiSeq platform (Illumina Inc., San Diego, CA) with v3 sequencing chemistry at The Centre for Integrative Genetics (CIGENE, Ås, Norway). Following DNA extraction, the

Table 1. Primers and probes

Bacteria[1]	Assay[2]	Forward/reverse primer and probe name	DNA sequence (5′–3′)
Total	TaqMan	Forward primer: Univ_F	TCCTACGGGAGGCAGCAGT
		Reverse primer: Univ_R	GGACTACCAGGGTATCTAATCCTGTT
		Probe: universal probe	FAM-GTATTACCGCGGCTGCTGGCAC-TAMRA
LGG	EvaGreen	Forward: L.rhamn1	CAATCTGAATGAACAGTTGTC
		Reverse: L.rhamn2	TATCTTGACCAAACTTGACG
La-5	EvaGreen	Forward: La-5–5L	TTACGCCAGTCCAAGGGTAG
		Reverse: La-5–5R	CAGAATGCCCGCAAGTTATC
Bb-12	TaqMan	Forward primer	AGA ACC ACG GCG GCG TC
		Reverse primer	CGC GGT CTT CTC GAG CAC T
		Probe	FAM-TGC GCT CGC CGA CG-MGB

[1]LGG = *Lactobacillus rhamnosus* GG; La-5 = *Lactobacillus acidophilus* La-5; Bb-12 = *Bifidobacterium animalis* ssp. *lactis* Bb-12.
[2]TaqMan (Thermo Fisher Scientific, Waltham, MA); EvaGreen (Biotium Inc., Fremont, CA).

V3 to V4 region of the 16S rRNA gene was amplified using PRK341F/PRK806R primers adapted for Illumina sequencing. The results were analyzed with the Quantitative Insights Into Microbial Ecology (QIIME) pipeline, utilizing AmpliconNoise to denoise the data and remove chimeras, uclust for operational taxonomic unit (**OTU**) clustering at 97% similarity and the Ribosomal Database Project classifier for assigning taxonomic identity to the identified OTU. A total of 1,712,182 sequences were generated, with a median of 4,517.6 reads per sample.

Statistical Analysis

Statistical analyses and graphics were conducted using Stata IC 13.1 (StataCorp, College Station, TX) and MATLAB 2016b (The MathWorks Inc., Natick, MA). The effect of probiotic supplementation on the presence of the administered bacteria in breast milk at each time point was estimated using the qPCR data and is presented as a risk ratio (**RR**) and 95% confidence interval, accompanied with a Fisher exact test. Samples were considered positive if the EvaGreen assays had a threshold cycle (**Ct**) value below 35 and a melting point of 86°C for LGG or 80°C for La-5. For Bb-12 and total bacteria, samples were considered positive if they had a Ct value below 35 on the TaqMan assay.

The 16S rRNA gene sequencing data was rarefied to 1,000 reads before descriptive and comparative analysis of the breastfeeding-associated microbiota. Samples with less than 1,000 reads were not included in these analyses. The effect of probiotic supplementation on the presence and relative abundance of individual genera at each time point was assessed using a χ^2 test (or a Fisher exact test as indicated) and a Wilcoxon rank-sum test, respectively. An OTU was considered "present" if it accounted for at least 1% of reads (10 reads) in a given sample in the rarefied data set. To avoid excessive multiple comparisons of uncommon or very low abundance OTU, comparisons were only estimated for OTU and genera that were present in at least 10% of the samples (i.e., $\geq$ 10 reads in $\geq$6 samples from 10 d or $\geq$9 samples from 3 mo). For each set of comparisons, a raw *P*-value of $\leq$0.05 was considered of potential interest. False discovery rates (**FDR**) were obtained using the Benjamini-Hochberg method in the multiproc Stata command and an FDR <0.1 was considered acceptable. Associations between the presence or relative abundance of the common genera and the following factors were also considered: stage of lactation, the development of atopic dermatitis, presence of older siblings, mode of delivery, maternal atopy, and sex of the child. The association between the presence of common genera and the development of atopic dermatitis was also assessed using multivariable logistic regression models including treatment allocation, presence of older siblings, maternal atopy, and sex of the child as additional covariates.

The effect of probiotic supplementation and stage of lactation on the α diversity was assessed using the Shannon and Simpson diversity indices, phylogenetic diversity, and species richness (observed species). Beta diversity was investigated using principal coordinates analysis (**PCoA**) derived from unweighted and weighted UniFrac, distance matrices, and Bray Curtis dissimilarity matrix. Each PCoA plot was reviewed with respect to stage of lactation, treatment allocation, and the previously mentioned mother-infant characteristics.

RESULTS

Participants

A total of 472 breast milk samples from 252 women were available for analysis (Figure 1). Due to low quantities of bacterial DNA, fewer samples could be assessed using 16S rRNA gene sequencing (Figure 1). The baseline family characteristics and allergy-related disease outcomes among children in the current study are representative of the original ProPACT study population

Table 2. Baseline characteristics of families and allergy-related disease in the children at 2 yr

	Treatment allocation				
	Probiotic		Placebo		Risk ratio (95% CI)
Item	n	Value	n	Value	
Characteristics					
Age, mother, yr mean (SD)	127	30.5 (3.9)	125	30.4 (4.1)	
Sex (male), child, no. (%)	127	66 (52.0)	125	51 (40.8)	
Siblings, no. (%)	127	60 (47.2)	125	53 (42.4)	
Atopy in family, no. (%)	127	90 (70.9)	125	92 (73.6)	
Maternal atopy, no. (%)	127	58 (45.7)	124	65 (52.4)	
Maternal smoking,[1] no. (%)	127	8 (6.3)	125	11 (8.8)	
Pet,[2] no. (%)	127	33 (26.0)	125	35 (28.0)	
Compliant,[3] no. (%)	123	111 (90.2)	123	110 (89.4)	
Mode of delivery, vaginal, no. (%)	123	108 (87.8)	124	108 (87.1)	
Allergy-related disease at 2 yr					
Atopic dermatitis,[4] no. (%)	127	29 (22.8)	125	43 (34.4)	0.66 (0.44–0.99)
Current asthma, no. (%)	127	7 (5.5)	125	12 (9.6)	0.57 (0.23–1.41)
ARC,[5] no. (%)	123	1 (0.8)	124	0 (0.0)	NA
Current sensitization,[6] no. (%)	120	18 (15.0)	119	14 (11.7)	1.28 (0.67–2.44)
Skin prick test positive, no. (%)	109	6 (5.5)	106	6 (5.7)	0.97 (0.32–2.92)
sIgE positive, no. (%)	80	17 (21.3)	89	12 (13.5)	1.58 (0.80–3.09)

[1]Maternal smoking reported during pregnancy, or 6 wk or 12 mo postpartum.

[2]Reported a household pet during pregnancy or the child's first year of life.

[3]Compliance with the study protocol was defined as consumption of the study milk on at least 50% of days from 36 wk gestation to 12 postpartum, no consumption of other products with probiotics and at least partial breastfeeding until 3 mo postpartum.

[4]Cumulative incidence of atopic dermatitis.

[5]ARC = allergic rhinoconjunctivitis (cumulative incidence).

[6]Allergic sensitization defined as positive skin prick test (wheal ≥3 mm) and or positive sIgE (≥0.35 kUL^{-1}). Not all children had both measured.

(Table 2 and Supplemental Table S1; https://doi.org/10.3168/jds.2017-13411). The baseline characteristics of the families with adequate reads on 16S rRNA gene sequencing analysis were also comparable; however, the preventative effect of probiotic supplementation on atopic dermatitis was not observed in this subgroup (Supplemental Table S1; https://doi.org/10.3168/jds.2017-13411).

Transfer of Administered Probiotic Bacteria Strains Via Breast Milk

Based on results from the qPCR assays, the majority of the breast milk samples had measurable quantities of bacterial DNA with 392 of 472 samples positive for total bacteria. Samples without measurable bacteria included 39 samples collected at 10 d postpartum (20 and 19 from the probiotic and placebo groups, respectively) and 41 samples from 3 mo postpartum (26 and 15 from the probiotic and placebo group, respectively). However, only 8 women from the probiotic group and 1 from the placebo group had detectable levels of the any of the administered bacteria. At 10 d postpartum, LGG was present in 2 of 114 samples collected from women in the probiotic group and 1 of 121 samples from the placebo group (RR 4.2, 95% CI 0.5–36.8, Fisher's exact $P = 0.21$). The La-5 was detected in 2 samples from the probiotic group only at 10 d postpartum (RR not applicable, $P = 0.49$). Neither LGG nor La-5 were found in breast milk samples collected 3 mo postpartum (n = 120 and 117 for the probiotic and placebo groups, respectively). The Bb-12 was present in breast milk samples from women in the probiotic group only, including 5 samples at 10 d postpartum (RR not applicable, $P = 0.03$) and 2 samples at 3 mo postpartum (RR not applicable, $P = 0.50$). Two women with Bb-12 in breast milk at 10 d also had one of the other administered bacteria in their milk sample, one with LGG and one with La-5. Additionally, the woman with both LGG and Bb12 at 10 d was also found to have Bb12 in her breast milk at 3 mo. Given that so few samples were positive for the administered probiotic bacteria, comparison of the relative abundance of these bacteria between the probiotic and placebo groups was not feasible. Similarly, it was not feasible to conduct any formal statistical assessment of the association between the presence of the administered bacteria in breast milk samples and previously analyzed mother and infant stool samples (Dotterud et al., 2015) or the development of atopic dermatitis in infants. A summary of these results for

mother-infant pairs with positive breast milk samples are presented in Supplemental Table S2 (https://doi.org/10.3168/jds.2017-13411).

Breastfeeding-Associated Microbiota at Different Stages of Lactation

Due to low quantity and quality of bacterial DNA in the breast milk samples, not all samples were sequenced and fewer samples had sufficient reads to be included in further analysis. Samples that were removed before sequencing had a median Ct value for total bacteria of 33.4 [interquartile range (**IQR**) 31.9–36.1, n = 128] and samples that were sequenced, but had insufficient reads, had a median Ct value of 31.7 (IQR 29.7–33.8, n = 199). Ultimately, sufficient reads were obtained for 142 samples from 125 women (median Ct value 30.2, IQR 27.9–32.0). These reads were attributed to a total of 307 OTU belonging to 147 genera. Considering the composition of the milk microbiota at the genus level, 45 and 69 genera were present (≥10 reads) in samples collected at 10 d and 3 mo, respectively. However, only 11 genera were present in at least 10% of samples at either time point and no genus was present in all samples (Table 3 and Supplemental Table S3 for OTU level summary; https://doi.org/10.3168/jds.2017-13411). Even at the phyla level, no phylum was present in all breast milk samples, although *Firmicutes* bacteria were present in all but one 3-mo sample (data not shown). The general breastfeeding-associated microbiota at 10 d and 3 mo postpartum was dominated by *Streptococcus* and *Staphylococcus* genera in most women (Supplemental Figure S1; https://doi.org/10.3168/jds.2017-13411). At both time points, a subgroup of women had a milk microbiota dominated by other genera, such as *Acinetobacter* and genera from the *Bacillaceae* family (Supplemental Figure S1). Additionally, some individual women had moderate to high relative abundances of uncommon genera, such as *Klebsiella*, *Lactobacillus zeae*, *Alkanindeges*, *Stenotrophononas*, and genera from the *Caulobacteraceae* family (data not shown, these genera fall into the category of "other" in Supplemental Figure S1).

When assessing the influence of the stage of lactation on specific genera, we found a higher presence and relative abundance of *Staphylococcus* in 10-d compared with 3-mo samples (Table 3 and Figure 2a). The samples from 10 d postpartum also had a lower prevalence and relative abundance of *Rothia* and *Veillonella* genera and a higher relative abundance of unassigned bacteria in 10-d samples. The *Granulicatella* and *Methylbacterium* had higher prevalence in 3-mo samples and *Granulicatella* had a higher relative abundance. The number of observed species, phylogenetic diversity, and Shannon's

Table 3. Presence and relative abundance of the 11 genera present in at least 10% of samples at either 10 d or 3 mo postpartum[1]

	10 d (n = 60)				3 mo (n = 82)				Prevalence: 10 d vs. 3 mo		RA: 10 d vs. 3 mo	
Taxonomic classification	Pos.	Median	IQR	Range	Pos.	Median	IQR	Range	*P*-value[2]	FDR[2]	*P*-value[3]	FDR[3]
Streptococcus	60	547	287–779	11–964	79	726	406–843	2–936	0.134	0.229	0.061	0.112
Staphylococcus	52	151	45–320	0–908	58	30	8–102	0–817	0.012	0.027	<0.001	<0.001
Gemellaceae family	36	18	3–48	0–555	52	15	2–44	0–179	0.679	0.679	0.598	0.598
Rothia	10	0	0–6	0–327	41	10	2–22	0–97	<0.001	<0.001	<0.001	<0.001
Veillonella	10	1	0–6	0–58	33	6	1–19	0–73	0.003	0.009	<0.001	<0.001
Unassigned	23	6	2–23	0–188	22	3	0–10	0–75	0.146	0.229	0.006	0.015
Acinetobacter	11	0	0–5	0–898	21	1	0–11	0–966	0.305	0.343	0.074	0.116
Hemophilus	6	0	0–1	0–211	13	1	0–5	0–244	0.311	0.343	0.112	0.137
Bacillaceae family	6	0	0–2	0–414	14	1	0–5	0–651	0.231	0.318	0.102	0.137
Granulicatella	1	0	0–0	0–82	21	1	0–10	0–75	<0.001	<0.001	<0.001	<0.001
Methylbacterium	1	0	0–2	0–13	12	1	0–4	0–195	0.008	0.022	0.162	0.178

[1]Pos. = number of samples with at least 10 reads for given genera; IQR = interquartile range; RA = relative abundance; FDR = false discovery rate.
[2]*P*-value and FDR for χ^2 test.
[3]*P*-value and FDR for Wilcoxon rank sum test.

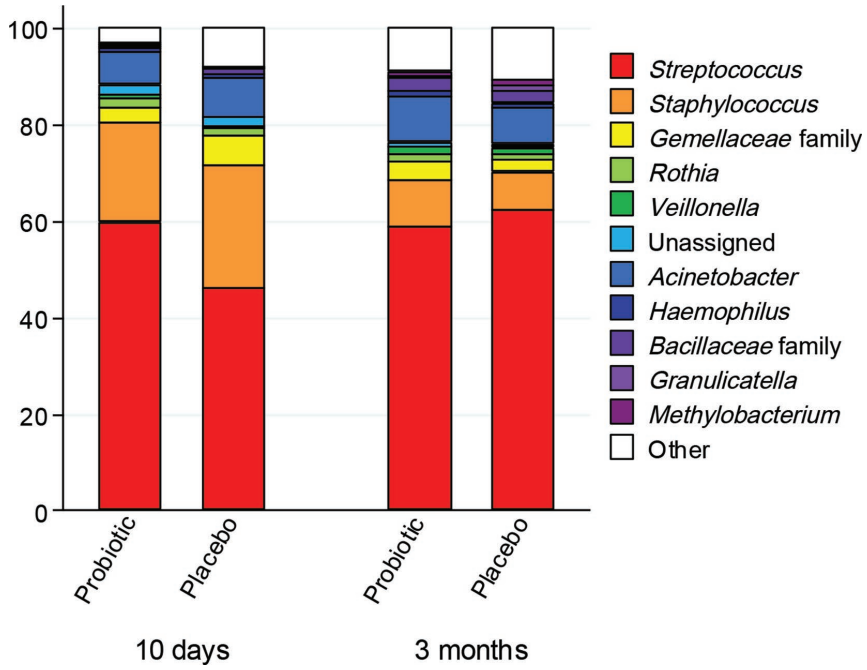

Figure 2. Overview of proportion of reads attributable to the 11 genera present in at least 10% of samples at 10 d or 3 mo in the probiotic and placebo groups.

diversity index of the breastfeeding-associated microbiota increased from 10 d to 3 mo postpartum (Shannon's index median (IQR): 1.70 (1.08–2.42) vs 2.15 (1.63–2.47), $P = 0.016$, Supplemental Figure S2; https://doi.org/10.3168/jds.2017-13411). The PCoA plots based on weighted and unweighted UniFrac distance matrices and Bray Curtis dissimilarity matrix did not indicate a clear separation in the breastfeeding-associated microbiota by stage of lactation (Supplemental Figure S3; https://doi.org/10.3168/jds.2017-13411), although 10-d samples were statistically more likely to be associated divergent microbiotas in the unweighted UniFrac and Bray Curtis analyses (data not shown).

An adequate number of reads was achieved in both 10-d and 3-mo samples from 17 women (Supplemental Figure S4; https://doi.org/10.3168/jds.2017-13411). Individually, these women had between 5 and 17 OTU (median 10, IQR 7–11) represented at either time point, with between 0 and 5 OTU present in both samples (median 3, IQR 2–4). These persistent OTU accounted for a large proportion of total reads in most women: median of 80.0% (IQR 36.7–93.2%, range 0.0–99.4%) of the total reads at 10 d and 72.5% (31.8–85.0, range 0.0–92.8) of the reads at 3 mo. However, 6 of 17 women had substantial changes in the OTU-level composition of their breast milk microbiota, with the persistent OTU accounting for less than 40% of reads at either or both time points. One of these women had no persistent OTU, suggesting a total replacement of the breastfeeding-associated microbial community between 10 d and 3 mo (M10 in Figure S4; https://doi.org/10.3168/jds.2017-13411).

At each time point, probiotics had no statistically significant effect on the presence or relative abundance of any of the individual genera that were present in at least 10% of the samples (Supplemental Table S4, https://doi.org/10.3168/jds.2017-13411, and Figure 2). Furthermore, probiotic supplementation was not found to have a statistically significant effect on the α or β diversity of the breast milk microbiota at either time point (Supplemental Figures S2 and S3; https://doi.org/10.3168/jds.2017-13411). In the subgroup of 17 women with 16S rRNA gene sequencing data, probiotic supplementation may have a positive influence on the stability of the breastfeeding-associated microbiota (RR of stable breastfeeding-associated microbiota after probiotic supplementation: 2.37, 95% CI 0.94–5.97, Fisher's exact $P = 0.050$).

Breastfeeding-Associated Microbiota and Atopic Dermatitis in Offspring

Finally, we considered the association between the general breastfeeding-associated microbiota and the development of atopic dermatitis. The presence of the *Staphylococcus* and *Veillonella* genera at 3 mo postpartum tended to be associated with a borderline greater risk of developing atopic dermatitis, although the FDR for these comparisons was unacceptably high (RR 1.93, 95% CI 0.92–4.06, $P = 0.052$ for *Staphylococcus*; RR 1.67, 95% CI 1.00–2.78, $P = 0.048$ for *Veillonella*; and FDR = 0.285 for both). Similarly, a higher relative abundance of *Staphylococcus* at 3 mo appeared to increase the risk of atopic dermatitis before considering the FDR [median number of reads per 1,000 (IQR): 40 (18–128) vs. 14 (5–63) for children with and without atopic dermatitis, respectively, $P = 0.045$, FDR: 0.50]. No other associations were found between the development of atopic dermatitis and the presence or relative abundance of the common genera in 10-d or 3-mo breast milk samples (data not shown). The relationship between the breastfeeding-associated microbiota and the development of atopic dermatitis is potentially confounded by other maternal-infant characteristics, such as the presence of older siblings, mode of delivery, maternal atopy, and the sex of the child. The *Gemellaceae* family was more common in 10-d samples from mothers with a personal history of allergy (23/31 vs. 13/29, $P = 0.020$, FDR = 0.081), whereas the *Rothia* genera was more commonly observed in 10-d samples from mothers with no allergic history (9/29 vs. 1/31, $P = 0.004$, FDR = 0.031). Samples collected at 3 mo from mothers of female infants also had a higher presence and relative abundance of the *Rothia* genera compared with mothers of male infants (present in 30/48 vs. 11/34 for female and male infants, respectively, $P = 0.007$, FDR = 0.079, and median (IQR): 14 (5–27) vs. 4 (0–12), respectively, $P = 0.005$, FDR = 0.055). Also at 3 mo postpartum, *Streptococcus* was more commonly present in breast milk samples after vaginal delivery, although the FDR was unacceptably high (65/66 vs. 23/14 in vaginal vs. cesarean delivery, $P = 0.022$, FDR = 0.246). No other statistically significant associations between mother-infant characteristics and the presence or relative abundance of individual bacterial genera were observed at either time point (data not shown). The presence and relative abundance of the *Staphylococcus* and *Veillonella* genera, which were highlighted for their potential association with the development of atopic dermatitis, do not appear to be related to any of these maternal-infant characteristics. Further, the strength of association between the presence of individual bacterial genera and the development of atopic dermatitis was not significantly altered by the addition of these maternal-infant characteristics as covariates in a multivariable logistic regression models (data not shown). Neither the development of atopic dermatitis nor these mother-infant characteristics were associated with a clear separation of samples when reviewing the PCoA plots, suggesting that the β-diversity of these samples was not defined by these characteristics (Supplemental Figure S3; https://doi.org/10.3168/jds.2017-13411).

DISCUSSION

Our findings indicate that pre- and postnatal maternal probiotic supplementation with LGG, La-5, and Bb-12 leads to the presence of these bacteria in breast milk samples for only a small subgroup of women. Thus, breast milk was not a meaningful source of LGG, La-5, or Bb-12 for the infants in the ProPACT trial, and the observed preventative effect of the ProPACT regimen on atopic dermatitis cannot be attributed to the transfer of these bacteria through breastfeeding. Using 16S rRNA gene sequencing, we also demonstrate that the general breastfeeding-associated microbiota of human milk is largely dominated by *Streptococcus* and *Staphylococcus* genera at both 10 d and 3 mo postpartum and that maternal probiotic supplementation does not have a substantial effect on this microbiota or its diversity.

One of the major strengths of this study is the use of samples collected in a randomized, placebo controlled trial with clinical follow-up. This allows us to conduct an unbiased assessment of the effect of probiotic supplementation on the presence of administered bacteria and the breastfeeding-associated microbiota. Furthermore, the qPCR analysis has a high sensitivity and specificity for the administered bacteria. Therefore, with only 9 of 252 women found to have any administered bacteria in their breast milk at either time point, breast milk is unlikely to be a substantial source of LGG, La-5, or Bb-12 for infants after maternal supplementation. Although we found a statistically significant increase in the presence of Bb-12 at 10 d postpartum in the probiotic group, over 95% of the samples had no detectable Bb-12 and we do not consider this statistical significance to imply a clinically meaningful transfer of Bb-12 during breastfeeding. In a previous study, we found that as many as 50 out of 129 (39%) infants in the probiotic group had LGG present in their stool samples at 10 d of age and 56 of 122 (46%) at 3 mo of age. This represented a statistically significant increase in the presence of LGG, which was found in only 7 of 133 (5%) and 23 of 121 (19%) stool samples from infants in the placebo group at 10 d and 3 mo, respectively. In contrast, the current study identified LGG in only 3 breast milk samples collected at 10 d postpartum

and none at 3 mo, suggesting that breastfeeding cannot have been the source of LGG for the majority of infants who had this bacteria present in their stool samples.

In previous randomized studies of maternal supplementation, *Lactobacillus* species have been recovered from breast milk in 12 to 53% of women after supplementation (Abrahamsson et al., 2009; Arroyo et al., 2010; Nasiraii et al., 2011; Hurtado et al., 2017). In a small trial, 4 of 10 women had viable cultures of *L. rhamnosus* LC705 isolated in breast milk samples after supplementation with that probiotic (Nasiraii et al., 2011). Also using culture-based techniques, Abrahamsson et al. (2009) demonstrated that 12% of women in their probiotic group had viable *L. reuteri* in colostrum samples, compared with 2% in the placebo group. Similarly, in a 3-arm trial, Arroyo et al. (2010) investigated *L. fermentum* CECT5716, *L. salivarius* CECT5713 or antibiotics in the treatment of mastitis and found that just over 50% of women in both probiotic arms, and none in the antibiotic arm, had lactobacilli cultured in breast milk samples after 21 d of supplementation. Results from subsequent trials suggest that maternal supplementation with *L. fermentum* CECT5716 may increase levels of that bacteria (Hurtado et al., 2017), but not the overall abundance of lactobacilli (Maldonado-Lobon et al., 2015; Hurtado et al., 2017). It is worth noting that both *L. fermentum* CECT5716, and *L. salivarius* CECT5713 were initially isolated from human milk samples, suggesting that these bacteria have a natural affinity for the breast milk microbiota. The low proportion of milk samples with either LGG, La-5, or Bb-12 in the current study may reflect strain-specific differences in their ability to be transferred to the milk microbiota. We also found that maternal probiotic supplementation did not significantly affect the general breastfeeding-associated microbiota, which was investigated using 16S rRNA gene sequencing. Furthermore, the development of atopic dermatitis was not convincingly associated with individual genera or the overall composition of this microbiota.

Over the past 5 yr, several studies have employed sequencing methods to characterize the breast milk or breastfeeding-associated microbiota and its association with mother-infant characteristics such as stage of lactation (Hunt et al., 2011; Cabrera-Rubio et al., 2012; Jost et al., 2013; Sakwinska et al., 2016; Urbaniak et al., 2016; Murphy et al., 2017), prematurity (Urbaniak et al., 2016), mode of delivery and elective versus nonelective caesarean section deliveries (Cabrera-Rubio et al., 2012, 2016; Kumar et al., 2016; Sakwinska et al., 2016; Urbaniak et al., 2016), infant sex (Urbaniak et al., 2016), maternal weight (Cabrera-Rubio et al., 2012; Collado et al., 2012), geography (Kumar et al., 2016; Li et al., 2017), and mastitis (Jiménez et al., 2015). Each of these mother-infant characteristics has been described to influence the microbiota composition, particularly in the smaller studies (n = 7–20). Three of the larger studies (n = 133, n = 90, and n = 39) report no clear distinction in the breast milk microbiota based on stage of lactation, prematurity, or mode of delivery (Sakwinska et al., 2016; Urbaniak et al., 2016; Li et al., 2017). Our results suggest that stage of lactation, sex, maternal atopy, and mode of delivery may influence the presence or relative abundance of specific genera. However, neither these characteristics, nor the presence of siblings, defined the overall composition of the breastfeeding-associated microbiota. Due to lack of information, we were unable to assess the effect of maternal antibiotic use and signs or symptoms of mastitis. Information regarding antibiotic use and the presence of mastitis would have been particularly useful with respect to the observation that probiotic supplementation may promote stability of the breastfeeding-associated microbiota.

Another recurrent theme among some of the previous smaller studies is the presentation of a "core" breast milk microbiota of between 6 and 12 OTU or genera, which are found in 90 to 100% of samples (Hunt et al., 2011; Jiménez et al., 2015; Murphy et al., 2017). In the present study we did not observe a "core" breastfeeding-associated microbiota, despite participants coming from a small geographic area. This is likely due to the high degree of individual variability and relatively large number of women included in our study. Indeed, the lack of a "core" microbiota is consistent with the conclusion of a recent systematic review, which identified *Staphylococcus* and *Streptococcus* as the only genera to be repeatedly reported as dominant breast milk-related genera (Fitzstevens et al., 2017). Methodological differences in sample collection and laboratory analysis are likely to influence the observed microbiota in our study and other published studies.

Our 16S rRNA gene sequencing data probably represent a combination of the breast milk and breast surface microbiotas because women were not provided with instructions for sterile collection. This has presumably resulted in contamination from the skin microbiota and possibly the infant oral microbiota, and may account for the higher relative abundance of *Staphylococcus* and *Streptococcus* genera in our results compared with previous sequencing studies that have used sterile collection protocols. Indeed, Sakwinska et al. (2016) demonstrate that the method of collection significantly influences the microbiota composition, although it was the higher presence and relative abundance of *Acinetobacter* species that appeared to characterize the nonsterile samples in their study. Due to the nonsterile sample collection method, we consider our results more rep-

resentative of the microbiota transferred while breastfeeding, rather than what infants receive from breast milk alone. We note here that we presume the relative abundance, but not the presence, of the administered bacteria would have been affected by the nonsterile collection. We therefore believe that the collection method is unlikely to have masked a significant transfer of the administered bacteria via breast milk and we consider the qPCR results to be a clear indication that breastfeeding was not a substantial route of probiotic transfer in the ProPACT study. These findings would also suggest that, even if probiotic supplementation altered the breast milk microbiota, the overall composition of the microbiota transferred during breastfeeding was not statistically significantly altered. The results of the 16S rRNA gene sequencing are also limited because only 30% of samples had enough reads to be included in further analysis and only 17 individuals had adequate reads at both time points. The baseline characteristics of the women with successful sequencing was representative of the original ProPACT population, and with 142 successfully sequenced samples, this currently represents the largest sequencing study of the breastfeeding-associated microbiota. Nonetheless, due to the high degree of individual variation, large studies are required to further investigate this microbiota and its association with mother-infant characteristics and infant health outcomes.

This study was motivated by the findings of the ProPACT trial, namely, that maternal probiotic supplementation reduced the cumulative incidence of atopic dermatitis in offspring at 2 yr (Dotterud et al., 2010) and led to a higher prevalence and relative abundance of LGG in infant stool samples up to 3 mo of age (Dotterud et al., 2015). In adults, continuous ingestion of LGG is required to maintain measurable quantities in stool samples (Alander et al., 1999). Given that ongoing transfer does not appear to come from breastfeeding, the observation of LGG in infant stool samples at 3 mo of age suggests that either early transfer is sufficient to establish a stable colonization with LGG in the relatively microbe-free newborn gut, or that children are receiving continued transfer of LGG via some other route from their mother. On a technical note, due to the low quantities of bacterial DNA in breast milk samples, future studies of the breast milk or breastfeeding-associated microbiota should consider starting with a greater volume of milk and establishing methods to maximize DNA extraction from milk.

In conclusion, we find that maternal supplementation with LGG, La5, and Bb-12 does not result in substantial transfer of these bacteria via breastfeeding. The breastfeeding-associated microbiota is largely dominated by *Streptococcus* and *Staphylococcus* genera at both 10 d and 3 mo postpartum.

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Supplementary Tables and Figures:

This supplementary file includes the following Tables and Figures:

Table S1: Baseline characteristics of families and allergy related disease in the children at 2 years – comparison of the whole ProPACT population, those included in the current study and those with sufficient reads from 16S rRNA gene sequencing.

Table S2: Combining information from stool samples[a] for mother-infant pairs with at least one administered bacteria present in at least one breast milk sample.

Table S3: Relative abundance of the 16 OTUs present in at least 10% of samples at either 10 days or 3 months postpartum

Figure S1: Relative abundance of 11 genera present in at least 10% of samples at either 10 days or 3 months

Figure S2: Box graphs displaying measures of diversity by time and treatment allocation where the red boxes represent samples from the placebo group and blue boxes represent samples from the probiotic group.

Table S4: Effect of probioitc supplemenation on the presence and relative abundance of common genera in breast milk samples at 10 days and 3 months postpartum.

Figure S3: Principle co-ordinate analysis (PCoA) analysis using unweighted UniFrac (left column) and weighted UniFrac (middle column) distances, and Bray Curtis dissimilarity (right column) matrices (n = 142).

Figure S4: Relative abundance of 11 common genera for each individual with paired 10 day and 3 month breast milk samples.

Table S1: Baseline characteristics of families and allergy related disease in the children at 2 years – comparison of the whole ProPACT population, those included in the current study and those with sufficient reads from 16S rRNA gene sequencing.

	Whole ProPACT Population (N = 415)					Included in current study (qPCR completed, N = 252)					Successful 16S rRNA gene sequencing (N = 125)				
	Treatment allocation					Treatment allocation					Treatment allocation				
	Probiotic		Placebo			Probiotic		Placebo			Probiotic		Placebo		
Characteristics	**n**		**n**			**n**		**n**			**n**		**n**		
Age, mother, yrs mean (SD)	191	30.1 (3.9)	189	30.3 (4.4)		127	30.5 (3.9)	125	30.4 (4.1)		58	30.7 (4.0)	67	30.7 (4.1)	
Sex (male), child, n (%)	193	96 (49.7)	191	80 (41.9)		127	66 (52.0)	125	51 (40.8)		58	32 (55.2)	67	28 (41.8)	
Siblings, n (%)	207	91 (44.0)	200	78 (39.0)		127	60 (47.2)	125	53 (42.4)		58	29 (50.0)	67	26 (38.8)	
Atopy in family, n (%)	207	152 (73.4)	200	148 (74.0)		127	90 (70.9)	125	92 (73.6)		58	44 (75.9)	67	50 (74.6)	
Maternal atopy, n (%)	205	102 (49.8)	199	100 (50.3)		127	58 (45.7)	124	65 (52.4)		58	26 (44.8)	67	32 (47.8)	
Maternal smoking[a], n (%)	204	16 (7.8)	200	19 (9.5)		127	8 (6.3)	125	11 (8.8)		58	6 (10.3)	67	8 (11.9)	
Pet[b], n (%)	207	52 (25.1)	200	53 (26.5)		127	33 (26.0)	125	35 (28.0)		58	13 (22.4)	67	20 (29.9)	
Compliant[c], n(%)	150	130 (86.7)	148	128 (86.5)		123	111 (90.2)	123	110 (89.4)		58	51 (87.9)	66	58 (87.9)	
Vaginal delivery, n(%)	134	118 (88.1)	139	120 (86.3)		123	108 (87.8)	124	108 (87.1)		56	47 (83.9)	66	55 (83.3)	
Allergy related disease to 2 yrs	**n**		**n**		**RR (95% CI)**	**n**		**n**		**RR (95% CI)**	**n**		**n**		**RR (95% CI)**
Atopic dermatitisd, n (%)	138	29 (21.1)	140	48 (34.3)	0.61 (0.41-0.91)	127	29 (22.8)	125	43 (34.4)	0.66 (0.44-0.99)	58	19 (32.8)	67	22 (32.8)	1.00 (0.60-1.65)
Current asthma, n (%)	138	8 (5.8)	140	12 (8.6)	0.68 (0.29-1.60)	127	7 (5.5)	125	12 (9.6)	0.57 (0.23-1.41)	58	4 (6.9)	67	8 (11.9)	0.58 (0.18-1.82)
ARC, n (%)	134	1 (0.8)	139	1 (0.7)	1.04 (0.07-16.4)	123	1 (0.8)	124	0 (0.0)	n.a.	56	0 (0.0)	66	0 (0.0)	n.a.
Current Sensitisatione, n (%)	131	20 (15.3)	133	15 (11.3)	1.35 (0.72-2.53)	120	18 (15.0)	119	14 (11.7)	1.28 (0.67-2.44)	54	8 (14.8)	65	12 (18.5)	0.80 (0.35-1.82)
SPT positive, n (%)	120	6 (5.0)	118	6 (5.1)	0.98 (0.33-2.96)	109	6 (5.5)	106	6 (5.7)	0.97 (0.32-2.92)	52	4 (7.7)	55	5 (9.1)	0.85 (0.24-2.98)
sIgE positive, n (%)	89	19 (21.4)	100	13 (13.0)	1.64 (0.86-3.13)	80	17 (21.3)	89	12 (13.5)	1.58 (0.80-3.09)	37	8 (21.6)	48	10 (20.8)	1.04 (0.45-2.37)

ARC: allergic rhinoconjunctivitis (cumulative incidence); [a]Maternal smoking reported during pregnancy, 6 weeks or 12 months postpartum; [b]Reported a household pet during pregnancy or the child's first year of life; [c]Compliance with the study protocol was defined as consumption of the study milk on at least 50% of days from 36 weeks gestation to 12 postpartum, no consumption of other products with probiotics and at least partial breastfeeding until 3 months postpartum; [d]Cumulative incidence of atopic dermatitis. [e]Allergic sensitisation defined as positive skin prick test (wheal ≥3mm) and or positive sIgE (≥ 0.35 kUL^{-1}). Not all children had both measured.

Table S2: Combining information from stool samples[a] for mother-infant pairs with at least one administered bacteria present in at least one breast milk sample.

			Lactobacillus rhamnosus GG					Lactobacillus acidophilus La-5					Bifidobacterium animalis spp lactis Bb-12				
Individual	Treatment Group	Atopic Dermatitis	Breast milk 10 days	Bresat milk 3 months	Mother stool, 3mo.	Infant stool, 10 days	Infant stool, 3 months	Breast milk 10 days	Bresat milk 3 months	Mother stool, 3mo.	Infant stool, 10 days	Infant stool, 3 months	Breast milk 10 days	Bresat milk 3 months	Mother stool, 3mo.	Infant stool, 10 days	Infant stool, 3 months
A	Placebo	No	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	Probiotic	Yes	-	-	-	-	?	-	-	-	-	?	+	-	-	-	?
C[b]	Probiotic	Yes	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
D	Probiotic	No	-	-	+	+	+	+	-	-	-	-	+	-	+	-	-
E	Probiotic	No	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
F	Probiotic	No	-	-	?	+	+	-	-	?	-	-	+	-	?	-	-
G	Probiotic	No	+	-	+	-	+	-	-	+	-	-	+	+	+	-	-
H[b]	Probiotic	No	+	?	+	+	+	-	?	+	-	-	-	?	+	-	-
I	Probiotic	Yes	-	-	+	-	+	-	-	+	-	-	-	+	+	-	-

? indicates a missing sample. [a]Stool sampels were previously analysed and presented in the paper: Dotterud CK, Avershina E et al. (2015) Does maternal perinatal probiotic supplementation alter the intestinal microbiota of mother and child? A randomised trial. Journal of Pediatric Gastroenterology & Nutrition; doi: 10.1097/MPG.0000000000000781.
[b]Subjects C and H had Ct values >35 for total bacteria, yet had detectable levels of one of the administered bacteria. We consider this to be a result of technical issues in the assessment of total bacteria.

This table is presented to demonstrate the presence of the administered bacteria in the stool samples from mothers and infants where at least one breast milk sample was positive for one or more of the administered bacteria. Only 9 of 472 breast milk samples had detectable levels of the administered bacteria, therefore breast milk cannot be considered a major source of these bacteria for the infants.

Table S3: Relative abundance of the 16 OTUs present in at least 10% of samples at either 10 days or 3 months postpartum

	Taxonomic classification / grouping		10 days (n = 60)			3 months (n = 82)			Prevalence: 10d vs 3mo.		Relative abund.: 10d vs 3mo.	
				Relative abundance (reads /1000)			Relative abundance (reads /1000)					
OTU	Family	Genus (species)	No. pos.	med.	IQR	No. pos.	med.	IQR	p-value	FDR	p-value	FDR
1	Streptococcaceae	Streptococcus	57	369	121 – 663	77	392	162 – 624	0.779	0.802	0.806	0.851
3	Staphylococcaceae	Staphylococcus (epidermidis)	52	151	45 – 320	58	30	8 – 102	0.025	0.041	<0.001	<0.001
5	Streptococcaceae	Streptococcus	42	32	4 – 75	34	4	0 – 42	<0.001	0.004	0.001	0.003
6	Gemellaceae		36	18	3 – 48	52	15	2 – 44	0.679	0.783	0.598	0.690
16	Unclassified		18	4	1 – 15	14	2	0 – 7	0.069	0.103	0.008	0.015
100	Streptococcaceae	Streptococcus	17	2	1 – 12	60	74	8 – 255	<0.001	<0.001	<0.001	<0.001
120	Streptococcaceae	Streptococcus	10	1	0 – 5	15	0.5	0 – 5	0.802	0.802	0.851	0.851
2	Morazellaceae	Acinetobacer (rhizosphaerae)	10	0	0 – 3	19	0	0 – 6	0.342	0.428	0.130	0.177
8	Micrococcaceae	Rothia (mucilaginosa)	10	0	0 – 3	37	9	1 – 19	<0.001	0.003	<0.001	<0.001
7	Streptococcaceae	Streptococcus	9	0	0 – 0	2	0	0 – 0	0.006	0.017	0.017	0.028
22	Pasteurellaceae	Haemophilus (parainfluenza)	4	0	0 – 1	12	0	0 – 3	0.138	0.188	0.113	0.169
25	Veillonellaceae	Veillonella (dispar)	3	0	0 – 1	21	2	0 – 3	0.001	0.005	<0.001	<0.001
28	Methylobacteriaceae	Methylbacterium	1	0	0 – 1	11	0	0 – 4	0.013	0.024	0.283	0.354
31	Carnobacteriaceae	Granulicatella	1	0	0 – 0	12	0	0 – 3	0.008	0.017	0.005	0.010
43	Carnobacteriaceae	Granulicatella	0	0	0 – 0	9	0	0 – 1	0.008	0.017	0.001	0.003

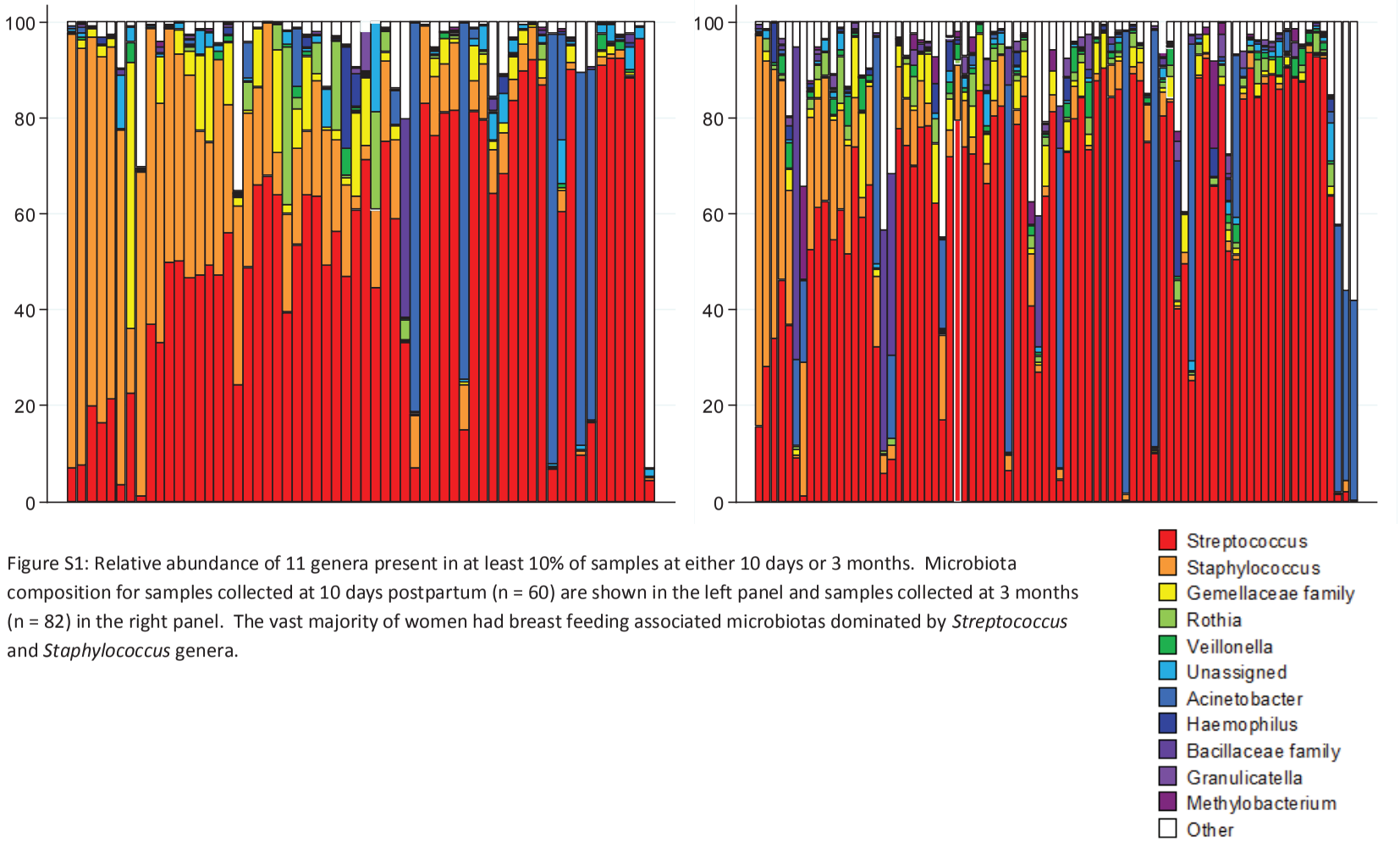

Figure S1: Relative abundance of 11 genera present in at least 10% of samples at either 10 days or 3 months. Microbiota composition for samples collected at 10 days postpartum (n = 60) are shown in the left panel and samples collected at 3 months (n = 82) in the right panel. The vast majority of women had breast feeding associated microbiotas dominated by *Streptococcus* and *Staphylococcus* genera.

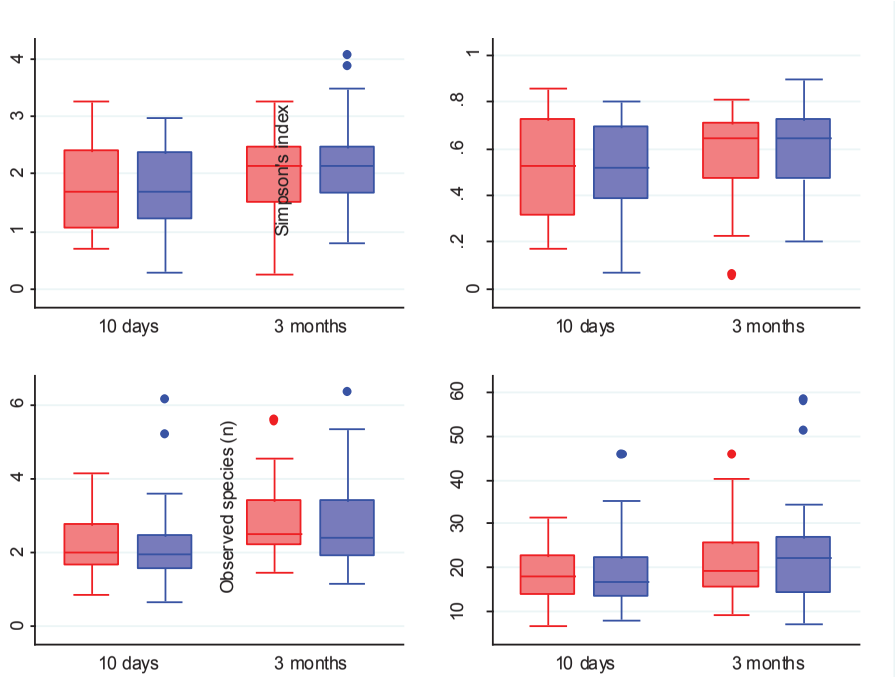

Figure S2: Box graphs displaying measures of diversity by time and treatment allocation where the red boxes represent samples from the placebo group and blue boxes represent samples from the probiotic group. When comparing all 10 day samples to all 3 months samples, all measures of diversity increased over time:

Measure of diversity	10 days (n = 60) median (IQR)	3 months (n = 82) median (IQR)	p-value
Shannon diversity index (top left)	1.70 (1.08-2.42)	2.15 (1.63-2.47)	0.016
Simpson diversity index (top right)	0.53 (0.36-0.70)	0.64 (0.47-0.72)	0.053
Phylogenetic diversity (bottom left)	1.98 (1.61-2.66)	2.45 (2.06-3.39)	≤ 0.001
Number of observed species (bottom right)	17.7 (13.6-22.6)	20.1 (15.7-26.6)	0.033

Probiotic supplementation had no effect on any of these measures of diversity overall or when considering each time point separately.

Table S4: Effect of probioitc supplemenation on the presence and relative abundance of common genera in breast milk samples at 10 days and 3 months postpartum.

Genus	Presence of genus				Relative abundance of genus					
					Probiotic		Placebo			
	Probiotic	Placebo	p-value	FDR	Med	IQR	Med	IQR	p-value	FDR
10 days	**(n = 28)**	**(n = 32)**								
Streptococcus	28	32	n.a.	n.a.	621	470 - 814	486	188 - 672	0.082	0.367
Staphylococcus	25	27	0.577	0.887	151	37 - 301	150	50 - 403	0.657	0.739
Gemellaceae family	14	22	0.139	0.557	11	3 - 29	22	3 - 68	0.148	0.389
Unassigned	11	12	0.887	0.887	5	1 - 18	7	4 - 27	0.204	0.389
Veillonella	7	3	0.105	0.557	1	0 - 9	0	0 - 4	0.065	0.367
Acinetobacter	6	5	0.562	0.887	0	0 - 8	0	0 - 1	0.216	0.389
Rothia	5	5	0.817	0.887	0	0 - 6	0	0 - 5	0.585	0.739
Bacillaceae family	3	3	0.863	0.887	0	0 - 3	1	0 - 2	0.942	0.942
Haemophilus	2	4	0.490	0.887	0	0 - 3	0	0 - 1	0.287	0.430
3 months	**(n = 39)**	**(n = 43)**								
Streptococcus	38	41	0.615	0.754	663	738 - 401	406	805 - 869	0.330	0.941
Staphylococcus	31	27	0.097	0.534	30	30 - 11	6	99 - 110	0.636	0.941
Gemellaceae family	26	26	0.560	0.754	16	12 - 2	2	64 - 40	0.324	0.941
Rothia	21	20	0.507	0.754	10	9 - 2	1	18 - 26	0.941	0.941
Veillonella	16	17	0.891	0.891	6	6 - 1	0	23 - 16	0.382	0.941
Acinetobacter	12	9	0.308	0.754	1	0 - 0	0	12 - 6	0.340	0.941
Unassigned	10	12	0.817	0.891	3	6 - 0	0	10 - 11	0.891	0.941
Granulicatella	9	12	0.617	0.754	1	0 - 0	0	5 - 12	0.909	0.941
Bacillaceae family	8	6	0.430	0.754	0	1 - 0	0	8 - 5	0.735	0.941
Haemophilus	5	8	0.474	0.754	1	0 - 0	0	5 - 7	0.913	0.941
Methylobacterium	3	9	0.090	0.534	1	0 - 0	0	3 - 6	0.678	0.941

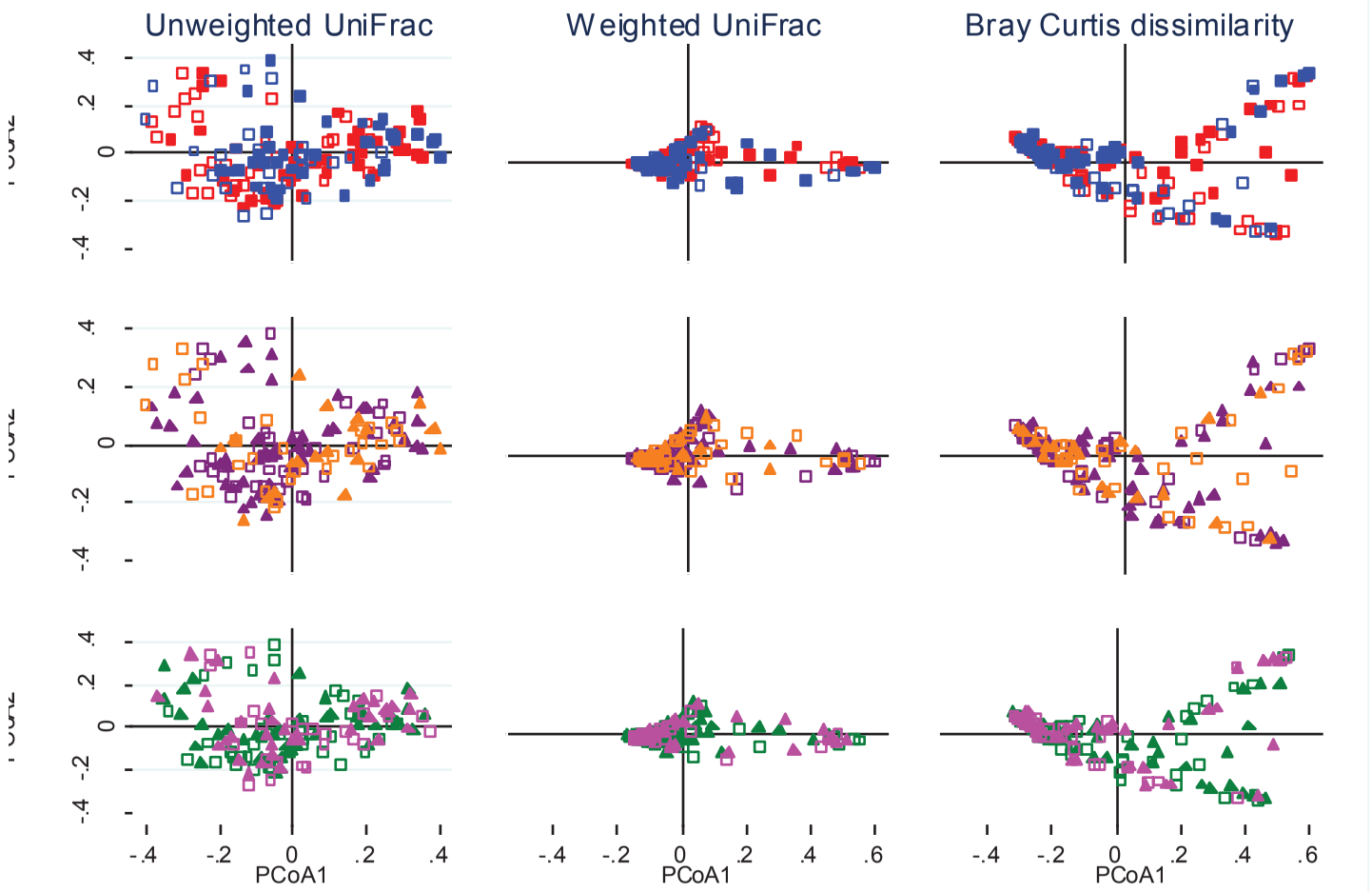

Figure S3: Principle co-ordinate analysis (PCoA) analysis using unweighted UniFrac (left column) and weighted UniFrac (middle column) distances, and Bray Curtis dissimilarity (right column) matrices (n = 142). Stage of lactation, treatment group and maternal-infant characteristics did not clearly contribute to separation of the breastfeeding microbiota in the first two principle co-ordinates. The colours and shapes of the markers represent the following:

- The upper row – Stage of lactation and treatment group: 10 days = open squares, 3 months = closed squares; probiotic = blue, placebo = red;
- Second row – maternal atopy and development of atopic dermatitis by 2 years of age in the infants: maternal atopy = open squares, no maternal atopy = closed triangles; Atopic dermatitis = purple, no atopic dermatitis = orange
- Lower row – presence of siblings and sex of the infant: no siblings = green, siblings = magenta; female = closed triangles, male = open squares.

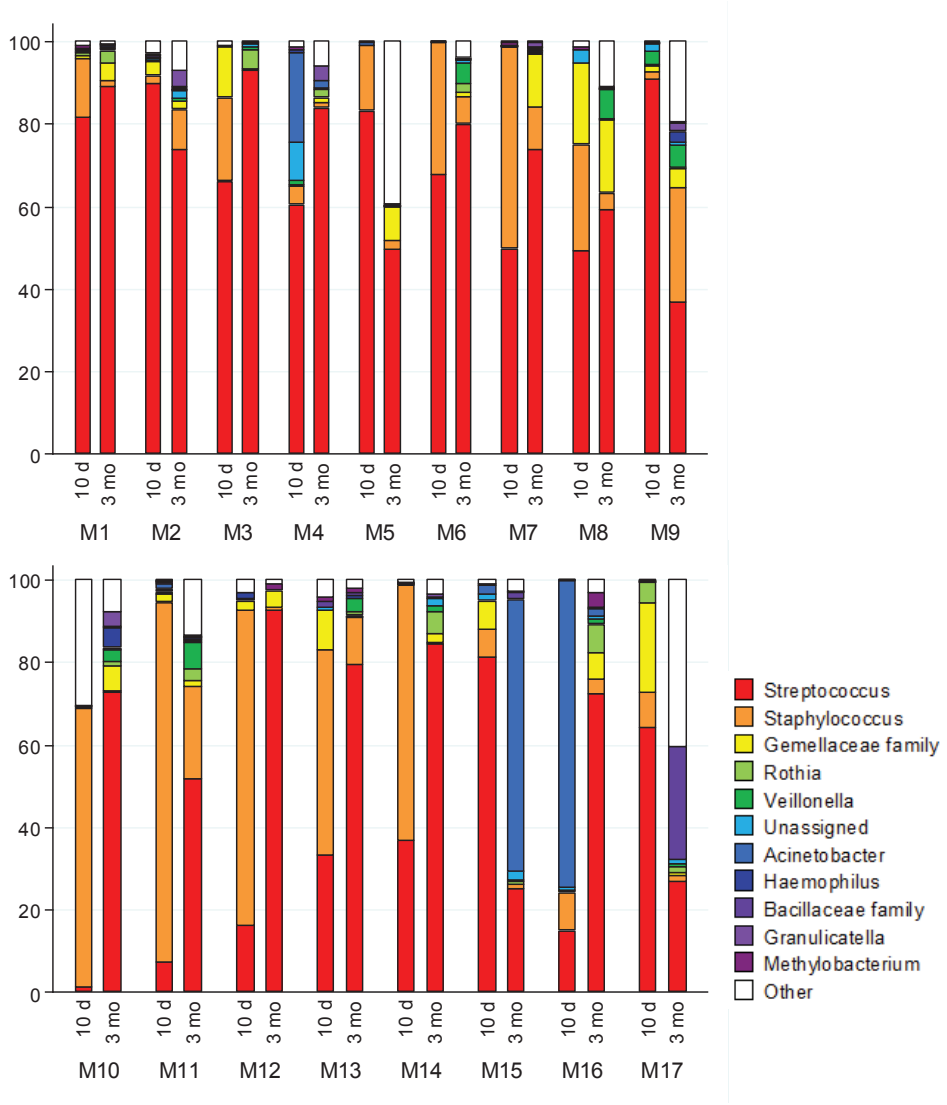

Figure S4: Relative abundance of 11 common genera for each individual with paired 10 day and 3 month breast milk samples. The breastfeeding associated microbiota was dominated by *Streptococcus* and *Staphylococcus* at both 10 days and 3 months postpartum. For some women *Streptococcus* genera was dominant at both time points (M1-9), whilst for other women there was a shift from a *Staphylococcus* dominated milk microbiota to *Streptoccocus* dominance (M10-M14). The remaining women had more variable composition of their milk microbiota. Some of these women displayed dominance of less common genera, such as *Acinetobacter* (M15 & M16), or *Bifidobacterium* genus and *Bacillaceae* family (M17) at either 10 days or 3 months postpartum, however the other time point was still dominated by *Streptococcus* and *Staphylococcus.* The *Bifidobacterium* genus is included in the "other" group.

Paper II

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RESEARCH Open Access

Atopic dermatitis prevention in children following maternal probiotic supplementation does not appear to be mediated by breast milk TSLP or TGF-β

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Abstract

Background: The Probiotics in Prevention of Allergy among Children in Trondheim (ProPACT) study, a randomised, placebo controlled trial, demonstrated that maternal supplementation with probiotic milk reduced the incidence of atopic dermatitis (AD) in infancy. The mechanisms behind this effect are incompletely understood and breast milk cytokines have been postulated as possible mediating factors. In this study we aimed to assess whether breast milk TLSP and TGF-β are affected by a maternal probiotic supplementation regime, and their contribution to the preventive effect of this regime on AD in the offspring.

Methods: TSLP and TGF-β isoforms (TGF-β_1, TGF-β_2 and TGF-β_3) were measured using ELISA and multiplex assays, respectively, in breast milk samples collected at 10 days and 3 months postpartum from women participating in the ProPACT trial (n = 259). The natural indirect and direct effects of maternal probiotics on AD, due to changes in breast milk cytokines, were estimated using causal mediation techniques.

Results: Probiotic supplementation tend to lead to high levels of breast milk TSLP at 10 days postpartum ($p = 0.062$), but this change did not contribute to the prevention of AD according to the mediation analysis. Probiotics had no apparent effect on TSLP at 3 months or TGF-βs at either time points. Thus, these are unlikely to be mediators of the effect of maternal probiotics on AD in offspring.

Conclusions: Whilst maternal probiotic supplementation resulted in higher breast milk concentrations of TLSP at 10 days postpartum, this does not appear to be a mechanism for prevention of AD by maternal probiotics.

Trial registration The original trial protocol is registered in ClinicalTrials.gov (identifier NCT00159523)

Background

Perinatal supplementation with probiotics has been shown to reduce the incidence of atopic dermatitis (AD) in infancy [1, 2]. Our own study, the Probiotics in the Prevention of Allergies among Children in Trondheim (ProPACT) trial, demonstrated a 40 % reduction in the development of AD following maternal probiotic supplementation [3]. However, the biological mechanisms behind this effect are incompletely understood. Using samples taken during the ProPACT trial, we have previously reported that the maternal intestinal microbiota is modified by probiotic supplementation, and that children born to mothers who received the probiotics have a higher abundance of *Lactobacillus rhamnosus GG* (LGG) which persists up until 3 months of age [4]. Whilst the transfer of such probiotic bacteria from mother to child may prevent AD, alterations in various components of breast milk have also been postulated as possible mediating factors.

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In addition to being a source of nutrition and hydration for newborn infants, breast milk contains a number of immunologically active cells and molecules, such as immunoglobulins, lactoferrins, growth factors and cytokines [5]. Among the cytokines found in breast milk are thymic stromal lymphopoietin (TSLP) and the transforming growth factor-β (TGF-β) cytokines. TSLP has been implicated in the establishment and maintenance of T helper type 2 (Th2) responses, and thus also in defence against helminthic infections and in the pathogenesis of allergy related diseases [6]. Genetic variants of TSLP have been associated with AD and asthma [7] and high levels of epidermal TSLP precede the clinical presentation of childhood AD [8], suggesting that TSLP may be particularly important in the establishment of AD. Other studies have demonstrated that TSLP activates skin dendritic cells promoting a Th2 response and interacts directly with skin-homing Th2 cells to enhance interleukin-4 (IL-4) production which is thought to contribute to the maintenance of inflammation in chronic AD [9, 10]. Consistent with these studies, higher concentrations of TSLP have been reported in both acute and chronic AD lesions [6]. Murine models have shown that over-expression of TSLP in keratinocytes induces an AD-like skin disease and can predispose to allergic airway inflammation after intranasal challenge. Thus, TSLP may also be involved in the progression to other allergy related diseases, a process often referred to as the atopic march [6]. However, the biological effects of breast milk TSLP for the mother and child are unknown [11], and this cytokine is previously unstudied within the context of maternal probiotic supplementation. Neither has the effect of maternal atopy on breast milk TSLP been investigated, nor the association between breast milk TSLP and the development of AD. In contrast to TSLP, the human isoforms of TGF-β (TGF-β_1, TGF-β_2 and TGF-β_3) are primarily implicated in inhibition of allergic inflammation through a wide range of immunoregulatory effects [12]. All three isoforms of TGF-β are found in breast milk. Studies investigating the effect of probiotic supplementation on breast milk TGF-β concentrations and or their association with later allergy related disease in offspring, have produced conflicting results [13–18].

Both TSLP and TGF-βs appear to have important roles in acute and chronic phases of allergy related disease, which makes them interesting as potential mediators of this preventive effect. The aims of the current study were to: (a) determine if perinatal maternal probiotic supplementation alters the concentration of TLSP, TGF-β_1, TGF-β_2 or TGF-β_3 in breast milk at 10 days and 3 months postpartum and (b) investigate if these breast milk cytokines contribute to the preventative effect of maternal probiotic supplementation on the development of AD at 2 years of age through causal mediation analysis.

Methods

Participant recruitment and sample collection

The ProPACT trial enrolled 415 pregnant women who were randomised to receive 250 mL per day of probiotic or placebo milk from 36 weeks gestation until 3 months postpartum [3]. The probiotic milk contained 5×10^{10} colony-forming units (CFUs) of *Lactobacillus rhamnosus GG* (LGG) and *Bifidobacterium animalis* subsp. *lactis* Bb-12 (Bb-12) and 5×10^9 CFU of *L. acidophilus* La-5 (La-5) per serving. The placebo was a fermented skim milk, pasteurised after fermentation and contained no probiotic bacteria. Participants completed lifestyle questionnaires at baseline (~30–36 weeks gestation), 6 weeks, 1 and 2 years postpartum and child health questionnaires at 1 and 2 years of age. The outcome of interest in the current study was the development of AD by 2 years of age as diagnosed by a paediatrician using the United Kingdom (UK) Working Party Diagnostic criteria [19]. Detailed descriptions of the ProPACT study design and clinical outcomes have been published previously [3, 20].

Breast milk samples were collected into a sterile container at 10 days and 3 months postpartum and stored in the participant's home freezer until transported to the laboratory in Styrofoam containers to prevent thawing. Samples were subsequently stored at −80 °C until analysis. All mother-infant pairs that attended the 2 year clinical follow-up and submitted at least one breast milk sample were eligible for inclusion in this study.

Cytokine quantification

Breast milk samples were thawed and centrifuged (16,100*g*, 10 min, 4 °C) to remove the lipids, cells and debris. The aqueous portion was used for subsequent cytokine quantification. TSLP concentrations were measured using a human TLSP ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. The standards and an internal control were conducted in triplicate. The intra-assay coefficient of variation (CV) was <13.5 % and inter-assay CV was 22 %. A large proportion of the samples had TSLP concentrations outside of the detection limits of the assay, both above and below. We have therefore categorised the TSLP concentrations into the following 4 categories: below detection, low detectable, high detectable and above detection. The internal control sample was chosen as the cut-off between the low and high detectable categories, because it was approximately the median concentration and maximised the inter-assay comparability. For the purpose of mediation analysis TSLP was categorised into a binary variable above and below the internal control.

TGF beta concentrations were measured using a multiplex assay (Bio-Plex Pro TGF-β assay, Bio-Rad Laboratories, Oslo, Norway) following the manufacturer's instructions which included an actvation step. Prior to analysis, 100 μL of the aqueous portion of breast milk was activated using 20 μL 1 N HCl for 10 min at room temperature, followed by neutralisation using 20 μL 1 N NaOH with 0.5 M HEPES. The standard and two internal control samples were conducted in duplicate. Concentrations were calculated using standard curves and scaled by 1.4 due to dilution in the activation-neutralisation process. The intra-assay CVs were <13.6, <13.4 and <14.6 % and the inter-assay CV were <9.9, <5.9 and <14.3 % for TGF-β_1, TGF-β_2 and TGF-β_3, respectively.

Statistical analysis

As described, TSLP concentrations were categorised into a four groups, and the effect of probiotics was assessed using ordinal logistic regression. The distributions of TGF-βs were right-skewed and concentrations are reported as medians and interquartile ranges. Wilcoxon matched-pairs signed-rank test was used to compare the concentration at 10 days and 3 months for the 243 women with TGF-β isoforms measured at both time points. The effect of probiotic supplementation on TGF-β concentrations was assessed using linear regression on log-transformed concentrations. Maternal atopy, maternal smoking during the first year of life and the presence of older siblings were considered to be potential moderators of the effect of probiotics on cytokine concentrations. The effect of probiotics on the breast milk cytokines were therefore also assessed in alternate regression models which included these covariates.

Causal mediation analysis was performed using the user written command, *paramed,* for breast milk cytokines which were found to be altered by probiotic supplementation. This analysis estimated the natural indirect effect (NIE) and natural direct effect (NDE) of maternal probiotic supplementation on the development of AD in offspring, mediated through breast milk cytokine concentrations, using counterfactual definitions of these effects and a log-binomial regression model for AD, given AD is not rare [21–23]. In this study, the NIE represents the effect of the probiotic regime on the development of AD which can be attributed to its effect on the breast milk cytokine. The NDE represents an estimation of what the relative risk (RR) of developing AD after maternal probiotic supplementation would have been, had there been no effect on the concentration of the breast milk cytokine. The *paramed* command does not currently support models with ordinal mediator variables and TSLP concentrations were therefore dichotomised for mediation analysis. Whilst the randomised design ensures that the effect of probiotics on breast milk cytokines and on AD can be estimated without confounding, the relationship between cytokine concentration in breast milk and the development of AD is not randomised. As such, the estimation of NIE and NDE may be confounded by factors which influence both breast milk cytokine concentrations and the risk of developing AD in offspring. The previously mentioned covariates were therefore included in the mediation analysis as potential confounders of the relationship between breast milk composition and AD. All statistical analyses were performed using Stata IC release 13 (StataCorp, College Station, Texas).

Results

Participants

Two hundred and fifty-nine mother-infant pairs were included in this analysis, 129 from the probiotic group and 130 from the placebo group (Fig. 1). This subgroup of participants is representative of the original study population with respect to distribution of baseline characteristics and allergy related disease outcomes [3]. Compliance was high and equivalent in both groups. At baseline, the probiotic group contained more male children and at 2 years of age there were fewer cases of AD (Table 1). In terms of maternal characteristics which have been previously suggested to influence breast milk cytokine concentration, fewer women in the probiotic group had a personal history of atopy (Table 1). There were also fewer women smoking at 6 weeks postpartum in the probiotic group, although the overall prevalence of smoking was very low (8 of 259, 3.2 %).

Thymic stromal lymphopoietin

A greater proportion of breast milk samples taken 10 days postpartum had high concentrations of TLSP compared with the samples taken at 3 months (Fig. 2). The concentration of TSLP was below the lower limit of detection (31.3 pg/mL) in 18.9 % and 26.7 % of samples collected at 10 day and 3 months postpartum, respectively, and above the limit of detection (2000 pg/mL) in 25.6 % and 17.3 %, respectively. Ordered logistic regression revealed a borderline non-significant effect of probiotics on TLSP concentration (OR 1.55 95 % CI 0.98–2.45, $p = 0.062$) at 10 days postpartum which was not sustained at 3 months postpartum (OR 1.35 95 % CI 0.84–2.16, $p = 0.219$). This effect was not significantly altered when adjusting individually or in combination for maternal atopy, maternal smoking or older siblings. The effect of probiotics on breast milk TSLP concentration at 10 days postpartum was marginally enhanced when considering only women compliant with the study protocol ($n = 209$, OR 1.74, 95 % CI 1.06–2.84, $p = 0.028$). We did not find evidence of an association between maternal

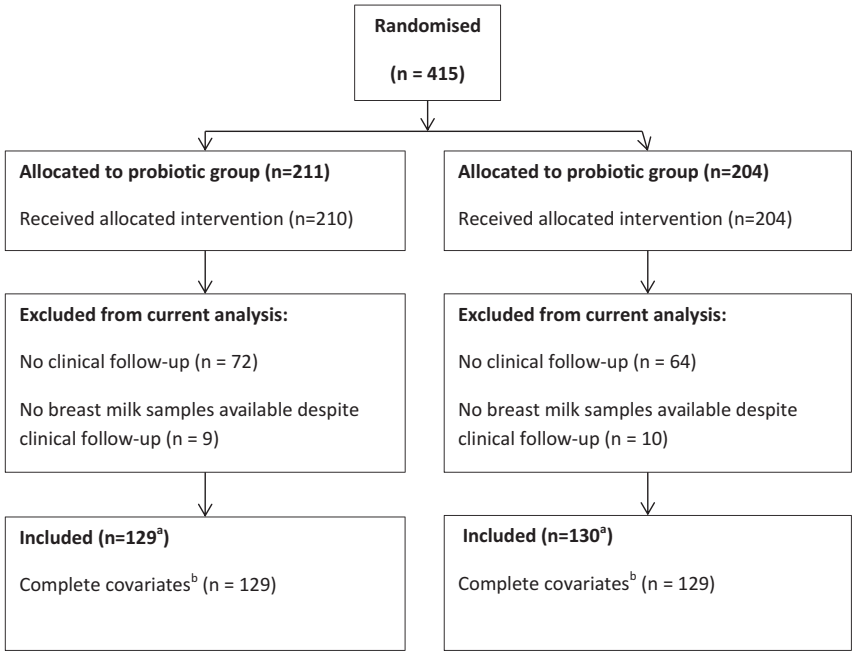

Fig. 1 Flow diagram of participants in the ProPACT study and cytokine analysis of breast milk samples. [a]The provided values represent the number of eligible women who had provided at least one breast milk sample. The precise number of samples analysed for each cytokine at each time point varies and is provided in association with the relevant results. [b]Complete covariates additionally required information on maternal atopy, maternal smoking and the presence of older siblings

Table 1 Baseline characteristics of participating families and allergy related disease in the children at 2 years

Characteristics	Probiotic		Placebo		
	n		n		
Age, mother, yrs mean (SD)	129	30.5 (3.9)	130	30.3 (4.1)	
Sex (male), child, n (%)	129	67 (51.9)	130	53 (40.8)	
Siblings, n (%)	129	60 (46.5)	130	54 (41.5)	
Atopy in family, n (%)	129	91 (70.5)	130	96 (73.9)	
Maternal atopy, n (%)	129	58 (45.0)	129	68 (52.7)	
Maternal smoking[a], n (%)	129	9 (7.0)	130	11 (8.5)	
Compliant[b], n(%)	125	113 (90.4)	128	115 (89.8)	
Allergy related disease at 2 years	**n**	**n (%)**	**n**	**n (%)**	**p-value[c]**
Atopic dermatitis, n (%)	129	29 (22.5)	130	45 (34.6)	0.031
Asthma, n (%)	129	7 (5.4)	130	12 (9.2)	0.240
Allergic rhinoconjunctivits, n (%)	125	1 (0.8)	129	0 (0.0)	0.492
Sensitisation[d], n (%)	122	19 (15.6)	124	15 (12.1)	0.429

[a] Maternal smoking reported during pregnancy, 6 weeks or 12 months postpartum; [b] Compliance with the study protocol was defined by consumption of the study milk on at least 50 % of days from 36 weeks gestation to 12 postpartum, no consumption of other products with probiotics and at least partial breastfeeding until 3 months postpartum; [c] p-value calculated using χ^2-test, except for allergic rhinoconjunctivits where a Fisher's exact *p* value is reported; [d] Allergic sensitisation defined as positive skin prick test (wheal $\geq$ 3 mm) and or positive sIgE ($\geq$ 0.35 kUL^{-1})

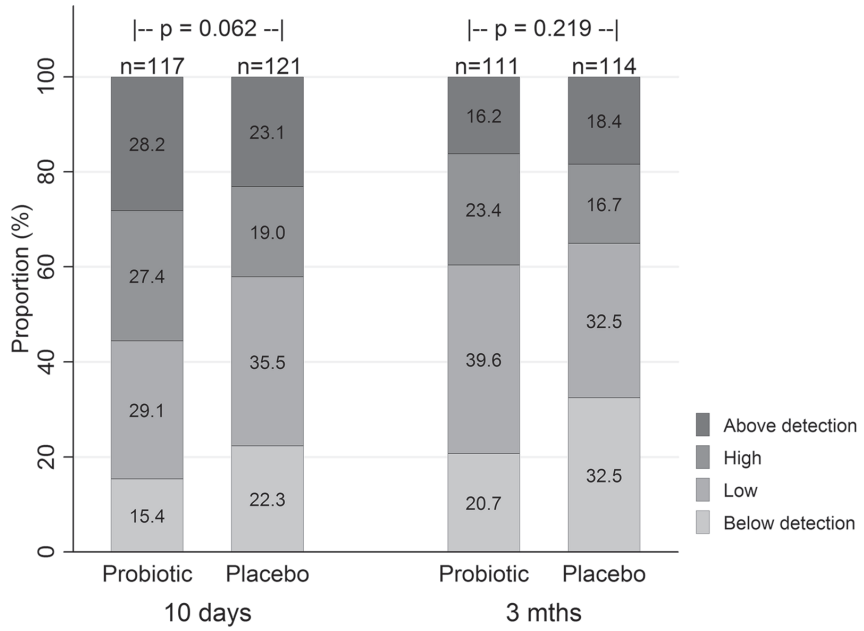

Fig. 2 Breast milk TSLP concentrations at 10 days and 3 months in the probiotic and placebo groups. Proportion of breast milk samples with categorised TSLP concentrations at 10 days and 3 months postpartum in the placebo and probiotic group. Percentages are provided within the *bars*. Overall, higher concentrations of TSLP were measured significantly more often in samples collected at 10 days postpartum ($p < 0.001$ from ordinal logistic regression clustered by individual). Also on subgroup analysis, TSLP concentrations were significantly higher at 10 days in both the probiotic group ($p < 0.001$) and placebo group ($p = 0.005$)

atopy, maternal smoking or the presence of older siblings and TSLP in univariate analysis.

Transforming growth factor β

All three TGF-β isoforms were detectable in all breast milk samples, with the exception of one sample collected at 3 months postpartum which had undetectable TGF-β_3. Similar to TSLP, the concentrations of TGF-β_1, TGF-β_2 and TGF-β_3 were higher at 10 days postpartum compared with 3 months postpartum (Fig. 3). Probiotic supplementation had no observed effect on the concentration of any of the TGF-β subtypes at either time point (Table 2). This lack of effect was not substantially altered when adjusting for potential moderators or when considering the subgroup of women compliant with the study protocol (n = 219–224).

Breast milk cytokines as potential mediators of preventative effect on atopic dermatitis

For the purposes of mediation analysis, TSLP concentrations at 10 days were dichotomised. Consistent with the ordinal logistic regression analysis, standard logistic regression on the dichotomised TSLP values also suggested that perinatal probiotic supplementation increased the odds of a high TSLP concentration at 10 days (OR 1.72, 95 % CI 1.03–2.87, $p = 0.039$). However, mediation analysis suggested that the effect of probiotics on TSLP did not result in a significant reduction, or increase, in the risk of developing AD [RR^{NIE} 1.04 (95 % CI 0.94–1.15, $p = 0.45$)], Fig. 4). Breast milk TSLP concentrations at 3 months postpartum and the TGF-β cytokines at both 10 days and 3 months postpartum were not demonstrably affected by probiotic supplementation, indicating that changes in these breast milk cytokines are unlikely to be responsible for the reduced risk of developing AD observed after maternal probiotic supplementation. As such, no estimation of NIE and NDE was conducted for these. Furthermore, none of the cytokines, including TSLP at 10 days, were found to be significantly associated with the development of AD when assessed independently of treatment allocation.

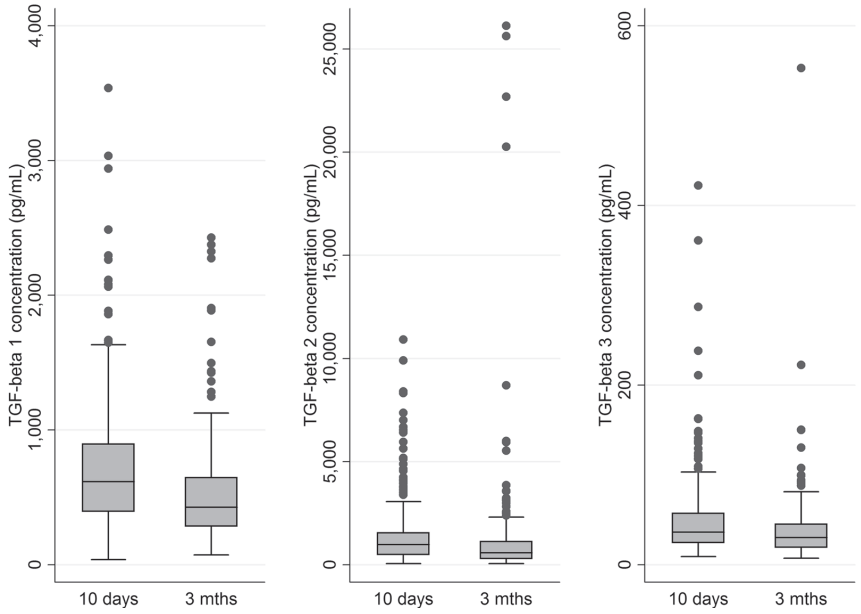

Fig. 3 Concentrations of TGF-β1, 2 and 3 at 10 days and 3 months. All three isoforms of TGF-β are found at statistically significantly lower levels at 3 months postpartum compared to 10 days postpartum ($p < 0.001$ for all isoforms using Mann–Whitney U-test)

Table 2 Breast milk TGF-β concentrations in the probiotic and placebo groups at 10 days and 3 months postpartum

	Days	Treatment				p[a]
		Probiotic		Placebo		
		n	Median (IQR) (pg/mL)	n	Median (IQR) (pg/mL)	
TGF-β_1	10	128	616.9 (407.5–950.4)	127	617.3 (369.7–853.9)	0.34 0.33
	90	123	438.5 (308.0–661.1)	124	417.4 (269.3–641.8)	0.27 0.23
TGF-β_2	10	128	909.8 (451.6–1653.8)	127	999.4 (519.0–1517.1)	0.90 0.83
	90	123	599.6 (311.0–1055.1)	124	532.5 (285.9–1144.2)	0.76 0.69
TGF-β_3	10	128	37.8 (25.3–58.7)	127	35.8 (22.9–57.2)	0.25 0.33
	90	123	29.9 (21.0–42.7)	123	30.8 (18.8–48.7)	0.95 0.86

[a] The upper p-value provided is from the regression analysis of the influence of probiotic ingestion on the log transformed TGF beta concentration by, whilst the lower represents the p-value calculated for the effect of treatment allocation after adjusting for maternal atopy, maternal smoking and presence of siblings

Discussion

Thymic stromal lymphopoietin was the only cytokine with a tendency to be affected by maternal probiotic supplementation, resulting in higher concentrations of TSLP at 10 days postpartum. Causal mediation analysis indicated that the higher concentrations of TSLP at 10 days did not appear to contribute to the beneficial effect of probiotics on the development of AD in offspring. TSLP concentrations at 3 months postpartum and TGF-βs at both time points were not affected by

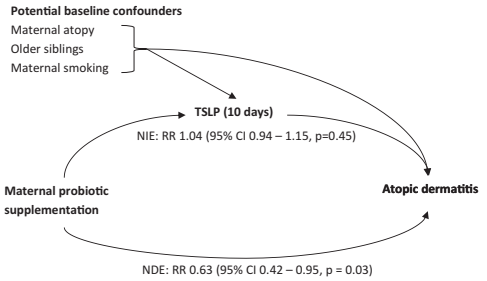

Fig. 4 Hypothetical mediation analysis and estimated natural indirect effect (NIE) and natural direct effect (NDE) of maternal probiotic supplementation. The NIE is an estimate of the effect of maternal probiotic ingestion on the development of AD in offspring mediated by increased TSLP levels in breast milk 10 days postpartum. The NDE is an estimate of the effect of maternal probiotic ingestion on the development of AD not mediated through changes in breast milk TSLP concentration 10 days postpartum. This analysis suggests that TSLP does not significantly contribute to, or oppose, the preventative effect of maternal perinatal probiotic supplementation on the development of atopic dermatitis

probiotic supplementation. As such, these breast milk cytokines are unlikely to be mediators of the beneficial effect of probiotics.

This study provides the first report of the influence of a maternal probiotic supplementation on breast milk TLSP concentrations and investigates its contribution to the prevention of AD. In experimental and observational studies, TSLP has been associated with promotion of a Th2 and allergic type inflammation, particularly in the lung and skin [6, 24]. Therefore, the finding that probiotic supplementation increased the breast milk TSLP concentrations was contrary to what one might have expected. At the same time, high breast milk TSLP may not be as detrimental in the intestinal system where it is described as having a more regulatory function [24, 25]. TSLP released by intestinal epithelial cells in response to commensal bacteria has been shown to promote tolerogenic properties in dendritic cells and macrophages which then produce IL-10 and retinoic acid and promote regulatory T helper (Treg) cell differentiation [25]. Thus the increased TSLP seen in the probiotic group may encourage intestinal immune homeostasis, which in turn is theoretically beneficial for the development of the neonatal immune system and prevention of AD. However, the current data does not suggest that breast milk TSLP contributes to the preventative effect of probiotics on AD, nor does it appear to be associated with the development of AD. It is unclear if the observed lack of mediating effect is due to a loss of information and power due to dichotomisation of TSLP concentration, TLSP degradation prior to reaching the intestines, or a more complex interplay between TSLP, the intestinal microbiota and the immune system. Further studies are required to confirm these findings.

The major strength of this study is that the randomised placebo controlled, double blind design of the ProPACT trial allows an unconfounded assessment of the effect of the probiotic regime on the selected breast milk cytokines. Furthermore, through causal mediation analysis techniques, we are able to estimate if and to what extent the prevention of AD is due to changes in TSLP. The randomised design also means that two of four assumptions required for this mediation analysis [21] are automatically satisfied, namely that there is no residual confounding of the effect of probiotics supplementation on either AD or breast milk TSLP. The other two required assumptions are that there is no unmeasured confounding of the relationship between breast milk TSLP and AD and that there is no confounder of the mediator-outcome relationship that is affected by treatment allocation. Whilst these cannot be excluded, we believe we have measured and included all variables which are likely to affect both breast milk cytokines and AD and that probiotics do not have a strong effect on any other confounders of the relationship between these variables. Another strength of this study is that it is the largest to investigate TSLP concentrations in breast milk and the only one to consider infant outcomes and maternal atopy status. The only other study to report breast milk TSLP concentrations involved 44 women who submitted samples at one of two time points [11]. Whilst they also found that mature milk tended to have lower TSLP concentrations, they reported concentrations which all fell within a relatively narrow range. Our results suggest that there may be a much greater variability between individuals than this small study managed to detect.

One of the limitations of this study is that the breast milk samples were stored between 6 and 8 years before analysis. This has potentially affected the measured concentrations of TSLP and TGF-βs, as has been previously reported for TGF-β_1 [26]. However, there was no difference in the average length of storage between the treatment groups and adjusting for length of storage did not significantly alter the estimated effect of probiotic supplementation on any of the measured cytokines (data not shown). Another limitation is that the time of sample collection was not standardised with respect to time of day and whether fore- or hindmilk was collected. Although diurnal and fore-/hindmilk variation has not been specifically investigated for TSLP and TGF-βs, previous studies suggest that diurnal variation exists in some breast milk cytokines [27] and that the quantity of total protein in the aqueous portion of fore- and hindmilk is reasonably

constant [28]. Whilst these factors may have influenced the cytokine concentrations, samples from the probiotic and placebo groups are presumably equally affected and we do not believe they have significantly affected the conclusions of this study. An underlying uncertainty surrounding the measure of breast milk cytokines is another limitation to this study. Previous studies of breast milk TGF-β and other cytokines have produced widely varying concentration measurements. Whilst some of this variability may be explained by factors such as diurnal and fore-/hindmilk variations, ethnicity, maternal atopy status and time point postpartum, it is likely that sample storage, preparation, analysis methods and inter-laboratory differences play a major role in the measured concentrations. As such, it is difficult to compare results between studies, particularly when these variables are inconsistently reported. In studies comparing two exposure groups, be that probiotic versus placebo or maternal atopy versus no atopy, it is reasonable to assume that technical variability between the groups are minimised. Thus, comparing groups within each study is valid and more generally the relative relationship between these groups can be roughly compared across studies.

Five previous studies have reported breast milk TGF-β_1 and or TGF-β_2 concentrations from similar randomised trials of probiotic supplementation. Each of these studies found a statistically significant effect of probiotics on at least one TGF-β isoform in colostrum samples and no effect on concentrations in mature milk. However, they provide conflicting evidence as to whether probiotics increase [14, 15] or decrease [16–18] colostrum TGF-β concentrations. The current study also found no effect of probiotic supplementation on TGF-β concentrations in mature milk, and provides no evidence to suggest either increased or decreased concentrations in breast milk at 10 days postpartum, a time when the milk is sometimes referred as transition milk before colostrum become mature milk. In addition to the previously mentioned sources of variability, the differing results may also reflect a probiotic strain specific effect as none of these studies used the same probiotic species in the same dose or combinations. In terms of methodological differences, it is interesting to note that the two studies reporting reduced TGF-β_2 after probiotic supplementation also reported the manufacturer recommended acid activation of the breast milk samples prior to measurement which means that the reported concentrations represent the entire pool of TGF-β which is naturally found in both a latent and active form. On the other hand, all three studies reporting higher TGF-β_1 or TGF-β_2 do not mention this activation step in their methods. One of these studies, however, used an alternate activation procedure which is thought to additionally release lipid bound TGF-β [17, 29]. All other studies have removed the lipid fraction prior to cytokine analysis. If the conflicting reports of positive or negative associations with probiotics are a reflection of a common effect of probiotics and sample preparation method, rather than strain specific effects, it may imply that probiotics affect the ratio between active and latent TGF-β in breast milk or the proportion of lipid bound TGF-β. Other factors are known to affect the proportion of active versus latent TGF-β, for example, mothers of premature infants having a higher proportion of latent TGF-β [30].

Conclusions

Whilst maternal probiotic supplementation appears to result in higher breast milk concentrations of TLSP at 10 days postpartum, this does not seem contribute to the preventative effect of the probiotic regime on the development of AD. Probiotics did not significantly alter the breast milk concentration of any TGF-β isoforms at 10 days or 3 months postpartum. Further studies are required to both confirm these findings and investigate the other potential mechanism behind the prevention of AD in infancy by perinatal probiotic supplementation.

Abbreviations

AD: atopic dermatitis; Bb12: *Bifidobacterium animalis* subsp. *lactis* Bb-12; ELISA: enzyme-linked immunospot assay; LGG: *Lactobacillus rhamnosus GG*; NDE: natural direct effect; NIE: natural indirect effect; OR: odds ratio; ProPACT: Probiotics in the Prevention of Allergy among Children in Trondheim; RR: risk ratio; TGF-β: transforming growth factor beta; Th2 cells: type 2 T helper cells; TSLP: thymic stromal lymphopoietin.

Authors' contributions

TØ, OS and RJ designed and directed the implementation of the ProPACT trial during which these breast milk samples were collected. TØ and ØG conceived and designed the breast milk analysis experiments. MS and ADBR analysed the data and wrote the initial manuscript draft. All authors have contributed to and approved the final draft of the manuscript.

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Competing interests

The authors declare they have no competing interests.

Availability of data and materials

The datasets supporting the conclusions of this article will be made available to all interested researchers upon request.

Ethics approval and consent to participate
The ProPACT trial and the collection of breast milk samples for cytokine analysis was approved by the Regional Committee for Medical Research Ethics for Central Norway (Ref. 097-03) and written consent was obtained from the participating families.

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Paper III

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RESEARCH ARTICLE

Human Breast Milk miRNA, Maternal Probiotic Supplementation and Atopic Dermatitis in Offspring

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Data Availability Statement: The filtered and normalised read counts, along with the participant characteristics, used to draw the conclusions presented in this study are available within the article and its additional files. Extensive results from the target gene and functional annotation predictions are also available in the additional files.

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Abstract

Background

Perinatal probiotic ingestion has been shown to prevent atopic dermatitis (AD) in infancy in a number of randomised trials. The Probiotics in the Prevention of Allergy among Children in Trondheim (ProPACT) trial involved a probiotic supplementation regime given solely to mothers in the perinatal period and demonstrated a ~40% relative risk reduction in the cumulative incidence of AD at 2 years of age. However, the mechanisms behind this effect are incompletely understood. Micro-RNAs (miRNA) are abundant in mammalian milk and may influence the developing gastrointestinal and immune systems of newborn infants. The objectives of this study were to describe the miRNA profile of human breast milk, and to investigate breast milk miRNAs as possible mediators of the observed preventative effect of probiotics.

Methods

Small RNA sequencing was conducted on samples collected 3 months postpartum from 54 women participating in the ProPACT trial. Differential expression of miRNA was assessed for the probiotic vs placebo and AD vs non-AD groups. The results were further analysed using functional prediction techniques.

Results

Human breast milk samples contain a relatively stable core group of highly expressed miRNAs, including miR-148a-3p, miR-22-3p, miR-30d-5p, let-7b-5p and miR-200a-3p. Functional analysis of these miRNAs revealed enrichment in a broad range of biological processes and molecular functions. Although several miRNAs were found to be differentially expressed on comparison of the probiotic vs placebo and AD vs non-AD groups, none

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Competing Interests: TØ, OS and MRS participated in seminars sponsored by Tine BA. All other authors declare that they have no conflict of interest. No authors received funds, sponsorship or other financial, professional or personal gain from Siemens Medical Solutions diagnostics AS. The stated competing interests do not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

had an acceptable false discovery rate and their biological significance in the development of AD is not immediately apparent from their predicted functional consequences.

Conclusion

Whilst breast milk miRNAs have the potential to be active in a diverse range of tissues and biological process, individual miRNAs in breast milk 3 months postpartum are unlikely to play a major role in the prevention of atopic dermatitis in infancy by probiotics ingestion in the perinatal period.

Trial Registration

ClinicalTrials.gov NCT00159523

Introduction

Breast milk is first and foremost a source of nutrition for the newborn infant. However, it is well established that breast milk also provides a direct immune defence against pathogens through immune cells and molecules such as immunoglobulin and lysozymes.[1–3] Additionally, observational studies have demonstrated a negative association between breastfeeding and the development of immune related diseases, including type 1 diabetes mellitus, ulcerous colitis and coeliac disease[4, 5]. This suggests that breastfeeding also has long lasting consequences through its early influence on the developing immune system. Allergy related diseases, such as atopic dermatitis (AD), asthma and allergic rhinoconjunctivitis (ARC), are also considered to be a result of an altered immune system development during infancy, however the association between breastfeeding and these diseases remains controversial.[3] Various components of breast milk have been suggested to contribute to its long term immunological effects, including growth factors, cytokines and more recently microRNAs.

MicroRNAs are a group of short, non-coding, RNA molecules (~22 nucleotides) that regulate gene expression at the post-transcriptional level.[6, 7] Extracellular miRNAs have been identified in several body fluids including serum, breast milk, amniotic fluid and urine.[8] These miRNAs are protected from RNase activity through their association with extracellular vesicles and proteins, such as Argonaute-2 (Ago2)[9], and they have come under particular attention because of their potential role in intercellular communication. Compared to other body fluids, breast milk has a large quantity of total-RNA[8], and a high proportion of miRNAs in breast milk are considered to be "immune-related".[8, 10–12] This has led to the hypothesis that breast milk miRNAs are one of the mechanisms that breastfeeding affects the early development of an infant's immune system. In support of the biological plausibility of this hypothesis, *in vitro* studies have demonstrated that breast milk miRNAs are stable under a variety of harsh conditions, including prolonged exposure to acidic solutions simulating the stomach environment[10, 11], and animal studies have suggested that the miRNA expression in milk is correlated with that of serum samples from suckling infants.[13, 14]

The breast milk samples analysed in this study were collected during the Probiotics in the Prevention of Allergy among Children in Trondheim (ProPACT) trial, which demonstrated that perinatal ingestion of probiotics by mothers reduced the cumulative incidence of AD by 40% at 2 years of age.[15] This is a reproducible finding with two meta-analyses demonstrating a beneficial effect of maternal and or infant probiotic supplementation in the primary

prevention of AD.[16, 17] These meta-analyses also indicate that postnatal supplementation is necessary to prevent AD, yet prenatal supplementation appears to strengthen the preventative effect. The biological mechanisms which mediate these effects are incompletely understood. In the current study we investigated the possibility that the prevention of AD is partially mediated by alterations in breast milk miRNAs.

The aim of this study was three-fold: first, to determine the miRNA profile of human breast milk samples in the largest collection of human milk samples to date, second, to examine if this profile is influenced by maternal probiotic supplementation and third, to assess if any changes in the miRNA profile are associated with the development of AD in offspring.

Methods

Participant recruitment

Breast milk samples were collected from women who participated in the ProPACT trial, a placebo controlled, randomised trial investigating the effect of maternal probiotic supplementation on the development of allergic diseases in early childhood as described elsewhere.[15] Briefly, 415 pregnant women living in Trondheim, Norway, and who intended to breastfeed were randomised to receive equivalently tasting study milk containing probiotic bacteria or sterile cultured milk as a placebo from 36 weeks gestation until 3 months postnatal. The probiotic milk contained 5 x 10^{10} colony-forming units (CFUs) of *Lactobacillus rhamnosus GG* (LGG) and *Bifidobacterium animalis* subsp. *lactis* Bb-12 (Bb-12) and 5 x 10^{9} CFU of *Lactobacillus acidophilus* La-5 (La-5) per day. Questionnaires detailing dietary habits, living conditions and symptoms of allergic disease were completed during pregnancy and 6 weeks, 12 months and 2 years postpartum. AD was diagnosed by a paediatrician at the 2 year clinical examination in accordance with the UK Working Party's diagnostic criteria for AD.[18]

To maximise the available information, mother-infant pairs were only eligible to be included in the current study if the child attended the 2 year clinical examination and the following biological samples collected 3 months postpartum were available: breast milk from the mother, a blood sample from the infant and stool samples from both. There were 124 eligible mother-infant pairs, from which 54 breast milk samples were randomly selected according to the criteria in Fig 1.

Breast milk sample collection

Participants were requested to collect breast milk samples at 10 days and 3 months post-partum. Only the 3 month samples were used in this analysis. Samples were collected into a sterile test container and frozen immediately in the participant's home freezer until transportation to the laboratory, where they were stored at -80°C until analysis. Length of storage ranged from 7–9 years. The timing of collection with respect to time of day and phase of lactation, ie whether fore- or hind-milk was collected, was not standardised.

Extracellular vesicle isolation

After thawing on ice, 1.5 mL of whole milk was centrifuged at 2000g for 15 mins at 4°C. The aqueous portion beneath the fat layer was aspirated and centrifuged twice at 16,000g for 40 mins and then 60 mins at 4°C. Subsequently, 500μL of the cell- and debris-free, defatted breast milk obtained after the third centrifugation was mixed with 250μL of ExoQuick Exosome Precipitation Solution™ (System Biosciences, CA, USA) and refrigerated for 12–14 hours at 4°C. The breast milk extracellular vesicles precipitated by this solution were pelleted at 1500g for 30mins at 4°C and resuspended in 100μL of RNAse free water. The breast milk extracellular vesicles solution was used immediately in the RNA isolation process.

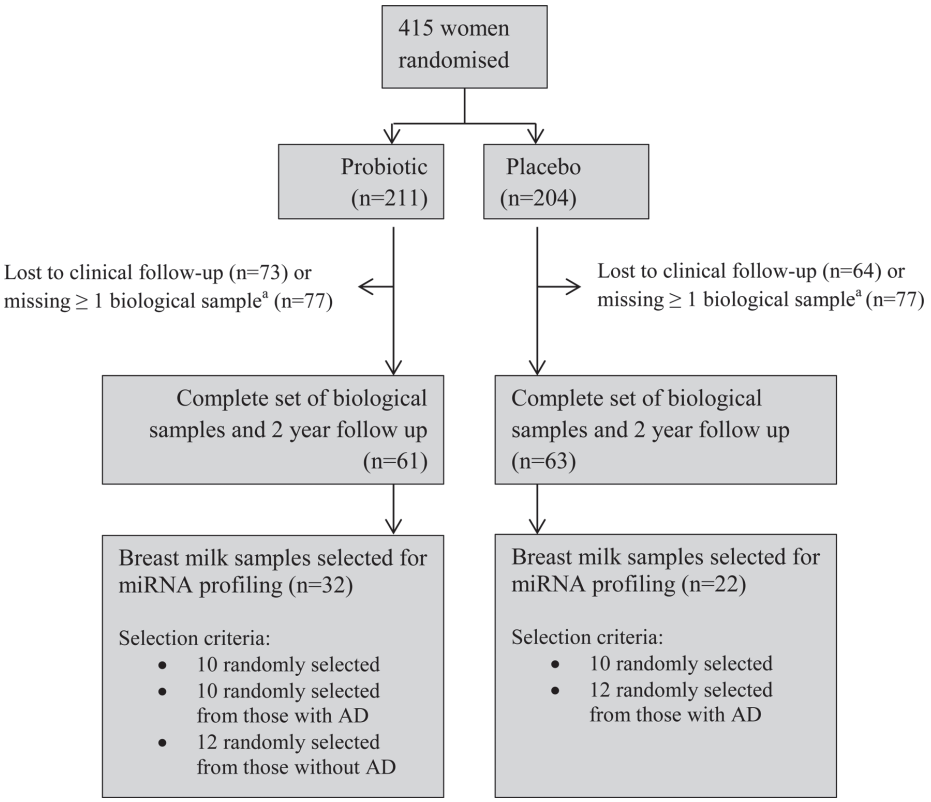

Fig 1. Patient flow and sample selection for ProPACT trial and miRNA sequencing project. [a]Missing 1 or more of the following biological samples collected at 3 months post-partum: breast milk and stool samples from mother, blood and stool samples from the infant.

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RNA isolation and quantification

The breast milk extracellular vesicles were lysed in 500μl QIAzol solution (Qiagen) for 5 mins at room temperature, and RNA isolation was conducted using the Qaigen miRNeasy kit, as per the manufacturer's instructions and without the optional Buffer RWT and second Buffer RPE washing steps. To ensure all RNA fragments were collected, the isolated RNA was eluted twice using 50μl RNase-free each time. The concentration of total RNA was measured using a Nano-Drop 1000 instrument (Thermo Scientific, Wilmington, USA) and selected samples were analysed with Agilent Bioanalyzer 2100 using the Agilent RNA 6000 Nano Kit for total RNA and Small RNA kit for a focused review of the small RNA.

Sequencing and bioinformatics pipeline

Small RNA sequencing was conducted by Ocean Ridge Biosciences (ORB, Palm Beach Gardens, FL). Library construction was performed using the ScriptMiner™ Small RNA-Seq Library Preparation Kit (Epicentre Biotechnologies, Madison, WI) on RNA fragments of 11–28 nucleotides (nt) in length from the total RNA from each sample (for more details see S1 Protocol).

The samples were sequenced using 50-base pairs (bp) single-end reads on the HiSeq 2000 sequencing system (Illumina Inc, San Diego, CA). Raw reads were processed with cutadapt. [19] Low-quality bases (<20) were removed from reads before adapter removal and the final reads were required to have a length of at least 17 bp. Mapping of reads to the human genome was done with STAR version 2.4.0[20], requiring a perfect match alignment. featureCounts version 1.4.0[21] was then used to count every hit of miRNAs in miRBase version 20, and finally a count matrix was produced by local scripting.

Statistical analysis and functional predictions

All statistical analyses were conducted in R version 3.03[22]. miRNA read counts were standardised and assessed for differential expression using the voom[23] and limma[24, 25] packages, respectively. Due to the varying proportions of miRNA in each sample, the mature miRNA reads were normalised to the total number of reads matched to mature miRNAs to create a count per million (cpm) value for each miRNA. Within the limma package, a linear model[25] was fitted to the data based on treatment allocation and development of AD. Subsequently, comparisons were made to assess the marginal effect of probiotic treatment and AD development on the expression levels for each miRNA. Comparisons were limited to miRNAs which had an expression level of $\geq$500 cpm in $\geq$4 samples (n = 125) in order to increase the likelihood of identifying biologically significant differences. The marginal effects of treatment allocation and AD diagnosis were also assessed in an alternate model which included maternal atopy and the presence of older siblings as covariates. A raw p-value of $\leq$0.05 was considered of potential interest and false discovery rate (FDR) was controlled for using the Benjamini-Hochberg method[26] with a FDR of $\leq$0.05 being considered acceptable.

Potential target genes were predicted for the 20 most highly expressed miRNAs and each of the differentially expressed miRNA lists using a locally executed TargetScan version 7.0 algorithm[27–30] with an upper threshold for the context score at -0.2. The lists of unique target genes were subsequently uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7[31, 32] to gain insight into potential functional consequences of these miRNAs through review of the functional annotation clusters and pathways enrichment using DAVID defined defaults for annotation categories. Additional functional annotation analysis was conducted on the subset of genes targeted by the 20 most highly expressed miRNAs and which were identified as up-regulated in epithelial tissue in the "UP_TISSUE" chart under the tissue expression category in DAVID. When few target genes were identified, they were assessed individually using the DAVID annotation table.

Trial registration and ethical approval

The clinical trial and this sequencing study were approved by the Regional Committee for Medical Research Ethics for Central Norway (Ref. 097–03) and written consent was obtained from the participating families. The original trial protocol is registered in ClinicalTrials.gov (identifier NCT00159523). The knowledge and technology required for this miRNA analysis was not available when the ProPACT trial commenced and thus is not described in the protocol.

Results

Participants

Fifty-four mother-infant pairs were included in this analysis, of which 32 had been randomised to receive probiotic milk, and 22 received placebo milk. The baseline characteristics and the clinical outcomes of the mother-infant pairs are provided in Table 1. There was no substantial

Table 1. Baseline characteristics and clinical outcomes for mother-infant pairs.

	Probiotic group (n = 32)	Placebo group (n = 22)
Mother-infant pair baseline characteristics:		
Age, mother (years), mean (SD)	30.51 (4.17)	31.18 (4.44)
Gestational age (days), mean (SD)	283.1 (10.51)	281.2 (14.54)
Birth weight (g), mean (SD)	3581 (402)	3537 (449)
Gender (male), n (%)	18 (56.3)	10 (45.5)
Premature[a], n (%)	0 (0.0)	2 (9.5)[b]
No siblings, n (%)	17 (53.1)	13 (59.1)
Atopy in family, n (%)	19 (59.4)	16 (72.7)[b]
Maternal atopy, n (%)	11 (34.4)	14 (63.6)
Post-randomisation characteristics[b]:		
Breastfeeding		
At least 3 mo., n (%)	31 (96.9)	21 (100)
Duration exclusive, mo., med (range)	4 (0–7)	5 (1–8)
Age of weaning, mo., med (range)	12 (2–24)	11 (4–22)
Sample collection, days (SD)	89.5 (8.1)	93.7 (15.5)
Age of samples, years (SD)	8.4 (0.6)	8.5 (0.6)
Infant clinical outcomes at 2 years of age:		
Atopic dermatitis, n (%)	11 (34.4)	18 (81.8)
IgE associated, n (%)	5 (15.6)	3 (13.6)
Non-IgE associated, n (%)	6 (18.8)	14 (63.6)
Sensitisation, n (%)	7 (21.9)	3 (13.6)
Asthma, n (%)	2 (6.3)	2 (9.1)
Allergic rhinitis, n (%)	1 (3.1)	0 (0.0)

[a]Defined as birth before 37 weeks gestation

[b]missing information for some individuals

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difference between the treatment groups with respect to maternal age, gestational age at birth, birth weight, or the presence of older siblings. The probiotic group had a higher proportion of male infants, a difference also observed in the total ProPACT population[15], and a lower proportion of participants with a family or maternal history of atopy, due to the selection criteria (Fig 1).

All mothers reported compliance with the research milk consumption from birth until 3 months postpartum and all but two were also compliant prenatally. None of the women included in the current study reported consumption of other probiotic supplements or probiotic enriched products.

Small RNA profile of human breast milk

The RNA isolates contained between 12.4 and 247.5 ng/μL of total RNA. Bioanalyzer analysis indicated that there exists significant amounts of RNA up to 1000 nt in length (Fig 2A) and focused analysis of small RNAs revealed concentration peaks at 22–23, 29–30, 33–34, 53–54, 90 and ~140 and 170 nt (Fig 2B). Small RNA sequencing of the 54 breast milk samples resulted in 1,938,162,564 raw reads with median number 34.7 million (range 17.3 M -163 M).

After trimming of low-sequence bases and adapter removal there were 1.3 billion reads left. Approximately 94.8% (1.25 billion) of these reads were perfectly matched to the human genome (hg 19 version) and used in subsequent analysis. Sequences were aligned to several

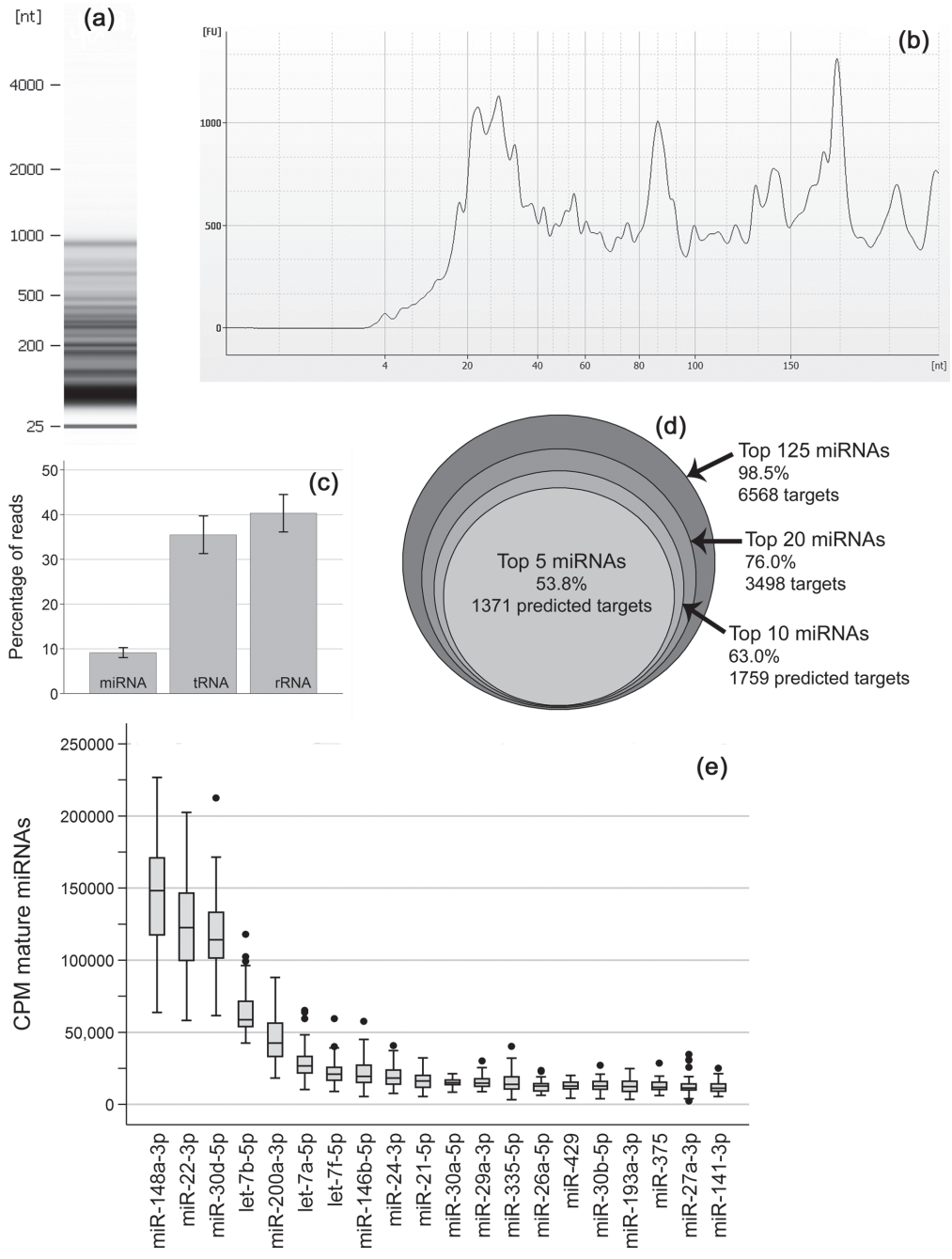
(a)
[nt]
4000
2000
1000
500
200
25
(b)
[FU]
1000
500
0
4
20
40
60
80
100
150
[nt]
(c)
Percentage of reads
50
40
30
20
10
0
miRNA
tRNA
rRNA
(d)
Top 125 miRNAs
98.5%
6568 targets
Top 20 miRNAs
76.0%
3498 targets
Top 5 miRNAs
53.8%
1371 predicted targets
Top 10 miRNAs
63.0%
1759 predicted targets
(e)
CPM mature miRNAs
250000
200000
150000
100000
50,000
0
miR-148a-3p
miR-22-3p
miR-30d-5p
let-7b-5p
miR-200a-3p
let-7a-5p
let-7f-5p
miR-146b-5p
miR-24-3p
miR-21-5p
miR-30a-5p
miR-29a-3p
miR-335-5p
miR-26a-5p
miR-429
miR-30b-5p
miR-193a-3p
miR-375
miR-27a-3p
miR-141-3p

Fig 2. Overview of RNA profile and relative abundance of highly expressed miRNAs from breast milk samples. (A) Bioanalyzer 6000 Nano gel from a representative sample showing abundant short RNAs up to 1000nt; (B) closer review of small RNAs using Agilent's Small RNA kit demonstrating peaks at 22-23nt, 29-30nt, 33-34nt, 53-54nt, 90nt and approximately 140nt and 170nt; (C) bar graph demonstrating average percentage of small RNA sequences aligned to different RNA species with 95% confidence intervals; (D) proportion of reads accounted for by the top 5, 10, 20 and 125 miRNAs along with the number of predicted target genes (excluding repeated target prediction for alternate transcripts of the same gene); (E) boxplot of counts per million (CPM) mature miRNA of the 20 most abundant miRNAs in the 54 samples.

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different RNA species including ribosomal RNA (rRNA), transfer RNA (tRNA), mature miRNA, and other RNAs (Fig 2C). There was no association between the proportion of total reads aligned to mature miRNA sequences and treatment group allocation or AD development in the children (data not shown).

miRNA profile of human breast milk and functional predictions

The average proportion of reads aligned to mature miRNA species was 9.1% (SD 3.82%, range 2.75–20.16%). The top 5 miRNAs were consistently highly expressed and included miR-148a-3p, miR-22-3p, miR-30d-5p, let-7b-5p and miR-200a-3p. These miRNAs accounted for 54% of all mature miRNAs (Fig 2D and 2E and S1 File). The miRNAs ranked 6^{th} to 20^{th} by overall mean expression showed a greater variability in individual ranking. Using these 20 most abundant miRNAs, 3498 unique potential gene targets were identified with the TargetScan algorithm. Functional annotation clusters of these predicted targets indicate that the breast milk miRNAs may have the greatest effect on a) the positive and negative regulation of metabolic processes involving nitrogen compounds, RNA, DNA and macromolecules and the positive regulation of transcription and gene expression, b) embryonic development, c) angiogenesis, d) catabolic processes and e) cell migration and localisation. The full results of the functional annotation clustering analysis, gene ontology and pathway analysis conducted in DAVID is provided in S2 File.

To date, it is unknown if, and in which tissues, breast milk miRNAs are active after ingestion. Based on tissues identified by DAVID to have upregulated expression of the predicted target genes, these miRNAs as a group have the potential to be most biologically influential in the brain (FDR = 3.32×10^{-31}) and epithelium (FDR = 5.01×10^{-12}). Other tissues where this gene set is highly expressed include female reproductive tissues, such as placenta, uterus and endometrium, foetal tissues, such as the foetal brain, kidney and lung, and haemopoetically involved tissues, such as platelets, T-cells, bone marrow, fibroblasts, and the thalamus (UP_TISSUE sheet in S2 File).

Functional clustering analysis conducted on the subset of predicated target genes identified as highly expressed in epithelial cells revealed that genes with activities located in the non-membrane-bounded organelles, cytoskeleton and nucleus were significantly enriched. The most highly enriched biological processes and molecular functions included chromatin and chromosome organisation, transcription and negative regulation of gene expression and biosynthetic processes (S3 File). The breast milk miRNA profile may also represent a beneficial collection of miRNAs for mammary gland development and or maintenance during lactation and in this context the mammary gland epithelium may also be a site of action. Healthy mammary tissues were otherwise not identified as being highly enriched with the potential target genes (UP_TISSUE sheet in S2 File).

miRNA profiles associated with maternal probiotic ingestion

Maternal probiotic supplementation was associated with upregulation of let-7d-3p and downregulation of miR-574-3p, miR-340-5p and miR-218-5p, although the estimated FDR for each

Table 2. Differentially expressed miRNAs.

miRNA	Fold change	p-value	FDR
Probiotic vs Placebo			
miR-574-3p	0.640	0.016	0.818
miR-340-5p	0.697	0.040	0.818
let-7d-3p	1.401	0.044	0.818
miR-218-5p	0.690	0.050	0.818
Atopic dermatitis vs no-atopic dermatitis			
miR-452-5p	0.660	0.001	0.107
let-7d-3p	1.615	0.005	0.308
miR-146b-5p	0.674	0.011	0.433
miR-21-5p	0.752	0.016	0.433
miR-22-3p	1.258	0.019	0.433
miR-375	1.247	0.023	0.433
miR-16-5p	0.686	0.026	0.433
miR-511-5p	1.323	0.028	0.433
miR-26b-5p	0.808	0.041	0.461
let-7f-5p	0.802	0.041	0.461
miR-30e-5p	0.844	0.042	0.461
miR-374a-5p	0.797	0.044	0.461
miR-335-5p	1.343	0.049	0.468

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is 0.818 (Table 2). Furthermore, these miRNAs showed relatively low levels of expression constituting 0.03 to 0.2% of mature miRNAs on average. Together they had 495 predicted target genes. Functional annotation clustering revealed no cluster directly involved with atopic dermatitis, asthma or allergic rhinoconjunctivitis (S4 File). On review of individual targets, the suppressor of cytokine signalling 3 (*SOCS3*) gene was found to be associated with atopic dermatitis in the Online Mendelian Inheritance in Man (OMIM) database. Other identified targets which may be related to the development of allergy related disease through their association with T cell differentiation and activation or transforming growth factor β (TGF-β) are listed in S4 File. These results were unaltered when maternal atopy and presence of siblings were included as covariates (data not shown).

miRNA profiles associated with infant atopic dermatitis

The development of AD by 2 years of age was associated with differential expression of several miRNAs (Table 2). None of these miRNAs had an acceptable FDR and were unaffected by the inclusion of additional covariates. However, a number of them are relatively highly expressed including miR-146b-5p, miR-21-5p, miR-22-3p, miR-375 and let-7f-5p. Functional analysis of the 2269 predicted target genes indicated that these genes were enriched in a diverse range of functional clusters from embryonic development to positive and negative regulation of metabolic processes involving RNA, macromolecules and of transcription and gene expression (S5 File). These functional clusters were particularly related to predicted targets of down-regulated breast milk miRNAs.

Discussion

In this study we present the miRNA sequencing results from breast milk samples collected 3 months postpartum from 32 women who received probiotic milk and 22 who received placebo

milk during the ProPACT trial.[15] Considering first the general miRNA profile of these samples, we found a relatively stable core group of highly expressed miRNAs which are predicted to have a wide range of potential biological effects. We also investigated the effect of maternal probiotic ingestion on the relative abundance of breast milk miRNAs and the association of individual miRNAs with the development of AD. On both of these accounts we observed no conclusive evidence of differentially expressed miRNAs. As such, individual breast milk miRNAs at 3 months postpartum are unlikely to play a major role in the mechanism behind the observed preventative effect of perinatal probiotics on AD.[15] Before further discussing the main findings, we first highlight the strengths and limitations of this study.

The major strength of this study is that it is the largest sequencing analysis of human milk small RNAs to date, allowing characterisation of the general profile of breast milk miRNAs at 3 months postpartum. In using a single collection time point we did not need to consider the temporal variation in breast milk miRNA[10, 13, 33, 34]. We were also able to investigate the relationship between miRNA expression levels and maternal probiotic ingestion and the development of AD in offspring because of the underlying RCT design. For this purpose, we acknowledge that one of the weaknesses of this study is that the sample size only allows us to detect large differences in expression levels. Furthermore, the sample selection criteria favoured the inclusion of mother-infant pairs where the infant had developed AD and there was a maternal or familial history of allergy related disease, particularly in the placebo group. We fitted an alternate model, which included maternal atopy and siblings as covariates, to investigate if the selection procedure had affected the results. The miRNAs identified as differentially expressed in association with treatment allocation and AD development were unchanged in this alternate model. Information regarding maternal antibiotic use was not recorded, however we do not believe that the lack of this information has substantially affected our conclusions.

Other weaknesses and methodological limitations of this study include the age of the samples, extracellular vesicle isolation, and functional analysis methods. The breast milk samples had been stored for between 7 and 9 years prior to analysis and, although breast milk miRNAs are reported to be stable, it is unclear whether prolonged storage alters the general miRNA profile of human milk. Reassuringly, we found no relationship between the amount of RNA isolated and the age of the samples (data not shown). Recent methodological papers suggest that our extracellular vesicle isolation method would have maximised the quantity of RNA isolated [35], but in doing so has captured protein-miRNA complexes which are both extracellular and extravesicular[35], as well as extracellular vesicles released by lysed maternal cells upon freezing.[36] The current article is primarily interested in the biologically available miRNAs in human breast milk and we consider our isolation procedure to be a reasonable approach given infants ingest breast milk in its entirety, extracellular vesicles, protein-miRNA complexes and maternal cells included. In support of this thinking, Gu et al[13] demonstrated relationships between several porcine milk miRNAs and their serum levels in suckling piglets using near identical preparation methods on previously frozen samples. Whilst Gu et al's findings imply that milk miRNAs are biologically available after ingestion, the investigation of the functional consequences of milk miRNAs is complicated and the available functional prediction tools have certain limitations. Specifically, gene targets are predicted rather than validated and no available tools simultaneously account for the relative abundance of the miRNAs, the number of miRNA-target interactions for any given miRNA or target, and the downstream effects of these interactions. We consider our functional annotation results to be speculative. All the same, evidence from experimental and animal studies suggests that breast milk miRNAs are stable[10, 11] and biologically active after ingestion[13, 14], which opens up the possibility that they may be involved in maternal guidance of the developing immune system and gastrointestinal tract.

The five most abundant miRNAs identified in the breast milk samples were miR-148a-3p, miR-22-3p, miR-30d-5p, let-7b-5p and miR-200a-3p. These 5 miRNAs were consistently highly expressed in all samples whilst other miRNAs showed greater variability in their relative ranking and are perhaps more influenced by individual characteristics, such as genetics, age, parity, diet or other environmental factors. Previous studies of human milk miRNA have also observed that a few highly expressed miRNAs are responsible for the majority of miRNA counts[11, 12], yet the list over the top 10 or 20 miRNAs varies between studies (Table 3).[8, 10–12] It would appear that the miRNA profile is particularly influenced by the miRNA quantification method, and may also depend on the milk fraction used for RNA isolation, time postpartum, ethnicity of the women and the dietary or environmental exposures of their cultures, and the bioinformatics pipeline used for filtering and alignment. Notably, the two sequencing analyses by Zhou et al[11] and Munch et al[12] showed the greatest similarity to the profile we observed despite the varying ethnic backgrounds. This would suggest that our results are generalisable beyond the predominantly Caucasian population included in the ProPACT study. Interestingly, the study by Munch et al[12] isolated miRNA from the lipid fraction of breast milk, yet observed a very similar miRNA profile to the extracellular vesicle enriched milk samples of the current study and Zhou et al[11]. The biological significance of this observation is uncertain, however it may suggest that even if miRNAs are preferentially selected into breast milk, their inclusion into extracellular vesicles or association with the lipid fraction may be less important for their biological actions.

Table 3. Comparison of timing, methods and results of the current study and previous studies of human breast milk miRNA.

Author (year)	n	Time postpartum	Method miRNA quantification	Breast milk portion	Comments and most highly expressed miRNAs[a]
Current study	54	3 mths	Illumina RNA seq, 50bp single-end reads	EV enrichment using Exoquick	Norwegian women participating in RCT investigating probiotics in prevention of allergy related diseases. Top 10 miRNAs: **miR-148a-3p, miR-22-3p, miR-30d-5p, let-7b-5p, miR-200a-3p, let-7a-5p, let-7f-5p, miR-146b-5p, miR-24-3p, miR-21-5p**
Zhou (2011)	4	60 days	Illumina RNA seq, 36bp single-end reads	EV enrichment using Exoquick	Chinese women. Top 10 miRNAs: **miR-148a-3p, miR-30b-5p, let-7f-5p, miR-146b-5p, miR-29a-3p, let-7a-5p, miR-141-3p**, miR-182-5p, **miR-200a-3p**, miR-378-3p.
Munch (2013)	3	6–12 weeks	Illumina RNA seq, 36bp single-end reads	Lipid fraction	Two (2) Breast milk samples were sequenced from 3 American of varying ethnic backgrounds. Study participants underwent pharmacological stimulation of breast milk production and submitted multiple samples at 3 hourly intervals. Top 10 miRNAs[b]: **mir-148a-3p, let-7a-5p,** mir-200c-3p, **mir-146b-5p**, **let-7f-5p**, **mir-30d-5p**, mir-103a-3p, let-7b-5p, let-7g-5p, **mir-21-5p**.
Kosaka (2010)	8	2–11 mths	MicroRNA microarray (Agilent)	Defatted, cell and debris free milk	Eight (8) Japanese women submitted up to 4 samples at varying time points. No quantitative results published beyond those for miRNA considered to be "immune related". Reported immune related miRNA with high expression[c]: miR-92a-3p, miR-155-5p, miR-181a-5p, miR-181b-5p, let-7i-5p, **miR-146b-5p**, miR-223-3p, miR-17-5p
Weber (2010)	5	Not reported	miScript Assay (incl. 714 miRNA produced by Qaigen)	Defatted, cell and debris free milk	Commercially available samples from 5 "healthy" women. Ethnicity and timing of sample collection is unspecified. Top 10 miRNAs[d]: miR-335-3p, miR-26a-2-3p, miR-181d-5p, miR-509-5p, miR-524-5p, miR-137, miR-26a-1-3p, miR-595, miR-580-3p, miR-130a-3p

EV: extracellular vesicles

[a]miRNAs in bold type were also found within the top 20 miRNAs of the current experiment

[b]miRNA names are converted to miRBase version 21.0 annotation version 16.0

[c]miRNA names converted from unspecified earlier version

[d]miRNA names converted from miRBase version 13.0. The miRNA names supplied in the study by Zhou et al (2011) required no conversion.

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The functional analysis of the breast milk miRNA profile revealed that the highly expressed miRNAs are potentially involved in the regulation of genes in many transcriptional, metabolic and biosynthetic processes. The ultimate effect of the cooperative regulation by breast milk miRNAs is difficult to decipher from this analysis. We have considered the intestinal epithelium to be the most likely first site of action in the infant and in support of this theory epithelial tissues were identified as having a gene enrichment profile that significantly overlaps with the predicted gene targets for the top 20 breast milk miRNAs. Following the birth of a healthy term infant, the intestinal tract matures resulting in a less permeable epithelium.[37] The early establishment of a well-functioning intestinal barrier is thought to be integral in the normal development of the immune system and a defective intestinal barrier in early infancy has been linked to several infant gastrointestinal diseases and a predisposition to autoimmune and inflammatory diseases later in life, including atopic dermatitis.[37] Breast milk appears to assist this maturation process through growth factors, hormones and cytokines, and studies have shown breastfeeding to be associated with reduced intestinal permeability[37, 38], morphological maturation[39, 40] and altered intestinal gene expression[41] in the early neonatal period. Additionally, the intestinal microbiota composition and diversity is also considered to be involved in promoting intestinal maturity and breast milk is thought to promote a "healthy" microbiota by transferring microbes, prebiotic milk oligosaccharides and by encouraging an anti-inflammatory, environment allowing microbial tolerance.[38, 42] We propose that breast milk miRNAs may be another factor contributing to intestinal maturation and microbiome establishment. Experimental evidence in support of this theory comes from mouse studies which have demonstrated improved epithelial barrier function associated with miR-146b[43], involvement of miR-375 in epithelium-immune system crosstalk[44] and promotion of innate immune tolerance in the neonatal period by miR-146a[45]. Although miR-146a was not highly expressed in breast milk samples, the more highly expressed miR-146b also targets interleukin 1 receptor associated kinase 1 (*IRAK1*), a Toll-like receptor (TLR) signalling molecule thought to be involved in the miR-146a mediated promotion of immune tolerance. Unfortunately, it is difficult to isolate the effects of breast milk miRNAs from other biologically active components of breast milk. In a pig experiment, Hu et al[40] found that weaning was associated with increased permeability and activation of mitogen-activated protein kinases (MAPK) in piglets, altered morphology and increased permeability. Interestingly, the analysis of gene targets of highly expressed breast milk miRNAs revealed enrichment of the MAPK signalling pathway. The results of Hu et al[40] would be consistent with a loss of a breast milk miRNA mediated down regulation of these pathways after weaning. On the other hand, gene expression analysis of intestinal epithelial cells from breastfed and formula fed human infants reveals that target genes of the top 20 miRNA are both up and down regulated in the breast fed infants[41], indicating that the biological consequences of breast milk miRNAs are either minimal or substantially more complex.

Looking to other mammalian species, there are similarities in the milk miRNA profile of pigs[13, 46], cows[47], rats[48] and tammar wallabies[33]. In particular, miR-148a, miR-30a, let-7a, let-7b and let-7f are reported to be expressed in moderate to high quantities across these species. The conservation of these miRNA, not only in their structural nature, but in their inclusion in breast milk over several mammalian species, implies that they are evolutionarily selected and have beneficial roles for the mother and or her offspring. With this in mind, it is perhaps not surprising that a four month dietary intervention did not convincingly affect the breast milk miRNA profile.

A number of differentially expressed miRNAs were identified on comparison between the probiotic and placebo group and between samples from mothers whose children did and did not develop AD. None of these miRNAs had an acceptable FDR after controlling for multiple

comparisons. Maternal probiotic ingestion was associated with four differentially expressed miRNAs with low abundance (miR-574-3p, let-7d-3p, miR-340-5p and miR-218-5p). As such, we found no conclusive evidence to suggest that maternal probiotic ingestion significantly alters the relative abundance of individual miRNAs and is therefore not a major mechanism by which the protective effect of probiotics is conveyed to the newborn infant. On the other hand, a number of breast milk miRNAs found to be associated with the development of atopic dermatitis were the relatively highly expressed miRNAs: miR-22-3p, miR-146b-5p, miR-21-5p, miR-375 and let-7f-5p. Curiously, the upregulation of let-7d-3p and miR-375 and downregulation of miR-21-3p and miR-146b-5p are opposite to what one would have expected based on a previously published review of miRNAs in allergic diseases[49]. It is unclear what the biological significance this observation has in terms of the impact of breast milk miRNAs and the development of allergic disease.

The functional annotation results are speculative and further research will need to investigate the biological availability and function of these miRNAs. It will be particularly useful to establish models to assess the cooperative gene regulation of breast milk miRNAs, and other non-coding RNAs, as a group. Such experiments and bioinformatics models may shed light on the biologically plausibility of a relationship between AD and the miRNAs differentially expressed in association with probiotic ingestion, something which is doubtful from this analysis. This study sequenced samples taken 3 months postpartum, at the end of the intervention period, however colostrum samples taken in the first 10–14 days postpartum may reveal a different profile with greater influence on the development of AD. Another line of future enquiry is to characterise and investigate the function of other short non-coding RNAs, such as fragmented tRNAs, which have also been reported in other extracellular vesicles isolated from other body fluids.[50]

In conclusion, there appears to be a stable group of core breast milk miRNAs, which are at least partially conserved across a number of mammalian species. The biological functions of this, presumably evolutionarily-driven, collection of miRNAs is uncertain. Functional analysis of the potential target genes of highly expressed breast milk miRNAs revealed enrichment in a broad range of biological processes and molecular functions. Although several miRNAs were found to be differentially expressed on comparison of the probiotic and placebo groups and AD vs non-AD, none had an acceptable FDR and their biological significance on the development of AD is not immediately apparent from functional analysis. Future experimental and bioinformatics techniques should investigate the biological consequences of highly and differentially expressed miRNAs as a group.

Supporting Information

S1 Checklist. CONSORT 2010 Checklist. Consolidated Stardards of Reporting Trials checklist.
(DOC)

S1 File. Expressed miRNA. Read processing and miRNA expression information
(XLSX)

S2 File. Top20 DAVID results. Highly Expressed breast milk miRNAs: Functional analysis for predicted targets of top 20 miRNA
(XLSX)

S3 File. Epithelium DAVID results. Functional analysis results of epithelial related genes targeted by highly expressed miRNAs.
(XLSX)

S4 File. Probiotic vs placebo DAVID results. Probiotic vs placebo: Functional analysis for differentially expressed breast milk miRNAs
(XLSX)

S5 File. AD vs nAD DAVID results. Atopic dermatitis versus no atopic dermatitis: Functional analysis for differentially expressed breast milk miRNAs.
(XLSX)

S1 Protocol. Supplementary methods. Additional details for methods.
(DOC)

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Author Contributions

Conceived and designed the experiments: GB TØ MRS. Performed the experiments: GB MRS. Analyzed the data: JJ PS MRS. Wrote the paper: MRS. Designed and directed the implementation of the ProPACT trial during which these breast milk samples were collected: TØ OS RJ.

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Appendices

Appendix I

Lifestyle questions asked during pregnancy, 6 weeks, 1 year and 2 years that are relevant to Papers I – III presented in this thesis.

	Pregnancy	6 weeks	1 year	2 years
How many children do you and the child's father have between you? (Include biological and step children, but not foster children or adoptive children)	✓	✓	✓	✓
Have you, the child's father or any of your children ever had asthma, eczema or eye/nose allergy (allergic rhinoconjunctivitis)? The associated check boxes indicated which family member had suffered from allergy related disease and was used to identify allergic mothers. For each family member, the subsequent questions detailed the age of diagnosis for asthma, symptoms of and medication use for asthma / eczema / allergic rhinoconjunctivitis over the past 12 months. This detailed information was not used in the papers presented in this thesis.	✓	✓	✓	✓
Do you or your partner currently smoke? The associated check boxes indicated if the person completing the questionnaire (presumed to be the mother) or their partner smoked.	✓	✓	✓	✓
Which of the follow household pets do you live with? Check boxes included: dog, cat, other furred animal (guinea pig, rabbit, etc.), bird, other animal or no animal.	✓	✓	✓	✓
Childs birthweight? (gm)		✓	✓	✓

The questionnaires completed during pregnancy and at 6 weeks, 1 year and 2 years included detailed questions about the living environment, diet of mother and child, vaccination of the child, illness during pregnancy and common illnesses for the children.

Appendix II

Diagnostic criteria for atopic dermatitis in children under 2 years of age:

Must have:

A skin condition that the child scratches or rubs.

Plus 3 or more of:

i. History of flexural involvement of arms and / or legs
ii. History of atopic disease (asthma, allergy or atopic dermatitis) in a first degree relative (parents or siblings)
iii. History of generally dry skin
iv. Visible dermatitis on cheeks and / or flexural surfaces of arms or legs and absence of rash in the axilla.

Translated from the form completed by the examining pediatrician. The Norwegian version is available in Dr. Christian Dotterud's PhD Thesis (Doctoral theses at NTNU,2016:151).

This criterion was modified from the UK Working Party diagnostic criteria for atopic dermatitis according to their recommendations for children under 4 years of age. (Reference: Willians HC, Burney PG et al. The UK Working Party's diagnostic criteria for atopic dermatitis. I. Derivation of a minimum set of discriminators for atopic dermatitis. *Br. J. Dermatology.* 1994. 131(3):383-396.)

Appendix III:

Individual miRNAs in fresh samples versus samples frozen as whole milk.

Table S1: Total count per million, fold change, p-value and FDR. Rows in bold indicate miRNAs that were identified in the top 20 in Paper III.

miRNA	Fresh (cpm)	Frozen as whole milk (cpm)	Log fold change	p-value	FDR
hsa-miR-148a-3p	**740683**	**1705430**	**1.191**	**6.80E-10**	**1.39E-07**
hsa-miR-125a-5p	90209	19499	-2.273	1.99E-09	1.92E-07
hsa-let-7d-3p	7044	2164	-2.031	2.82E-09	1.92E-07
hsa-let-7b-5p	**376198**	**182487**	**-1.146**	**3.21E-08**	**1.64E-06**
hsa-miR-375	**164783**	**73241**	**-1.218**	**1.49E-07**	**4.65E-06**
hsa-miR-200c-3p	277153	103110	-1.435	1.52E-07	4.65E-06
hsa-miR-19b-3p	4512	21167	2.388	1.81E-07	4.65E-06
hsa-miR-29c-3p	3147	9203	1.712	1.82E-07	4.65E-06
hsa-miR-19a-3p	500	1954	2.268	2.25E-07	5.10E-06
hsa-miR-140-3p	10783	20902	0.960	2.59E-07	5.27E-06
hsa-miR-125b-5p	71580	23833	-1.670	1.00E-06	1.86E-05
hsa-miR-193a-3p	**909**	**3846**	**2.332**	**2.20E-06**	**3.52E-05**
hsa-miR-452-3p	1183	3723	1.541	2.25E-06	3.52E-05
hsa-miR-106b-5p	4865	11685	1.328	3.68E-06	5.37E-05
hsa-miR-378c	5702	17054	1.857	5.10E-06	6.93E-05
hsa-miR-30a-3p	17238	32200	0.905	6.55E-06	8.19E-05
hsa-miR-193b-5p	2826	1027	-2.065	6.83E-06	8.19E-05
hsa-miR-193b-3p	14643	34398	1.382	1.47E-05	1.62E-04
hsa-miR-7704	3850	1094	-1.960	1.51E-05	1.62E-04
hsa-miR-148b-3p	23196	43130	0.905	1.74E-05	1.73E-04
hsa-miR-378d	1005	3103	1.883	1.83E-05	1.73E-04
hsa-miR-101-3p	30755	85451	1.595	1.88E-05	1.73E-04
hsa-miR-361-3p	24057	12579	-0.917	1.95E-05	1.73E-04
hsa-miR-3607-3p	699	112	-2.173	3.21E-05	2.73E-04
hsa-miR-130b-5p	1982	719	-1.566	3.40E-05	2.75E-04
hsa-miR-193a-5p	40993	16913	-1.436	3.59E-05	2.75E-04
hsa-miR-452-5p	5480	12028	1.228	3.64E-05	2.75E-04
hsa-miR-29a-3p	**51768**	**95766**	**0.911**	**9.21E-05**	**0.001**
hsa-let-7e-5p	7333	1395	-2.203	9.51E-05	0.001
hsa-miR-3178	1725	510	-3.236	9.79E-05	0.001
hsa-miR-1307-5p	593	1825	1.810	1.07E-04	0.001
hsa-miR-151a-5p	36443	19735	-0.856	1.13E-04	0.001
hsa-miR-30d-5p	**484712**	**320696**	**-0.648**	**1.30E-04**	**0.001**
hsa-miR-200b-5p	10329	5699	-0.892	1.31E-04	0.001
hsa-miR-28-5p	6157	3514	-0.825	1.98E-04	0.001

Table S1: continued

miRNA	Fresh (cpm)	Frozen as whole milk (cpm)	Log fold change	p-value	FDR
hsa-miR-197-3p	1385	3079	1.767	2.16E-04	0.001
hsa-miR-6510-3p	1061	234	-2.351	2.42E-04	0.001
hsa-miR-23b-3p	24258	46130	0.950	3.30E-04	0.002
hsa-miR-92a-3p	179343	122484	-0.551	4.17E-04	0.002
hsa-miR-152-3p	4787	8104	0.857	4.19E-04	0.002
hsa-miR-23a-3p	60476	107193	0.868	4.62E-04	0.002
hsa-miR-200b-3p	128375	46527	-1.473	4.71E-04	0.002
hsa-miR-22-3p	**167173**	**325413**	**1.024**	**0.001**	**0.003**
hsa-miR-96-5p	879	2203	1.395	0.001	0.003
hsa-miR-423-5p	157896	77115	-1.261	0.001	0.003
hsa-miR-30c-5p	78674	51658	-0.604	0.001	0.003
hsa-miR-103a-3p	61592	32026	-0.819	0.001	0.003
hsa-miR-141-3p	**37763**	**81065**	**1.102**	**0.001**	**0.003**
hsa-miR-378a-3p	28362	59070	1.242	0.001	0.003
hsa-miR-2110	1724	940	-1.341	0.001	0.003
hsa-miR-99b-3p	2342	4315	0.816	0.001	0.004
hsa-miR-320a	328110	168321	-1.181	0.001	0.004
hsa-miR-3196	1032	165	-2.880	0.001	0.004
hsa-miR-511-5p	5068	3047	-0.762	0.001	0.004
hsa-miR-497-5p	1400	2959	1.394	0.001	0.005
hsa-miR-146b-3p	14627	7147	-1.048	0.002	0.006
hsa-miR-185-5p	1147	3044	1.297	0.002	0.006
hsa-miR-30b-5p	**33541**	**54310**	**0.749**	**0.002**	**0.006**
hsa-miR-192-5p	1051	1863	0.814	0.002	0.008
hsa-miR-24-3p	**43119**	**74781**	**0.772**	**0.002**	**0.008**
hsa-miR-4449	966	342	-2.095	0.003	0.009
hsa-let-7c-5p	40001	21676	-0.795	0.003	0.009
hsa-miR-224-5p	22809	14732	-0.703	0.003	0.009
hsa-miR-4488	2606	882	-2.714	0.003	0.010
hsa-miR-339-3p	2779	4602	0.861	0.004	0.012
hsa-miR-4516	5795	894	-3.084	0.004	0.013
hsa-miR-1301-3p	1366	677	-0.925	0.004	0.013
hsa-miR-30c-2-3p	831	577	-0.816	0.005	0.014
hsa-miR-181a-2-3p	4750	2900	-0.622	0.005	0.014
hsa-miR-3615	3818	2176	-1.210	0.005	0.015
hsa-miR-877-5p	534	206	-1.224	0.007	0.020
hsa-miR-425-5p	27810	15708	-0.778	0.008	0.024
hsa-miR-335-5p	**82404**	**145627**	**0.849**	**0.012**	**0.033**
hsa-miR-4492	761	213	-2.233	0.012	0.033
hsa-miR-146a-5p	72934	127096	0.787	0.012	0.033

Table S1: continued

miRNA	Fresh (cpm)	Frozen as whole milk (cpm)	Log fold change	p-value	FDR
hsa-miR-574-3p	9547	14533	0.697	0.013	0.033
hsa-miR-17-5p	1076	1689	0.786	0.013	0.033
hsa-miR-30d-3p	385	715	0.988	0.015	0.039
hsa-let-7a-5p	**200294**	**73592**	**-1.111**	**0.015**	**0.039**
hsa-miR-29b-3p	561	953	0.883	0.015	0.039
hsa-miR-199a-3p	812	1543	0.765	0.018	0.044
hsa-miR-199b-3p	812	1543	0.765	0.018	0.044
hsa-miR-181c-5p	505	887	1.162	0.018	0.044
hsa-miR-183-5p	15663	22331	0.533	0.018	0.044
hsa-miR-4443	1734	1353	-1.132	0.018	0.044
hsa-miR-25-5p	343	62	-1.674	0.019	0.046
hsa-miR-99b-5p	81481	58656	-0.455	0.020	0.047
hsa-miR-15b-5p	7632	10390	0.530	0.022	0.050
hsa-miR-340-5p	1749	4635	1.168	0.022	0.050
hsa-miR-26a-5p	**185526**	**105773**	**-0.732**	**0.022**	**0.050**
hsa-miR-660-5p	1872	2641	0.533	0.023	0.051
hsa-miR-107	5456	6821	0.478	0.025	0.056
hsa-miR-671-3p	757	434	-1.000	0.026	0.057
hsa-miR-26b-5p	14700	7295	-1.268	0.027	0.057
hsa-let-7f-5p	**84624**	**16760**	**-1.658**	**0.027**	**0.057**
hsa-miR-320e	1244	1722	1.264	0.032	0.069
hsa-miR-148a-5p	15545	22628	0.532	0.034	0.071
hsa-miR-191-5p	188969	124716	-0.551	0.035	0.074
hsa-miR-3653-3p	385	134	-1.444	0.037	0.075
hsa-let-7d-5p	20616	12352	-0.618	0.045	0.091
hsa-miR-7706	437	133	-1.287	0.045	0.092
hsa-miR-132-3p	521	386	-0.878	0.046	0.092
hsa-miR-27a-3p	**13260**	**18964**	**0.507**	**0.047**	**0.092**
hsa-miR-23b-5p	457	260	-1.216	0.047	0.092
hsa-miR-500a-3p	3550	4725	0.484	0.049	0.095
hsa-miR-128-3p	6951	4825	-0.420	0.051	0.099

Appendix IV:

Individual miRNAs in fresh samples versus samples centrifuged prior to freezing.

Table S2: Total count per million, fold change, p-value and FDR. Rows in bold indicate miRNAs that were identified in the top 20 in Paper III.

miRNA	Fresh (cpm)	Centrifuged then frozen (cpm)	Log fold change	p-value	FDR
hsa-miR-200c-3p	277153	142555	1.007	5.33E-05	0.004
hsa-miR-502-3p	4175	8298	-1.020	5.51E-05	0.004
hsa-miR-378d	1005	3054	-1.942	1.93E-05	0.004
hsa-miR-378c	5702	13187	-1.466	1.44E-04	0.005
hsa-miR-500a-3p	3550	7164	-1.079	9.62E-05	0.005
hsa-miR-1287-5p	1068	2217	-1.188	1.39E-04	0.005
hsa-miR-26a-5p	**185526**	**79124**	**1.341**	**2.63E-04**	**0.006**
hsa-miR-200b-3p	128375	50145	1.614	2.27E-04	0.006
hsa-let-7d-5p	20616	8340	1.304	2.20E-04	0.006
hsa-miR-185-5p	1147	3475	-1.558	3.64E-04	0.006
hsa-miR-3607-3p	699	161	1.983	3.41E-04	0.006
hsa-miR-1307-5p	593	1740	-1.717	3.46E-04	0.006
hsa-miR-19a-3p	500	1020	-1.398	0.001	0.010
hsa-miR-22-3p	**167173**	**315081**	**-1.024**	**0.001**	**0.011**
hsa-let-7i-5p	32616	12516	1.524	0.001	0.011
hsa-miR-182-5p	32098	11336	1.402	0.001	0.011
hsa-miR-19b-3p	4512	10969	-1.332	0.001	0.011
hsa-let-7a-5p	**200294**	**56280**	**1.585**	**0.001**	**0.013**
hsa-miR-186-5p	95471	174667	-0.986	0.001	0.013
hsa-miR-365a-3p	6014	2446	1.595	0.001	0.013
hsa-miR-365b-3p	6014	2446	1.595	0.001	0.013
hsa-miR-20a-5p	1209	403	1.698	0.001	0.013
hsa-miR-135a-5p	876	200	1.966	0.002	0.015
hsa-let-7c-5p	40001	21659	0.826	0.002	0.018
hsa-miR-378a-3p	28362	55761	-1.142	0.002	0.018
hsa-miR-874-3p	621	1077	-1.608	0.002	0.018
hsa-let-7g-5p	105271	39835	1.274	0.005	0.030
hsa-miR-26b-5p	14700	4172	1.896	0.004	0.030
hsa-miR-345-5p	5097	7874	-0.799	0.005	0.030
hsa-miR-29c-3p	3147	4958	-0.793	0.005	0.030
hsa-miR-499a-5p	2738	1057	1.559	0.005	0.030
hsa-miR-99b-3p	2342	3793	-0.719	0.004	0.030
hsa-miR-193a-3p	**909**	**2080**	**-1.298**	**0.005**	**0.030**
hsa-miR-769-5p	767	1395	-0.993	0.005	0.030
hsa-let-7e-5p	7333	2427	1.470	0.006	0.032
hsa-miR-140-3p	10783	14267	-0.425	0.006	0.034
hsa-miR-320a	328110	557280	-0.900	0.006	0.035
hsa-miR-30e-3p	4586	2600	0.886	0.007	0.037

Table S2: continued

miRNA	Fresh (cpm)	Centrifuged then frozen (cpm)	Log fold change	p-value	FDR
hsa-miR-505-3p	991	1602	-0.978	0.007	0.038
hsa-miR-146a-5p	72934	125546	-0.872	0.008	0.039
hsa-miR-452-5p	5480	8570	-0.733	0.008	0.039
hsa-miR-339-3p	2779	4177	-0.810	0.008	0.039
hsa-miR-497-5p	1400	2403	-1.181	0.008	0.039
hsa-miR-16-5p	10342	3810	1.524	0.009	0.040
hsa-miR-1307-3p	2689	4464	-0.739	0.009	0.040
hsa-miR-21-5p	**247404**	**132929**	**1.078**	**0.009**	**0.042**
hsa-miR-106b-5p	4865	7467	-0.647	0.011	0.048
hsa-miR-197-3p	1385	2122	-1.200	0.012	0.052
hsa-miR-200a-3p	**109273**	**70166**	**0.822**	**0.013**	**0.053**
hsa-miR-193b-3p	14643	22880	-0.708	0.013	0.053
hsa-miR-27b-3p	45072	33328	0.589	0.014	0.054
hsa-miR-320b	24571	41202	-0.934	0.014	0.054
hsa-miR-148a-5p	15545	23322	-0.629	0.014	0.054
hsa-miR-320d	8227	13295	-0.957	0.015	0.054
hsa-miR-181c-5p	505	1254	-1.272	0.014	0.054
hsa-miR-423-5p	157896	251783	-0.812	0.016	0.060
hsa-miR-423-3p	44000	62437	-0.636	0.017	0.060
hsa-miR-205-5p	36523	51737	-0.646	0.017	0.060
hsa-miR-339-5p	4957	7185	-0.754	0.017	0.060
hsa-miR-23a-3p	60476	82700	-0.569	0.019	0.063
hsa-miR-151a-3p	23264	30275	-0.397	0.019	0.063
hsa-miR-193b-5p	2826	4396	-0.794	0.021	0.071
hsa-miR-28-5p	6157	4352	0.477	0.023	0.074
hsa-miR-30e-5p	29201	18177	0.886	0.024	0.077
hsa-miR-484	12637	16022	-0.464	0.025	0.078
hsa-miR-425-5p	27810	41736	-0.632	0.028	0.081
hsa-miR-429	**18455**	**10970**	**0.865**	**0.027**	**0.081**
hsa-miR-320c	12904	20687	-0.852	0.028	0.081
hsa-miR-195-5p	2159	1046	1.278	0.026	0.081
hsa-miR-452-3p	1183	1936	-0.659	0.027	0.081
hsa-miR-21-3p	455	770	-1.194	0.027	0.081
hsa-miR-320e	1244	1579	-1.373	0.030	0.084
hsa-miR-374b-5p	2888	1093	1.221	0.032	0.090
hsa-miR-181a-5p	139838	188828	-0.495	0.033	0.092
hsa-miR-103a-3p	61592	42232	0.480	0.037	0.094
hsa-miR-24-3p	**43119**	**59154**	**-0.527**	**0.036**	**0.094**
hsa-miR-361-3p	24057	18952	0.389	0.037	0.094
hsa-miR-183-5p	15663	11154	0.480	0.036	0.094
hsa-miR-22-5p	8598	10437	-0.481	0.037	0.094
hsa-miR-29c-5p	1002	1623	-0.982	0.037	0.094
hsa-miR-362-5p	642	1151	-1.128	0.035	0.094