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INFANT COLIC CRYING AND GASTROINTESTINAL TRACT

Causes, Consequences and Cure

by

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To my Family

ABSTRACT

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Infant Colic Crying and Gastrointestinal Tract – Causes, Consequences and Cure
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Despite over 50 years of investigation, the precise cause of infant colic crying remains unresolved and the long-term consequences unrevealed, and an effective treatment is lacking. Indeed, a more profound understanding of the complex nature of infants' excessive crying is needed. The purpose of this series of studies was to investigate the association between gut microbiota composition and infant crying, to evaluate the impact of colic crying on children's later health and to study the possibilities of treating and preventing excessive crying with pro- and prebiotics.

The material comprised three on-going, prospective randomized controlled trials of the probiotic *Lactobacillus rhamnosus* GG (ATCC 53103, LGG) or a mixture of prebiotics administered in early infancy. The study populations consisted of term infants (n=89), preterm infants (n=94) and term colic infants (n=30).

Early crying was found to be inversely associated with the number of *Bifidobacterium* and *Lactobacillus*. Furthermore, at the age of 13 years functional gastrointestinal disorders (FGID) were manifested more frequently among children with previous colic crying than in those without. In preterm infants pro- and prebiotic supplementation during the first months of life reduced the frequency of excessive crying when compared to placebo. In parallel, probiotic LGG in tandem with a cow's milk elimination diet and behavioral counseling reduced the daily crying amount among term colic infants when compared to placebo.

In conclusion, the composition of the gut microbiota is associated with infant crying and colic, and probiotic LGG might provide a safe and effective treatment or preventive option to alleviate excessive crying in early infancy in term and preterm infants. Furthermore, early colic crying might be associated with the later development of FGID.

Keywords: Colic, crying, fussing, gut microbiota, *Bifidobacteria*, *Lactobacilli*, probiotics, prebiotics, functional gastrointestinal disorders

TIIVISTELMÄ

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Imeväisen koliikki-itku ja gastrointestinaalikanava – syyt, seuraukset ja hoito
Lastentautioppi, Turun yliopisto ja Turun yliopistollinen keskussairaala
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Koliikki-itkulle ei ole pystytty yli 50 vuoden tutkimuksesta huolimatta löytämään yksiselitteistä syytä, eikä kontrolloiduissa tutkimuksissa ole pystytty osoittamaan tehokasta hoitomuotoa. Koliikin pitkäaikaisvaikutukset terveydelle ovat myös yhä osittain tuntemattomia. Näin ollen tarkempaa tutkimustietoa koliikin syystä, pitkäaikaisvaikutuksista sekä hoidosta tarvitaan vielä. Tämän tutkimuksen tarkoituksena oli selvittää suolistomikrobiston ja itkun yhteyttä, tutkia koliikki-itkun pitkäaikaisia vaikutuksia terveydelle sekä selvittää pro- ja prebioottien tarjoamaa mahdollisuutta vähentää ja ennaltaehkäistä koliikki-oireita.

Väitöskirjan aineisto koostui kolmesta etenevästä, kaksoissokkoutetusta probiootti- tai prebiootti-interventiotutkimuksesta. Tutkimuspotilaat olivat täysiaikaisena syntyneitä lapsia (n=89), ennenaikaisina syntyneitä lapsia (n=94) sekä täysiaikaisina syntyneitä koliikki-lapsia (n=30).

Tämän väitöskirjan tulokset osoittivat, että lapsen varhaisen itkun määrä on käänteisesti yhteydessä suoliston bifidobakteerien ja laktobasillien määrään. Varhainen koliikki-itku ennakoi teini-iässä esiintyviä toiminnallisia vatsavaivoja. Keskoslapsilla varhainen pro- ja prebioottilisä vähensi itkun esiintymistä ensimmäisen kahden elinkuukauden aikana. Myös täysiaikaisilla koliikkilapsilla probiootti *Lactobacillus rhamnosus* GG (ATCC 53103, LGG) vähensi päivittäistä koliikki-itkun määrää verrattuna lumevalmisteeseen.

Tutkimuksen johtopäätöksenä voidaan todeta, että suolistomikrobiston koostumus on yhteydessä varhaiseen itkun määrään ja koliikkiin. Probiootti LGG tarjoaa mahdollisesti turvallisen ja tehokkaan tavan vähentää tai ennaltaehkäistä imeväisen itkuisuutta sekä täysiaikaisilla että ennenaikaisina syntyneillä lapsilla. Lisäksi varhainen koliikki-itku saattaa ennakoida myöhemmällä iällä ilmeneviä toiminnallisia vatsavaivoja.

Avainsanat: Koliikki, itku, kitinä, suolistomikrobisto, bifidobakteerit, laktobasillit, probiootti, prebiootti, toiminnalliset vatsavaivat

TABLE OF CONTENTS

ABBREVIATIONS

ADHD	Attention deficit and hyperactivity disorder
AS	Asperger syndrome
CFU	Colony-forming unit
CMA	Cow's milk allergy
Cy3	Carbocyanine 3
DGGE	Denaturing gradient gel electrophoresis
FAO	The Food and Agriculture Organization
FGID	Functional gastrointestinal disorder
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
GER	Gastroesophageal reflux
GERD	Gastroesophageal reflux disease
GI	Gastrointestinal
GOS	Galacto-oligosaccharide
ICD-10	International Classification of Diseases and Related Health Problems, Tenth Revision
Ig	Immunoglobulin
LGG	*Lactobacillus rhamnosus* GG
NAMI	Nutrition, Allergy, Mucosal immunology and Intestinal microbiota project
PBS	Phosphate buffered saline
PCR-DGGE	Polymerase chain reaction denaturing gradient gel electrophoresis
PDX	Polydextrose
qPCR	Quantitative polymerase chain reaction
RAP	Recurrent abdominal pain

RAST	Radioallergosorbent test
RCT	Randomized clinical trial
SD	Standard deviation
SDS	Sodium dodecyl sulphate
WHO	World Health Organization

DEFINITIONS

Infant colic: A colic infant is defined as an "otherwise healthy and well-fed infant that has paroxysms of irritability, fussing or crying lasting for a total of more than three hours a day and occurring on more than three days in any one week" (Wessel, 1954).

Prebiotic: Prebiotics were originally defined in the year 1995 as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health" (Gibson, 1995).

Probiotic: Probiotic has been defined by The Food and Agriculture Organization (FAO) and World Health Organization (WHO) as a "live microorganism, which when administered in adequate amounts confers health benefit on the host" (FAO, 2009).

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I–IV, and on some supplementary unpublished data.

I. Pärtty A, Kalliomäki M, Akihito E, Salminen S, Isolauri E. Compositional development of *Bifidobacterium* and *Lactobacillus* microbiota is linked with crying and fussing in early infancy. PLoS One, 2012; 7:e32495.

II. Pärtty A, Kalliomäki M, Salminen S, Isolauri E. Infant distress and development of functional gastrointestinal disorders in childhood: is there a connection? JAMA Pediatrics, 2013; 167:977-978.

III. Pärtty A, Luoto R, Kalliomäki M, Salminen S, Isolauri E. Effects of early prebiotic and probiotic supplementation on development of gut microbiota and fussing and crying in preterm infants - a randomized, double-blind, placebo-controlled trial. J. Pediatrics, 2013; 163:1272-1277.

IV. Pärtty A, Lehtonen L, Kalliomäki M, Salminen S, Isolauri E. Probiotic *Lactobacillus rhamnosus* GG therapy and microbiological programming in the colic infant - a randomized, controlled trial. Submitted.

1 INTRODUCTION

The word “colic” is probably one of the most widely used terms to describe the infant crying problem. The word colic originates from Greek “kolikos”, the adjective of “kolon”, suggesting that infant crying has been considered due to gastrointestinal disturbance for hundreds of years (St James-Roberts, 2012). Despite more than 50 years of research into the origins of infant crying, the exact chain of pathological events remains unclear. However, the latest advances in molecular techniques have opened up new possibilities to investigate, for example, the relationship between infant crying and gut microbiota.

The most widely cited criteria for colic is Wessel’s Rule of Three. According to this criterion infant colic is characterized by paroxysmal, excessive and inconsolable crying, with a duration exceeding three hours a day for three or more days in any one week in an infant otherwise healthy and well fed (Wessel 1954). However, this rule is not without limitation and even Wessel and associates did not argue that this definition is anything other than a convenient cut-off point for the purpose of comparing “fussy” and “content” babies. Moreover, the average daily crying time varies substantially between different cultures and countries, indicating that the number of “problem cases” also varies between societies by definition, if a single rule such as the Rule of Three is applied all over the world. As an example, 6-week-old infants in Boston cry an average of 2 hours 45 minutes and at the same age infants in Copenhagen only 1 hour 20 minutes; thus more Bostonian infants would have a crying problem according to the Rule of Three definition (Brazelton, 1962; Alvarez, 2004).

One infant in four is brought for medical evaluation because of excessive crying during the first months of life (Alvarez, 1996). However, no more than 5% of excessively crying infants taken to professionals prove to have some organic disturbance and thus most such infants are healthy, put on weight and develop normally (Freedman, 2009). Infant crying as a clinical problem is characterized foremost by parental concern rather than as a problem of the infant (St James-Roberts, 2012). One of the most prominent causes increasing parental concern is the lack of an explanation for infant crying (St James-Roberts, 2012). On the other hand, benign prolonged crying can become a problem equally for the infant, if it begins to disturb the parent’s ability to take care of the baby. Infant crying is a source of frustration to parents and is associated with maternal depression (Stifter, 1998; Vik, 2009). Nevertheless, it is true that the behavior of the excessively crying infant is different from that of most other babies, and it is

therefore justifiable to question why are these infants crying? Does it have any long-term consequences? How could we treat these infants? Or could we prevent these problems?

2 REVIEW OF THE LITERATURE

2.1 Crying pattern in infancy

During the first three months of life the daily amount of crying manifests a developmental transition pattern. Western studies have shown that the daily crying time peaks around five to six weeks of age and decreases significantly by the age of 12 weeks (Brazelton, 1962; Hunziker, 1986). The average daily crying time during the first three months of life is around two hours, varying considerably between different studies from 1 hour 20 minutes up to 2 hours 45 minutes (Brazelton, 1962; Hunziker, 1986; Lehtonen, 1995; Alvarez, 2004). Diverse cultures and inconsistent definitions for fussing, crying and total crying might be one of the reasons for these differences.

Delineation of colic as a clinical entity is not justified in view of the many similarities between the crying behavior of infants with and without colic. While colic infants have proven to cry longer than other infants, the age at the crying peak, the evening cluster of the crying, and the numbers of daily crying bouts have been shown to be similar between colic and other infants (Brazelton, 1962; Hunziker, 1986; Barr, 1992; St James-Roberts, 2012). Consequently, colic infants might be considered to be at the arbitrary upper limit of otherwise normal crying rather than to constitute a distinct clinical entity. Lack of consistent clinical findings and the self-limiting nature of colic also support such a conception. The prevalence of problematic infant crying i.e. colic varies widely because of the diversity of definitions. According to Wessel's Rule of Three the figure varies from 8% up to 24% (Alvarez, 1996; van der Wal, 1998; Reijneveld, 2001; Clifford, 2002a). In Finland, the prevalence has been reported to be around 13% (Lehtonen, 1995).

2.2 Etiological theories on colic crying related to the GI tract

The precise etiology of infant colic is not yet well understood and the underlying cause is considered to be multifactorial, including behavioral and medical origins (Savino, 2007a). Distended abdomen, gas problems, abdominal discomfort and increased tone of colicky infants have led researchers to suspect the gastrointestinal tract as the source of this common problem. Consequently, possible causative agents can also be divided into gastrointestinal and non-gastrointestinal, and the following section focuses on the former (Gupta, 2002).

2.2.1 Gastroesophageal reflux

Gastroesophageal reflux (GER), a normal physiologic process in infants, has received much attention as a cause of infant excessive crying, and thus irritable infants are often treated with anti-reflux medications on an empirical basis (Heine, 2006). However, only a minority of infants has clinical gastroesophageal reflux disease (GERD), i.e. frequent passage of gastric contents into the esophagus causing troublesome symptoms and/or complications (Hyman, 2006). There are only a few studies available exploring the role of gastroesophageal reflux as a cause of infantile colic during the peak period of crying (Heine, 1995; Miller-Loncar, 2004; Heine, 2006).

A study of 151 excessively crying infants, whose daily crying time was measured with cry charts and reflux time with esophageal 24-hour pH monitoring, showed that non-regurgitant "silent" GER is uncommon in irritable infants. The study also indicated that there was no association between total daily crying time and reflux, when measured as the number of reflux episodes and the proportional reflux time (Heine, 2006). An earlier study also suggested that pathological GER is an unlikely cause of infant irritability during the first months of life (Heine, 1995). However, these studies were not planned to investigate the etiology of colic, but rather to examine the association between these two problems.

Similarly the recently published Pediatric Gastroesophageal Reflux Clinical Practice Guidelines, jointly sponsored by the North American and the European Society for Pediatric Gastroenterology, Hepatology and Nutrition states that GER is an uncommon cause of unexplained crying, irritability, or distress behavior in otherwise healthy infants, and there are no data to support the empirical use of acid suppression therapy in crying infants (Vandenplas, 2009). This conception is also supported by a recent systematic review concluding that proton-pump inhibitors are not effective in reducing the symptoms of gastrointestinal reflux disease such as crying and irritability in infants (van der Pol, 2011). In the same vein, another recent review concluded that proton-pump inhibitors reduce the acidity of the stomach, but have no effect on symptoms such as crying or irritability in infants under the age of 1 year (Blokpoel, 2010). As a conclusion GER is a very rare cause of infantile colic, being a significant factor in only approximately 5% of colic infants, and proton-pump inhibitors are not recommended on an empirical basis (St James-Roberts, 2012).

2.2.2 Allergy

Over 20 years ago a group under Lothe demonstrated that breast- and formula-fed colic infants evince increased absorption of the macromolecule alpha-lactalbumin from their intestine compared to healthy controls, and proposed that the gut mucosa is affected in infant colic (Lothe, 1990a). This notwithstanding, serum IgE levels, radioallergosorbent test (RAST) and skin prick test for cow's milk have proven to be within normal limits in most colic infants (Liebman, 1981; Moravej, 2010). However, negative skin prick tests do not rule out cow's milk allergy (CMA), and the diagnostic gold standard for food allergy is the oral food challenge.

If allergy were a significant cause of infantile colic, a change or modification in formula or the breast-feeding mother's diet would reduce colicky behavior, as the diagnosis of CMA is based on elimination of and exposure to a cow's milk diet. A high-quality randomized clinical trial (RCT) (n=90) on the effects of maternal diet on colic has shown an absolute risk reduction of 37% when dairy foods, eggs, nuts, wheat, soy and fish were eliminated from the breast-feeding mother's diet (Hill, 2005). However, in this study no challenges were performed to demonstrate whether symptoms relapsed after re-introduction of these foods. The results are supported by three out of five other RCTs (Evans, 1981; Oggero, 1994; Hill, 1995; Lust, 1996; Taubman, 1998). Overall, a recent comprehensive review concludes that changing the maternal diet to low-allergen may provide some benefit in reducing symptoms in breast-fed colic infants (Iacovou, 2012). To emphasize that the evidence is not absolutely unambiguous, one recent study has posited that cow's milk elimination from the breast-feeding mother's diet is not beneficial for infants negative to the skin prick test, but that infants positive to it would rather benefit from it (Moravej, 2010).

Eleven RCTs of good quality have assessed the effect of extensively hydrolyzed formulas or amino acid based formulas, most commonly indicated for cow's milk allergies, on colic symptoms (Iacovou, 2012). One of the largest studies (n=70) demonstrated an over two hours' reduction in daily crying time in 7 days in colic infants receiving extensively hydrolyzed formula compared to colic infants on a standard cow's milk formula (Arikan, 2008). These findings are supported by those of 10 other studies, using whey- or casein-based extensively hydrolyzed formulas or amino acid based formulas (Lothe, 1982; Taubman, 1988; Forsyth, 1989; Lothe, 1989; Oggero, 1994; Hill, 1995; Estep, 2000a; Estep, 2000b; Jakobsson, 2000; Lucassen, 2000). However, again the re-introduction of cow's milk based formula is missing in most of these studies.

To conclude, the present findings and the results of three separate reviews (Lucassen, 1998; Garrison, 2000; Iacovou, 2012), breast-fed colic infants seem to benefit from a maternal low-allergen diet and formula-fed infants from the use of hydrolyzed formulas. However, diversity of definitions of colic crying and the inconsistent estimates of a clinically relevant crying time reduction call for appropriate standardization.

2.2.3 Feeding difficulties

During the first two months of life colic infants have been shown to have more functional feeding problems, including problems with feeding and sucking and discomfort after feeding recorded in questionnaires and parental diaries compared to healthy controls (Miller-Loncar, 2004). A selection bias might have contributed to the results of the study in question and the sample size (n=19) was too small to generalize the results. There is a need for larger studies to replicate these findings and to establish whether feeding problems are the cause or a consequence of excessive crying.

More recently an alternative hypothesis as to the etiology of colic crying has also been proposed by a neurosurgeon, envisaging infant colic as a pain syndrome resulting from continuous sucking of bottle or nipple, pain thus originating from a heavy work load on a small digastricus muscle located on the under-side of the jaw (Gudmundsson, 2010).

2.2.4 Gut microbiota

Normal development of gut microbiota

After birth, the gastrointestinal tract is rapidly colonized by vaginal and intestinal microbes from the mother, especially facultative anaerobic bacteria such as *Enterococci* and *Enterobacteria* (Adlerberth, 2009; Marques, 2010). Thereafter an exclusive anaerobic population such as *Bifidobacteria*, *Clostridia* and *Bacteroides* begin to dominate the intestine (Adlerberth, 2009; Marques, 2010). However, gestational age, mode of delivery, antibiotic use, type of feeding and environment set their own pattern on the gut microbiota colonization processes (Penders, 2006; Di Mauro, 2013; Figure 1). For example, in breast-fed infants *Bifidobacteria* become abundant, whereas formula-fed infants harbor raised levels of *Clostridia* and *Bacteroides* (Harmsen, 2000; Penders, 2006). During the first years of life the process of maturation of the infant gut microbiota is established and it begins to resemble adult-like microbiota, where the number of

bacterial cells exceeds by 10-fold the number of cells forming the human body (Wall, 2009; Sekirov, 2010; Simren, 2013). The intestinal microbiota has an important role in human health in producing nutrients as well as taking part in a variety of metabolic functions, preventing colonization of the gut by potentially pathogenic bacteria and modulating and activating the immune system (Guarner, 2003).

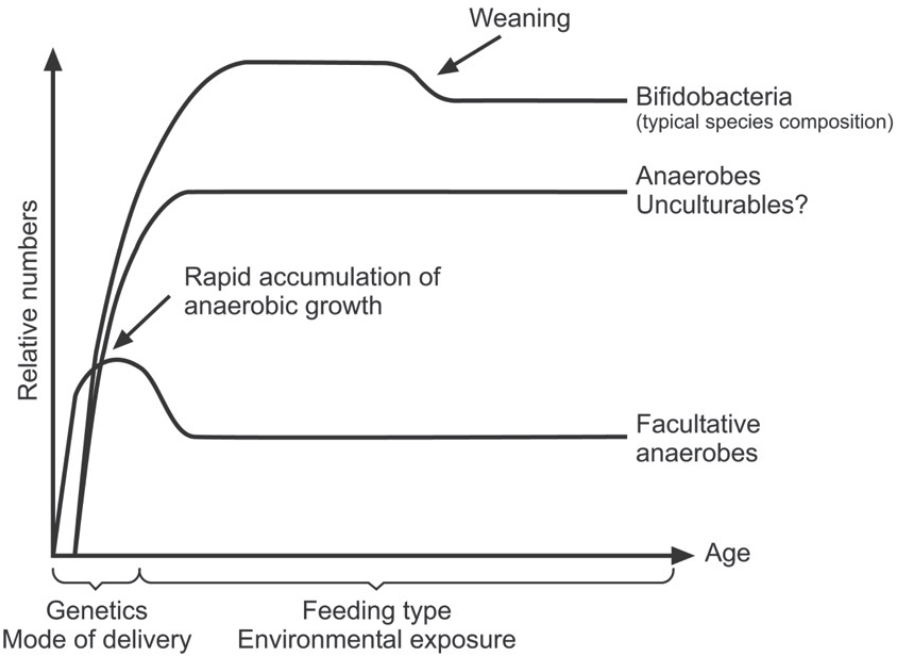


Figure 1. Gut microbiota colonization. Modified from Mitsuoka, 1984.

Gut microbiota and crying

Recently the gut microbiota composition in colic infants has been studied intensively. However, it is challenging to arrive at an explicit conclusion as to the gut microbiota composition of colic infants, this by reason of variation in study populations, methodologies, investigated microbes and results obtained (Table 1).

Table 1. Studies analyzing the gut microbiota composition of colic infants.

Author	Study population	Methods	End-point	Main outcome
Lehtonen 1994b	55 colic and 49 healthy infants	Culture methods and gas-liquid chromato-graphy	Intestinal microbiota	Colic infants were more frequently colonized with *Clostridium difficile* and had aberration in fatty-acid profiles compared to controls.
Savino 2004	42 colic and 29 non-colic infants	Culture methods	Intestinal microbiota	Colic infants were more frequently colonized by gram-negative bacteria and less frequently by *Lactobacilli*.
Savino 2005a	30 colic and 26 non-colic infants	Culture methods	*Lacto-bacillus* species	*Lactobacillus brevis and Lactobacillus lactis lactis* were only found in colic infants.
Mentula 2008	9 colic and 9 non-colic infants	Culture methods, cellular fatty acid analysis	Intestinal microbiota	Colic infants were more frequently colonized with *Escherichia coli* and *Klebsiella* and had aberrations in fatty acids profiles compared to controls.
Savino 2009	41 colic and 39 healthy infants	PCR and biochemical characterization technique	Coliform bacteria	Colic infants had more coliforms than healthy controls.
Rhoads 2009	19 colic and 19 control infants	DGGE	Intestinal microbiota, calprotectin levels	Calprotectin levels were higher, diversity of total bacteria lower and *Klebsiella* were more common in colic infants than in controls.
Ali 2012	55 colic and 30 healthy infants	*Helicobacter pylori* stool antigen testing	*H. pylori* antigen	*H. pylori* antigen was positive more often in colic infants than in references.
Roos 2013	29 colic infants	454 pyro-sequencing	Intestinal microbiota	Colic infants showed high inter-individual variability, but on an individual basis the microbiota was relatively stable.
de Weerth 2013b	12 colic infants and 12 age-matched controls	Phylogenetic microarray	Intestinal microbiota	The diversity and stability of microbiota was lower, *Proteobacteria* increased and number of *Lactobacilli* and *Bifidobacteria* decreased in colic infants compared to controls.

Lehtonen and colleagues introduced the first evidence of the association between colic crying and gut microbiota composition as far back as 1994. They demonstrated differences in bacterial cellular fatty acid profiles between infants with severe colic and healthy controls (Lehtonen, 1994c). Similar findings in the cellular fatty acid profiles of bacteria are also reported in a subsequent study (Mentula, 2008).

More recently, the complexity of the gut microbiota in colic infants has been revealed by high-throughput and other culture-independent approaches (de Weerth, 2013b; Roos, 2013). Colic infants evince a decreased fecal bacterial diversity when compared to non-colic infants (Rhoads, 2009; de Weerth, 2013b), their gut microbiota being composed mainly of four phyla including *Proteobacteria, Firmicutes, Actinobacteria and Bacteroides* (Roos, 2013). Furthermore, the gut microbiota composition in colic infants shows high inter-individual variability, but on an individual basis is relatively stable over time (Roos, 2013). Nonetheless, the stability of the microbiota is significantly lower in infants with colic than in controls (de Weerth, 2013b).

Colic infants have higher counts of gram-negative bacteria, especially coliform bacteria, mainly species belonging to *Escherichia coli*, when compared to non-colic infants (Savino, 2004; Mentula, 2008; Savino, 2009; de Weerth, 2013b). Furthermore, three studies have reported the number of *Klebsiella* species to be increased in colicky when compared to healthy infants (Mentula, 2008; Rhoads, 2009; de Weerth, 2013b). On the other hand, the numbers of *Lactobacilli* and *Bifidobacteria* have been demonstrated to be reduced in colic when compared to healthy infants (Savino, 2004; de Weerth, 2013b). Also the colonization patterns of different *Lactobacilli* species have been shown to be distinct between colicky and healthy infants; *L. brevis* and *L. lactis lactis* were found only in colicky infants, *L. acidophilus* only in controls (Savino, 2005a).

A study from Saudi Arabia has reported that 82% of colic infants, but only 23% of healthy controls, proven positive to a *Helicobacter pylori* stool antigen test; however the concentration of the antigen was not associated with the daily crying amount (Ali, 2012). Thereafter a Dutch group found a two-fold increase in the level of *Helicobacter*-related bacteria in colic infants when compared to controls (de Weerth, 2013a). In light of these results *Helicobacter*-related bacteria might be considered one of the possible etiological agents in infantile colic. On the other hand, *Helicobacter pylori* is nowadays very rare in Western infants and consequently might be of greater significance in developing countries as a factor in infantile colic (Alarcon, 2013).

As a conclusion, there is accumulating evidence to indicate that the gut microbiota composition in colic infants differs from that in healthy controls, although no more specific conclusions can be drawn.

2.2.5 Other theories

A subset of normal infants may have partial malabsorbtion of dietary carbohydrates, which typically resolves by the age of three months, the same age as colic subsides (Barr, 1984). Carbohydrate malabsorbtion, as well as deviating gut microbiota colonization, might be reflected in increased breath hydrogen levels (Gupta, 2002). A sudden production of hydrogen in the bowel might distend colon and cause pain (Savino, 2007b). In three separate studies have infants with colic proven to have increased breath hydrogen levels when compared to healthy infants (Moore, 1988; Miller, 1989; Rhoads, 2009), while one study found no such association (Hyams, 1989).

In the same vein, functional lactose overload might take place when the breast milk fat concentration is reduced and the milk transit time through the gut is thus decreased (Douglas, 2013). Undigested lactose might be fermented in the colon by bacteria increasing gas and explosive frothy stools and causing pain and crying (Douglas, 2011; Douglas, 2013). However, there is no evidence that lactase supplementation or low-lactose milk reduce the symptoms of colic, although a subset of colic infants might benefit from it (Stahlberg, 1986; Miller, 1990; Kanabar, 2001; Hall, 2012).

The gastrointestinal tract produces a number of hormones, for example motilin, vasoactive intestinal peptide, gastrin and ghrelin, which are all involved in the regulation of intestinal motility. Serum motilin concentrations at birth and during the colic period have been shown to be increased in colic infants when compared to healthy controls (Lothe, 1987; Lothe, 1990b; Savino 2006). It has been suggested that motilin might improve gastric emptying, increase small-bowel peristalsis, and reduce bowel transit time and thus might lie behind colic symptoms (Lothe, 1987; Savino, 2007b). Furthermore, Shenassa and associates have introduced an interesting theory positing that maternal cigarette smoking increases the risk of infant colic by elevating motilin levels (Shenassa, 2004). Similarly to motilin levels, the serum ghrelin concentration has proven to be increased in colic infants when compared to healthy controls (Savino, 2006). However, the levels of vasoactive intestinal peptide and gastrin have been demonstrated to be raised in children with some gastrointestinal disorders (vomiting, diarrhea) other than colic (Lothe, 1987).

Another etiological theory suggests that colic infants have decreased levels of cholecystokinin, a hormone released from small-intestine endocrine cells in response to feeding (Huhtala, 2003). Cholecystokinin mediates pacifying behavioral effects after feeding (Marchini, 1992) and contributes to postprandial gallbladder contraction (Meyer, 1989), which in turn has been shown to be lower in colic infants than healthy controls (Lehtonen, 1994c; Huhtala, 2003). Consequently, low cholecystokinin levels might predispose the infant to colic.

Altered intestinal motility has been suggested to lead to abdominal cramping and colicky behavior. Particularly during the first weeks of life transient dysregulation of the nervous system may cause colonic motility problems in colic infants (Savino, 2007a). This conception is supported by the beneficial effect of an antispasmodic drug, dicyclomine hydrochloride, on colic crying (Hwang, 1985). However, whether the mechanisms of action of this drug is mediated through a direct relaxation of colonic smooth muscle or via sedative effects on central nervous system, remains unknown (Savino, 2007a). Furthermore, probiotics, another potential treatment option for colic, have evinced to improve gut function and feeding tolerance in infants (Indrio, 2008; Indrio, 2013). Probiotics, as a part of the normal gut microbiota, might balance the intestinal motility by regulating enteric nervous system via Toll-like receptor signaling (Brun, 2013).

One further possible etiological theory of infantile colic supports the idea of gut inflammation behind this problem. Levels of fecal calprotectin, a gut inflammation marker, have been 2-fold higher in infants with colic compared to healthy infants (Rhoads, 2009). However, this theory remains open to doubt since another study found no differences in fecal calprotectin values between colicky and non-colicky infants (Olafsdottir, 2002).

2.3 Consequences of colic crying

Traditionally colic crying has been described as a benign and transient condition. However there is some evidence from prospective and retrospective follow-up studies to indicate that it might have short- and long-term consequences for later health, including gastrointestinal and allergic disorders, migraine, developmental, behavioral, eating and sleeping problems as well as with family functioning (Rautava, 1995; Kirjavainen, 2001b; Savino, 2005b; Hemmi, 2011; Romanello, 2013).

2.3.1 Functional gastrointestinal disorders

Based on the Rome III criteria, infantile colic has been classified as an early manifestation of functional gastrointestinal disorder (FGID) (Hyman, 2006), but there is only sparse and contradictory evidence as to the association between colic crying and the development of other FGID later in life. As far back as 1984 Joseph and colleagues explored a possible linkage between infantile colic and recurrent abdominal pain (RAP) and found that only 1 out of 30 colicky infants developed RAP at the age of 13 years (Joseph, 1984). In contrast, in another study the relative risk of developing constipation or RAP in childhood was two and three times greater for those with a prior history of colic than those without. In the same study colic infants carried a four and six times greater risk of developing constipation or RAP when compared to the risk of developing inflammatory bowel disease (Groffie, 1998). Both of these studies were retrospective, whereas there is one study which has evaluated the association prospectively. There RAP was diagnosed at the age of 10 years in 33% of ex-colicky and in 4% of the ex-non-colicky children, but there were no differences in the frequency of constipation between the groups (16% vs. 16%, respectively). Moreover, a family history of gastrointestinal diseases was more common in infants with colic than among controls (33% vs. 13%), this possibly explaining some of the findings (Savino, 2005b).

2.3.2 Allergic disease

As noted above, cow's milk allergy has been implicated as one of the possible causes of infant colic, but some studies have also investigated whether colic is related to the development of allergic disease later in life. In a prospective 10 years' follow-up study of 48 children with previous colic and 48 healthy children, the colic group manifested a higher frequency of allergic rhinoconjunctivitis (27% vs. 4%), asthmatic bronchitis (23% vs. 6%), atopic eczema (31% vs. 6%) and food allergy (23% vs. 6%) than non-colicky children (Savino, 2005b). Furthermore, the same study revealed a family history of allergic disease in 48% of ex-colicky infants, but in only 25% of ex-non-colicky infants. Kalliomäki and associates showed in a prospective follow-up study of 116 high-allergy-risk children that children with atopic disease at the age of two years had manifested more fussing and colic type crying during the first months of life than those who remained healthy (Kalliomäki, 2001). On the other hand, a prospective study of 90 colic children from an unselected population showed no increased risk of the development of eczema, allergic rhinitis, positive skin prick test or higher levels of total serum IgE in children with a history of colic crying

than controls during the first 11 years of life (Castro-Rodriguez, 2001). These results were supported by a prospective three-year follow-up study of 124 colic infants, which found no association between colic and allergic disease (Rautava, 1995). As a conclusion, the association of infant colic with the later allergic disease is far from unambiguous and further studies are needed to clarify this interconnection.

2.3.3 Migraine

The exact etiology of migraine is partly unknown, but some retrospective studies demonstrate that children suffering from migraine have a history of infant colic more often than other children (Bruni, 1997; Jan, 2001; Romanello, 2013). One recent study found this linkage specific only to migraine with or without aura, not to other types of headache such as tension-type (Romanello, 2013). However, the diagnosis of infantile colic was set retrospectively in all of these studies and there is the possibility of a recall bias. Moreover, another interesting linkage between colic and migraine was recently established by Gelfand and associates They found that infants with a maternal history of migraine were 2.6 times more likely to have colic than those without such a background (Gelfand, 2012). The authors concluded that as migraine has a significant genetic base, this linkage suggests that colic might be an early manifestation of the disorder.

2.3.4 Cognitive development

Infants with colic crying have been demonstrated to obtain lower scores on the Mental and Psychomotor scales of the Bayley Scales of Infant Development at the age of six months, while at later ages infant colic had no effect on test scores (Sloman, 1990). The authors suggested that these results might be due mainly to less favorable patterns of parent-infant interaction during the first months of life (Sloman, 1990). Moreover, a prospective five years' follow-up study of 48 typical colic infants, 15 prolonged crying infants and 264 controls evaluated whether crying impairs children's intelligence, motor abilities and behavior (Rao, 2004). Children with excessive crying continuing over three months of age had adjusted mean intelligence quotient (IQ) significantly lower and poorer fine motor abilities compared to controls. However, transient infant colic (< three months) had no effect on cognitive development during the five-year follow-up (Rao, 2004). Furthermore, Rautava and associates found no differences in cognitive development at the age of three years between ex-colic and non-colic children (Rautava, 1995). On the other hand, a study of very low birth weight

infants revealed that both mental and psychomotor indexes at two years of corrected age were significantly lower in excessively crying infants than controls (Munck, 2008). However, these results cannot be generalized to all excessively crying infants, since prematurely born infants are at increased risk of developmental problems (Munck, 2008). Although the data on the long-term consequences of infantile colic on children's cognitive development is somewhat complex, they would imply that the nature of colic crying is benign and transient and not disturbing to the normal cognitive development in term infants.

2.3.5 Behavioral problems

A recent comprehensive meta-analysis has demonstrated that children with early crying problems have more behavioral problems, especially aggressive or destructive behavior, conduct problems, temper tantrums and attention deficit hyperactivity disorders (ADHD) later in childhood (Hemmi, 2011). Such an association was also found in early multiple regulatory problems, including sleeping and eating disorders (Hemmi, 2011). However, the mean age of infants at the time of the crying problems was 2.6 months, which is close to the end of colic age. It is also worth noting that three out of nine cry studies included in the meta-analysis had infants with a persistent crying problem.

2.3.6 Eating problems

Excessive crying and early feeding problems, both referred to as an infant regulatory problem, might have some common etiological causes, since both problems are related to difficulties in coping with change (Hemmi, 2011). A four-year follow-up study of 50 Swedish children with previous colic crying examined the eating habits of these children later in life. Former colicky children, according to the parental questionnaire, were less likely to enjoy meals and like eating than the controls at the age of four years. Moreover, these previous colic children also refused to eat certain foods more often than the control children, while other eating habits were similar between the groups (Canivet, 2000). On the other hand, only persistent prolonged crying (> five months) has been shown to predict eating problems at 20 months of age (Schmid, 2010). Likewise another study demonstrated no remaining feeding problems in a group of children with colic crying combined with some early feeding problem, although the number of children in this study was only nine (Dahl, 1987). There are not enough data to draw any conclusions regarding early crying and the development of later eating problems.

2.3.7 Sleep

Early excessive crying has been proposed to be a manifestation of infants' incompetence to control behavioral states, and thus disrupted sleep-wake behavior might also be involved (St James-Roberts, 1998). Consequently, sleeping patterns during the colic crying period and thereafter have been intensively studied (Taubman, 1984; Rautava, 1995; St James-Roberts, 1997; Canivet, 2000; White, 2000; Kirjavainen, 2001b; Schmid., 2010). Colic infants have been demonstrated to sleep one to two hours less per day than healthy controls during the first two months of life (Taubman, 1984; St James-Roberts, 1997; White, 2000; Kirjavainen, 2001b). Kirjavainen and associates showed that daily sleeping time was shorter in infants with colic compared with controls at five weeks of age. However, at the age of two and seven months polygraphic data showed a similar sleep structure between the study groups (Kirjavainen, 2001b). Furthermore, the total duration of night sleep, the number and duration of night wakenings, and sleeping patterns have been demonstrated to be similar between colic and non-colic infants after the peak period of colic crying, at the age of 8 and 12 months (Lehtonen, 1994a). Similarly, a group under Canivet showed no difference in sleeping habits between colic and control groups at the age of four years (Canivet, 2000). On the other hand, one three years' follow-up study of colic children demonstrated that crying infants had more sleeping problems than controls at the age of three years (Rautava, 1995). Lastly, Schmid and associates established that only prolonged (> five months) excessive crying is a single predictor of sleeping problems at the age of four years (Schmid, 2010).

In conclusion, most of the available data on the role of colic crying in the long-term consequences for children' sleep patterns suggests that infants who have transient crying problems, i.e. colic, do not have sleeping problems later in life, but there might be a group of children who are an exception to this finding.

2.3.8 Family functioning

Although the nature of colic crying is considered to be self-limiting and benign, it imposes a heavy strain on many parents and may be an underlying cause of child abuse. Lee and associates found convergent indirect evidence that crying, especially during the first months of life, was a significant stimulus in shaken baby syndrome (Lee, 2007).

The literature is contradictory regarding long-term effects of colic crying on parental mental health. Two earlier studies have reported that mothers of infants

with previous colic felt less competent, tended to have more separation anxiety and had higher maternal depression scores compared with mothers of non-colic infants (Stifter, 1998; Vik, 2009). On the other hand, two other studies did not find an increased risk of later parental depression once colic had resolved (Clifford, 2002a; Wake, 2006). Furthermore, a prospective three years' follow-up study of 338 colic families evaluated whether family functioning differed within families with and without previous colic child. The families with previously colic infants demonstrated more dissatisfaction with the arrangement of daily family responsibilities and with the amount of both leisure time and shared activities in families than the control group three years after colic (Rautava, 1995). Furthermore, at the end of the three-year follow-up period, fewer parents in the severe colic group than in the control group had decided to have more infants. In practice infantile colic should be seen in the perspective of the whole family, and families with complaints about infant crying need more attention for possible psychosocial problems and family functioning later in life.

3 AIMS OF THE STUDY

The main purpose of this thesis was to evaluate the association between gut microbiota composition and infant crying, to assess the impact of colic crying on children's later health, and to study the possibilities of treating and preventing excessive crying with pro- and prebiotics.

The specific aims were:

1. to evaluate the association between gut microbiota composition and infant crying (**I**[1]**, III, IV**).

2. to determine the influence of infant crying on subsequent health in childhood (**II**[1]).

3. to assess the impact of pro- and prebiotic supplementation on preterm infant crying and gut microbiota development (**III**).

4. to establish the influence of early probiotic supplementation on infant colic crying and gut microbiota composition (**IV**).

[1]The questions were evaluated in Study I and II on a population that received probiotics or placebo.

4 MATERIALS AND METHODS

4.1 Subjects and study design

The thesis consists of four original studies (I, II, III, IV) and the data are derived from three randomized, double-blind, placebo-controlled trials named the **Crying study** (I, II), the **Preterm study** (III) and the **Colic study** (IV). The profiles and trial flows of these studies are presented in Figures 2-4.

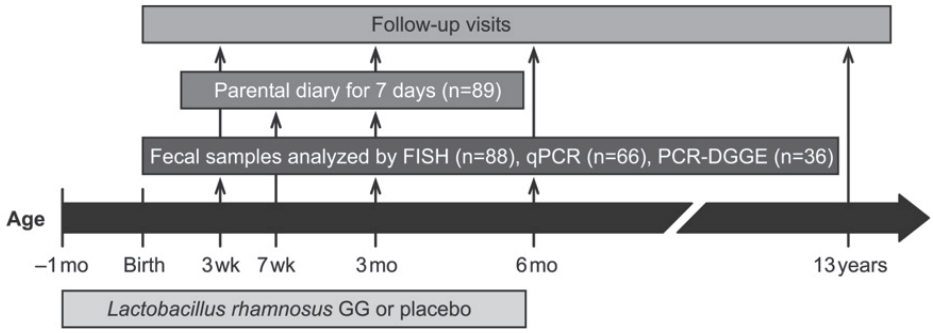


Figure 2a. Study design in the Crying study (I, II).

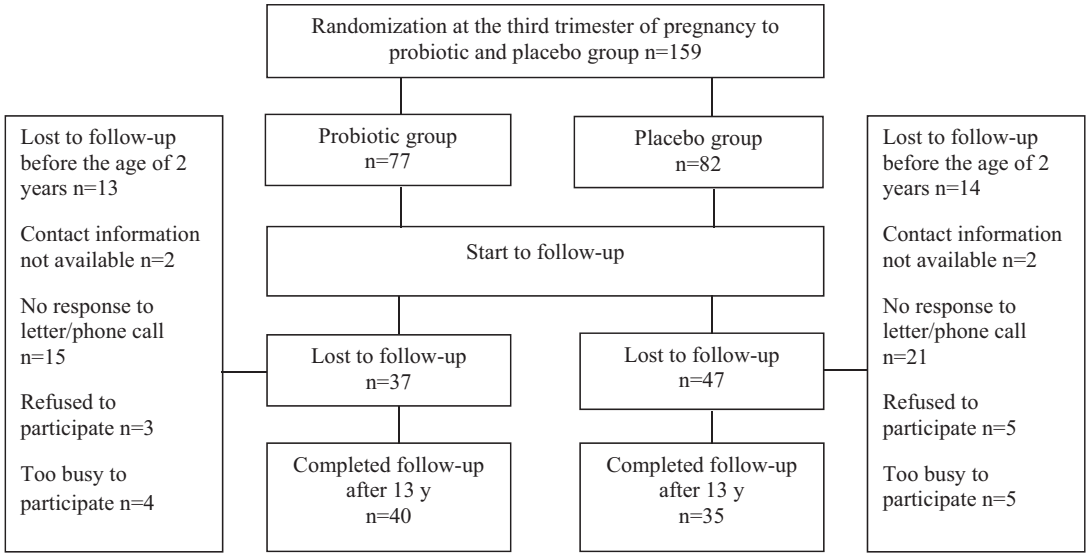


Figure 2b. Trial flow in the Crying study (I, II).

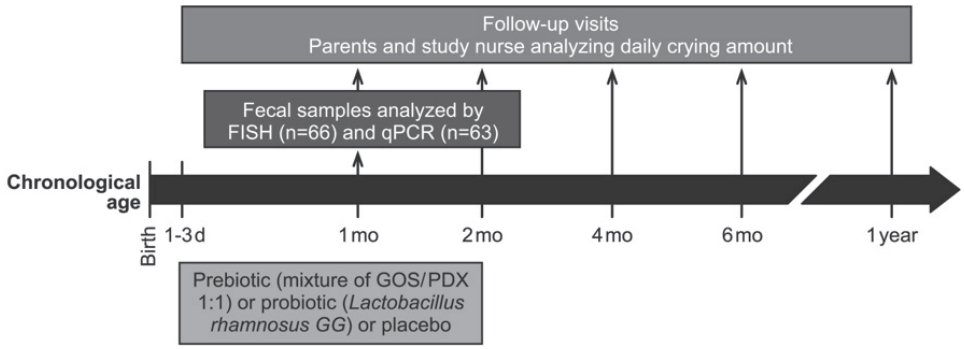


Figure 3a. Study design in the Preterm study (III).

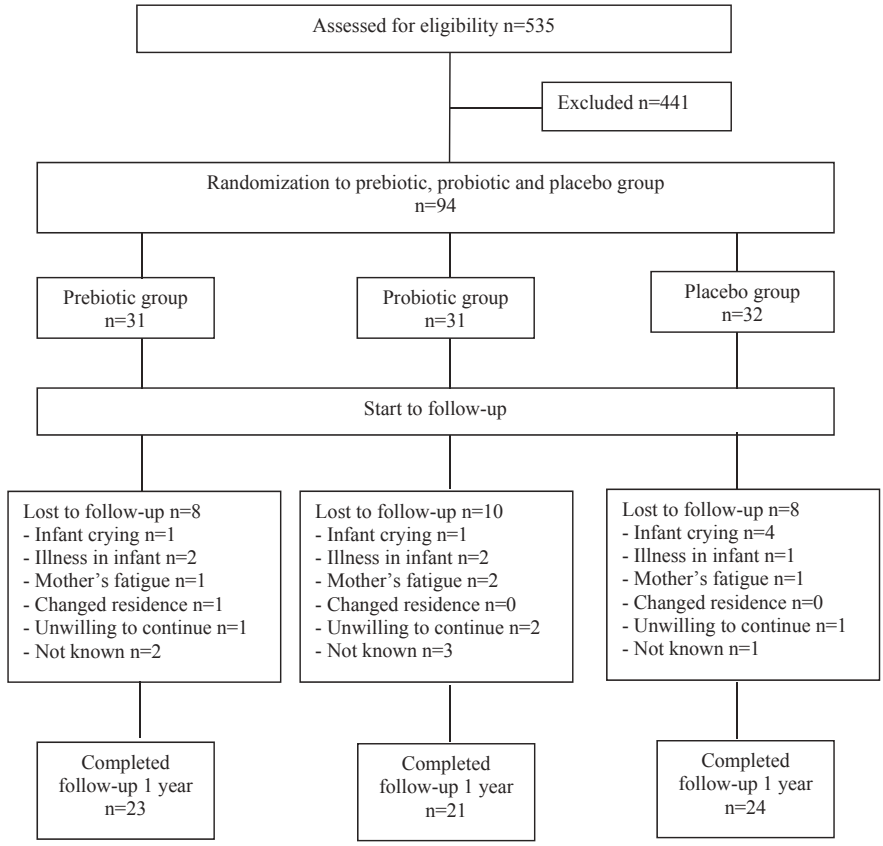


Figure 3b. Trial flow in the Preterm study (III).

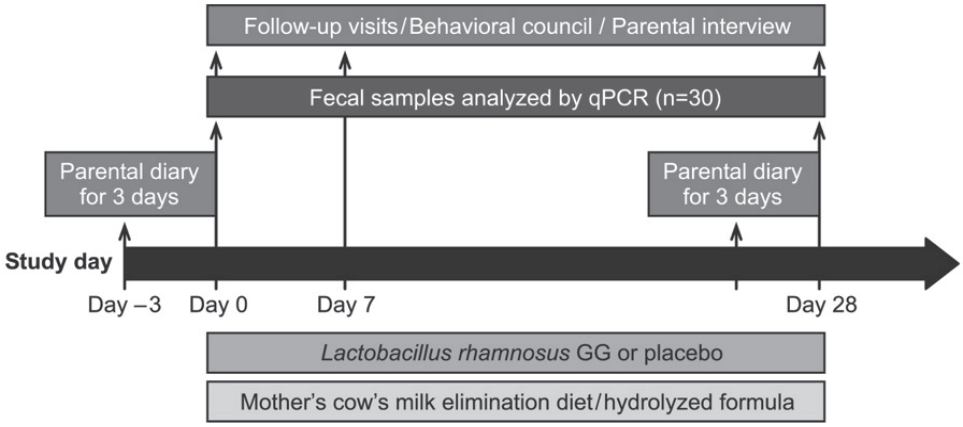


Figure 4a. Study design in the Colic Study (IV).

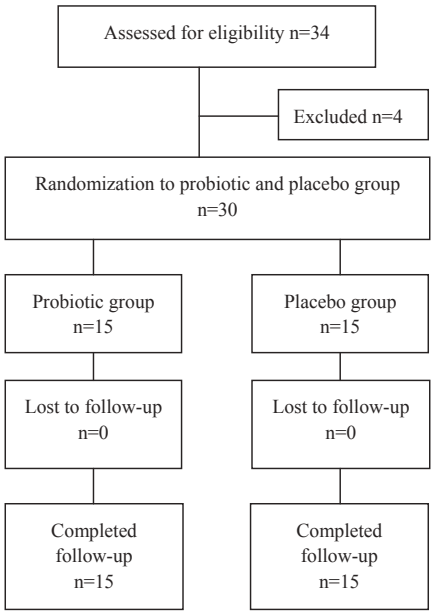


Figure 4b. Trial flow in the Colic Study (IV).

4.1.1 The Crying study (I, II)

Altogether 159 mothers-infant pairs from high-allergy-risk families were recruited in maternal welfare clinics in Turku, Finland, between February 1997 and January 1998, to participate in an on-going randomized, double-blind prospective follow-up study of probiotic LGG (ATCC 53103) (*http://www.clinicaltrials.gov/ct/gui/show/NCT167700*). The inclusion criteria for infants in Study I was a correctly filled parental diary of the infant's behavior during the 7th and/or 12th week of life (n=89). Study II included all children completing the 13-year-olds' follow-up visit (n=75).

4.1.2 The Preterm study (III)

A total of 94 preterm infants were recruited between the 1st and 3rd days of life in the Department of Pediatrics in Turku University Hospital between March 2008 and May 2011 to participate in a prospective, randomized, double-blind placebo-controlled study (*http://www.clinicaltrials.gov/ct/gui/show/NCT00167700*). The inclusion criteria were the following: gestational age between 32+0-36+6 weeks, birth weight over 1500g, absence of any congenital defects of the gastrointestinal system or other defects which prevent enteral nutrition.

4.1.3 The Colic study (IV)

Altogether 30 colic infants were recruited in the maternity ward, well-baby clinics and pediatric outpatient clinics in the catchment area of Turku University Hospital between February 2011 and January 2013 to participate in this randomized, double-blind, placebo-controlled clinical study of infantile colic (*http://www.clinicaltrials.gov/ct/gui/show/NCT00167700*). The participants comprised of full-term infants under six weeks of age, who had paroxysmal, unsoothable crying at least three hours per day on three days per week raising concern in parents. The reference population consisted of healthy, vaginally term-delivered breast-fed infants (n=11).

4.2 Methods

4.2.1 Administration of probiotics and prebiotics

In the Crying study (I, II), the mothers were randomized to receive $1x10^{10}$ colony-forming units (cfu) of LGG (ATCC 53103) (Valio Ltd., Helsinki, Finland) or placebo (microcrystalline cellulose, Chr. Hansen, Hoersholm, Denmark) capsules once a day for two to four weeks before expected delivery. After delivery, the capsule contents were given either to the children, mixed with water, or continuously to the mother, if breast-feeding, for six months.

In the Preterm study (III) infants were randomly assigned to receive a prebiotic mixture of polydextrose (Danisco Sweeteners, Surrey, UK) and galacto-oligosaccharides (Friesland Foods Domo, Zwolle, Netherlands) in a 1:1 ratio; 1 x 600 mg/day for 1.-30. days and 2 x 600 mg/day for 31.-60. days, probiotic LGG (ATCC 53103) (Mead Johnson & Co, Evansville, Indiana, USA) 1 x 10^9 cfu/day for 1.-30. days and 2 x 10^9 cfu/day for 31.-60. days or placebo (microcrystalline cellulose and dextrose anhydrate, Chr. Hansen, Hoersholm, Denmark). The study product was mixed with breast milk or formula and given to the infants by spoon or bottle.

In the Colic study (IV) infants were randomized to receive once a day probiotic LGG (ATCC 53103) (Mead-Johnson, Evansville, Indiana, USA) $3.4x10^9$ cfu/day or placebo (microcrystalline cellulose, Tamro Ltd, Finland) for 28 days. Breast-fed colic infants received the product mixed with breast milk or water given by spoon or bottle, and formula-fed infants received the product mixed with extensively hydrolyzed casein formula (Nutramigen-LGG®, Mead-Johnson, Evansville, Indiana, USA).

4.2.2 Clinical evaluation

In the Crying study (I, II) clinical evaluation was made by a physician at the ages of 3 weeks, 3 months, 6 months, and 13 years. In the Preterm study (III) follow-up visits were scheduled at the age of 1, 2, 4 and 6 months by the same study nurse, and at 12 months, and when needed, by the physician. In the Colic study (IV) infants were evaluated by the same study nurse and physician after enrolment (day 0), on day 7 and 28 and when needed. At enrolment parents were interviewed on the baseline characteristics. During the study visits a physician made a clinical examination of the children, including inspection of skin, eyes,

ears, nose, auscultation of heart and lungs, palpation of abdomen and femoral pulsations, and evaluation of growth and nutritional status and neurological development.

In the Colic study (IV) all the families received support by means of general information on infant colic, supportive discussion, and the continuous possibility to contact the study nurse and physician. Families were also offered a possibility to bring the infant to the hospital for an overnight stay in a case of parental exhaustion. During the study visit, the feeding technique of breast- and bottle-fed infants was evaluated.

Evaluation of allergy (III, IV)

At the age of one year in the Preterm study (III) and at enrolment in the Colic study (IV) atopic sensitization to cow's milk, egg white and wheat was assessed by skin prick test as previously described (Nermes, 2013).

In the Colic study (IV), to eliminate the effect of food allergy behind colic crying, all breast-feeding mothers were asked to avoid dietary products containing cow's milk and egg in their diet and formula fed-infants received extensively hydrolyzed casein formula (Nutramigen® or Nutramigen-LGG®, Mead-Johnson, Evansville, Indiana, USA) during the study period. At the end of the intervention, breast-feeding mothers returned to their normal diet and standard formula was re-introduced for formula-fed infants. In the case of suspicion of cow's milk allergy, a formal cow's milk challenge was implemented at the Department of Pediatrics, Turku University Hospital.

Evaluation of FGID (II)

The modified Rome III diagnostic questionnaire for pediatric FGID was used to describe the symptoms associated with the disorder according to the international pediatric Rome III criteria (Rasquin, 2006; Rome Foundation). The questionnaire comprises five sections: pain or discomfort in the upper abdomen above the umbilicus, pain or discomfort in the lower abdomen around and/or below the umbilicus, bowel habits, other gastrointestinal symptoms (nausea and vomiting), and impairment. The questionnaire was translated from English into Finnish and was completed by the children with parental help at home before the 13 years follow-up visit. At the study clinic the doctor assisted in completing missing items. The diagnosis of FGID was based on the questionnaire data.

Evaluation of neuropsychiatric disorders (unpublished)

The clinical diagnosis of ADHD and Asperger syndrome (AS) was made by a child psychiatrist or neurologist not involved in the study or follow-up and

blinded to the randomization. The diagnostic criteria of ICD-10 were used (WHO, 1992). According to these criteria, children with ADHD must have at least six of the listed symptoms of inattention and at least three of the listed symptoms of hyperactivity and impulsivity. These symptoms have to persist for at least six months to a degree which is maladaptive and inconsistent with the child's development level. The symptoms must have begun before the age of seven years, must be manifested in at least two places, negatively influence school, social or occupational functioning, and are not better explained by some other disorder. According to ICD-10, a person with Asperger syndrome shows no clinically significant general delay in spoken or receptive language or cognitive development, but has qualitative abnormalities in reciprocal social interaction and an unusually intense circumscribed interest or restrictive, repetitive and stereotyped patterns of behavior, interests and activities. The disorder is not attributable to other varieties of pervasive developmental disorder.

4.2.3 Assessment of infant behavior patterns

The Crying study (I, II)

The parents recorded their infants' behavior patterns using a modified 24-hour Barr chart on seven consecutive days during the 7th and 12th week of life (Barr, 1988; Kalliomäki, 2001). The caregivers recorded infant behavior patterns such as sleeping time, time awake and content, fussing, colic-type cry, other cry, as well as feeding times, vomits and stools with their consistency. The 24-h day was divided into 15-minute sections.

Fussing was defined as a state of irritability, "not quite crying but not awake and content". Other cry was defined as crying responsive to intervention (feeding, diaper change, carrying, sucking a pacifier), and colic-type cry as a cry not responsive to such intervention. Total distress, the amount of crying and fussing, was the sum of these various modes as reported by the parents.

The Preterm study (III)

During all study visits parents reported their infants' behavior patterns, including sleeping patterns, fussing, crying, irritability, feeding, vomits, stools (consistency and frequency), infection and other diseases and medication use. An infant was classified as an excessive crier if parents and study nurse reported excessive crying and irritability (> three hours/day) causing clinical concern without underlying medical causes during the 1- and 2-month study visits and resolving by the age of six months.

The Colic study (IV)
The caregivers were asked to fill in the validated 24-hour Baby Day Diary for three consecutive normal days before the commencement of the intervention to confirm the entry criteria, and at the end of it (Barr, 1988). The parents were given different symbols to record both infant behavioral status (sleeping time, time awake and content, fussing, crying, feeding) and parental behavior related to the infant (carrying/holding infant with body contact, taking care of the infant (i.e. changing, dressing), moving the infant without body contact (i.e. in the car or a baby carriage)). The smallest recording unit on the chart was five minutes and parents were instructed to fill in the diary when convenient, usually using feeding intervals. The same research nurse instructed them in the use of the diary.

In addition, during all study visits parents reported the infant's behavior patterns, including sleeping patterns, crying characteristic (daily crying time (min/day), number of days per week of infant cry over three hours), feeding, regurgitation, vomits, stools, infection and other diseases, medication use and adverse events as well as parental coping. Crying was defined as continuous crying, and fussing as non-continuous negative vocalization which is not crying. Total distress, the amount of crying and fussing, was the sum of these two modes as reported by the parents.

4.2.4 Gut microbiota analysis (I, III, IV)

A fecal specimen was taken at the ages of 3 weeks (I), 1 (III), 3 (I), and 6 (I) months and in the Colic study (IV) on day 0 and 28 either by nursing staff at a scheduled visit or by the parents immediately prior to the visit. If a sample was taken before a visit, it was stored at 4°C and delivered to the hospital within 24 hours.

FISH (I, III)
Homogenized fecal samples were fixed overnight in 4% paraformaldehyde and stored in phosphate-buffered saline (PBS)-ethanol at -20^{o}C (maximum three months) until analyzed. Fluorescent *in situ* hybridization (FISH) with microscopic detection was performed as previously described (Kalliomäki, 2001). In short, samples were hybridized at specific temperatures in hybridization buffer with specific probes. After overnight hybridization they were washed with buffer without SDS, pelleted, and re-suspended in PBS. An EUB 338 probe was covalently linked at the 5´ end with fluorescein isothiocyanate (FITC) and other probes with carbocyanine 3 (Cy3) (Thermo Biosciences, Ulm, Germany). Probes included were Bac303

(CCAATGTGGGGGACCTT) specific for *Bacteroides-Prevotella*, Bif164 (CATCCGGCATTACCACCC) for *Bifidobacterium*, His150 (TTATGCGGTATTAA TCT(C/T)CCTTT) for *Clostridium histolyticum*, Lab158(GGTATTAGCA(T/C)CTGTTTCCA) for *Lactobacillus-Enterococcus* and Muc 1437 (CCTTGCGGTTGGCTTCAGAT) for *Akkermancia muciniphila* (sequence 5′→3′) as previously reported (Rigottier-Gois, 2003). Total cell numbers were counted using a nucleic acid stain, 4′,6-diamidino-2-phenylindole.

qPCR (I, III, IV)

Fecal samples were stored at -80ºC until analyzed for quantitative PCR (qPCR). Samples were pre-treated and DNA extracted using an automated KingFisher DNA extraction system (Thermo Fisher Scientific Oy, Vantaa, Finland) and InviMag Stool DNA kit (Stratec Molecular, Berlin, Germany), as previously described (Nylund, 2010). The standard DNA for qPCR was prepared as previously described (Nylund, 2010). The DNAs, extracted from bacterial suspensions of *Bifidobacterium adolescentis* DSM 20083, *B. bifidum* DSM 20456, *B. breve* DSM 20213, *B. catenulatum* DSM 16992, *B. dentium* DSM 20436, *B. infantis* DSM 20090, *B. lactis* Bb-12, *B. longum* DSM 20219 (also for *Bifidobacterium* genus), *Bacteroides fragilis* DSM 2151^T, *Clostridium coccoides* DSM 935, *C. difficile* DSM 1296, *C. leptum* DSM 753^T, *C. perfringens* DSM 756, *S. aureus* DSM 20231, *and Escherichia coli* K-12 (for total bacteria) were used as standards for quantification. All DNA samples were stored at -20ºC until analyzed. Quantitative PCRs were conducted as described elsewhere (Collado, 2008; Scalabrin, 2012). PCR amplification and detection were performed with an ABI PRISM 7300-PCR sequence detection system (Applied Biosystems, Foster City, CA).

PCR-DGGE (I)

In the fecal samples the composition of *Lactobacillus* species and *Bifidobacterium* species was analyzed by PCR-DGGE. PCR amplification of the DNA of each bacterial group was as described elsewhere (Endo, 2010). Amplification was confirmed by gel electrophoresis in 1.0% agarose. DGGE analysis of each PCR product was conducted with a DCode System (Bio-Rad Laboratories, Hercules, CA, USA), as previously described (Endo, 2005). DNA bands were re-amplified using the same primer set as for generating the DGGE samples. The PCR products were purified and sequenced according to a method described elsewhere (Endo, 2005). Sequences were compared with known sequences at the GenBank by BLAST analyses. The detection limit was approximately 10^5 cfu/g of feces.

4.2.5 Statistical analysis

Data analysis was performed using commercially available software SAS (version 9.2; SAS Institute, Cary, North Carolina) or SPSS (version 20; SPSS Inc, Chicago, Illinois). A *P*-value of <0.05 was considered statistically significant. The clinical characteristics of the study subjects are reported as mean values with standard deviations (SD) or medians with range for continuous variables and as numbers and proportions for categorical variables. Differences between study groups were compared using Chi-square test or Fisher's exact test, as appropriate, for categorical variables, independent samples t-test for normally distributed continuous variables, and Mann-Whitney *U* or Kruskal-Wallis test for non-normally distributed continuous variables, if not otherwise stated. Statistical consultation was provided by statistician, MSc, Jaakko Matomäki.

The Crying study (I)
Associations between amount of total crying and predictor variables were analyzed by mixed model repeated measures analysis. Univariate associations between the amount of total crying during the first three months of life (mean of the total crying at 7^{th} and 12^{th} weeks of life) and numbers of gut microbiota bacteria at six months were analyzed using linear regression. The variables of total distress, proportion of *Bifidobacterium* counts to total bacterial counts (%) and proportion of *Lactobacillus* counts to total bacterial counts (%) were square-root transformed in the statistical analyses.

The Crying study (II)
Univariate associations between the response variable (FGID, neuropsychiatric disorders) and continuous variables were studied by logistic regression analysis.

The Preterm study (III)
All analyses were carried out on data from the intention-to-treat population. Univariate associations between clinical characteristics and the dichotomous outcome variable of excessive crying were analyzed using logistic regression.

The Colic study (IV)
Alterations in the amounts of microbiota between the two evaluation points were categorized into three groups (an increase, no change, a decrease) and then compared between the intervention groups using Chi-square test for trend, because a large proportion of observations were below the detection limit.

4.2.6 Ethics

The studies were conducted according to the guidelines laid down in the Declaration of Helsinki. The studies were found ethically acceptable by the Ethics Committee of the Hospital District of South-West Finland. Written informed consent was obtained from the infants' parents (I, II, III, IV) and children (II).

5 RESULTS

5.1 Baseline characteristics (I-IV)

The baseline and clinical characteristics of the study populations (I-IV) are presented in Tables 2a and 2b. All the variables were comparable among the study groups except birth weight ([a] p=0.02) and birth length ([b]p=0.03) in Study II, birth weight ([c]p=0.02, [d]p=0.01) and gestational age ([e]p=0.01) in Study III and gestational age ([f]p=0.03) in Study IV.

Table 2a and 2b. The baseline characteristics of the participants.

	Study I	**Study II**	
	n=89	Healthy n=59	FGID n=15
Early LGG supplementation	42 (47)	31 (53)	9 (60)
Vaginal delivery	73 (82)	47 (80)	14 (93)
Male	59 (66)	30 (51)	10 (67)
Birth weight (g)	3601 (480)	3654 (596)[a]	3364 (438)[a]
Birth length (cm)	51 (0.5)	51 (2.0)[b]	50 (1.8)[b]
Gestational age (wk)	40 (35-42)	40 (35-42)	39 (36-41)
Apgar score (5min)	9 (8-10)	9 (8-10)	9 (8-10)
Exclusive breast-feeding (mo)	2.5 (1.7)	3.0 (1.7)	3.7 (1.7)
Total duration of breast-feeding (mo)	5.5 (3.6)	6.8 (3.6)	7.9 (5.0)

	Study III			**Study IV**	
	Probiotic n=31	Prebiotic n=31	Placebo n=32	Probiotic n=15	Placebo n=15
Perinatal antibiotic exposure	10 (32)	6 (19)	10 (31)	9 (60)	10 (67)
Vaginal delivery	24 (77)	20 (65)	20 (63)	13 (87)	12 (80)
Male	19 (61)	16 (52)	23 (72)	7 (47)	5 (33)
Birth weight (g)	2494 (409)[c]	2175 (463)[c,d]	2505 (523)[d]	3490 (430)	3320 (520)
Birth length (cm)	47 (2.0)	46 (2.1)	47 (3.1)	51.2 (2.0)	50.3 (2.2)
Gestational age (wk)	35 (32-36)	34 (32-36)[e]	35 (32-36)[e]	41 (38-42)[f]	39 (37-41)[f]
Apgar score (5min)	8 (4-10)	8 (0-10)	8 (5-9)	9 (7-9)	9 (7-10)
Exclusive breast-feeding (mo)	1.6 (2.2)	1.3 (1.9)	1.9 (2.1)	-	-
Total duration of breast-feeding (mo)	7.2 (4.4)	5.5 (3.3)	5.9 (4.5)	-	-
Exclusive breast/ formula-feeding	-	-	-	6 (40)/ 2 (13)	5 (33)/ 2 (13)

Results are given as mean (SD) or median (range) or as number (%) of subjects.

5.2 The association between gut microbiota composition and infant crying (I, III, IV)

In the Crying study (I) the association between gut microbiota composition and daily crying time was analyzed as a continuous variable focusing on the entire spectrum of crying, not only colic crying, among the whole study population, since the use of LGG during the first six months of life had no effect on infant total distress during the 7th or 12th weeks of life (p=0.74 and p=0.82, respectively).

In the Crying study (I) at the age of three weeks the prevalence of *Lactobacillus* species was inversely associated with the daily crying amount (crying and fussing) during the 7th week of life (p=0.02). In the same vein, the proportion of *Bifidobacterium* at the age of three months was inversely associated with the daily crying time during the first three months of life (p=0.03). On the contrary, the number of *Bifidobacterium breve* at the age of three months was positively associated with the total distress during the first three months of life (p=0.02).

Furthermore, these gut microbiota differences were still seen at the age of six months, after the peak period of crying as the total number of *Bifidobacterium* and *Lactobacillus* were inversely associated with the daily crying amount experienced during the first three months (r=-0.27 and p=0.03; r=-0.32 and p=0.008, respectively; Figure 5a and Figure 5b).

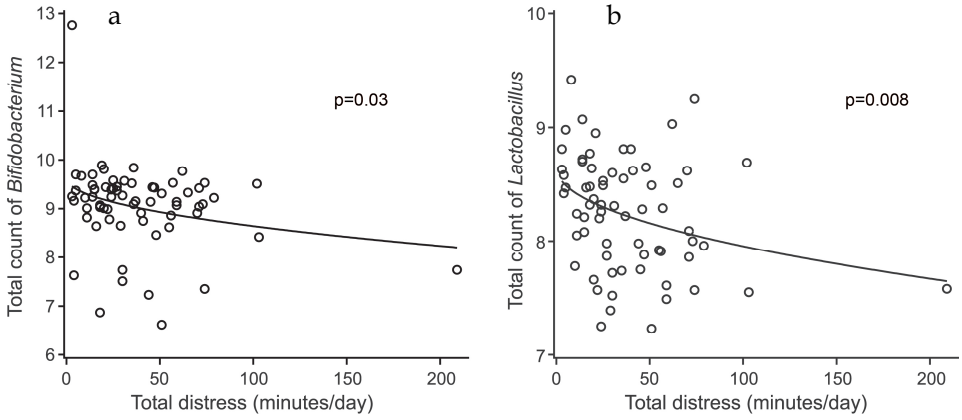


Figure 5a and 5b. A correlation curve between the daily total crying amount (crying and fussing) during the first three months of life and the number of *Bifidobacterium* (Figure 5a) and *Lactobacillus* (Figure 5b) at six months of age.

In the Preterm study (III) the composition of gut microbiota was analyzed between excessive criers and content infants by FISH and qPCR at the age of one month. Excessive criers had higher proportion of *Lactobacillus -Enterococcus*-group and *Clostridium histolyticum*-type of bacteria than content infants (Figure 6).

In the analysis of the gut microbiota composition by qPCR, the number of *Bifidobacterium infantis* was seen to be lower among excessive criers when compared to contented infants (1.3×10^7 vs. 2.5×10^8, respectively; p=0.04). However, there were no other statistically significant differences in gut microbiota composition between the groups by qPCR (data not shown).

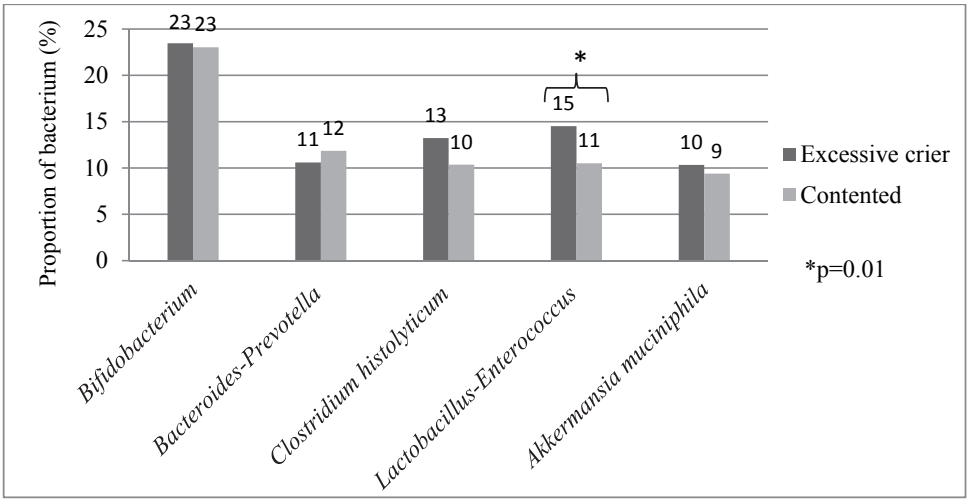


Figure 6. The mean proportion (%) of different bacterium counts to total bacterial count by FISH at the age of 1 month among excessive criers and contented infants in the Preterm study (III).

In the Colic study (IV) the gut microbiota of the colic infants was compared with that in healthy vaginally-delivered breast-fed controls. Colic infants had lower numbers of *Bifidobacterium* genus than healthy controls (1.1×10^9 vs. 4.6×10^{10}, respectively; p=0.04). Furthermore, the colic infants were colonized less frequently with *Bifidobacterium breve*, although the difference did not reach statistical significance (13% vs. 50%, respectively; p=0.07). Interestingly, this difference remained throughout the four weeks' study-period despite the fact that half of the colic infants received probiotics (27% vs. 64%, respectively; p=0.06).

5.3 The association between infant crying and the development of functional gastrointestinal and neuropsychiatric disorders later in childhood (II, unpublished data)

The infant crying study (II) assessed the association between early crying and children's later health outcomes, functional gastrointestinal and neuropsychiatric disorders. By the age of 13 years 20% of the study population had been diagnosed with FGID (Table 3), 4% with ADHD, 1% with AS and 3% with both ADHD and AS.

Table 3. Distribution of FGID diagnoses by the age of 13 years (II).

Diagnose	**n=74**
Abdominal migraine	7 (9.5)
Irritable bowel syndrome	4 (5.4)
Functional constipation	2 (2.7)
Aerophagia	2 (2.7)
Functional abdominal pain	1 (1.4)
Adolescent rumination syndrome	1 (1.4)

Results are given as numbers (%) of subjects.

Functional gastrointestinal disorders were manifested by the age of 13 in 6% and 28% of children without and with colic-type crying during the 7^{th} week of life, respectively (p=0.05). Similarly, children with FGID at the age of 13 had had more colic-type crying (min/day) during the 7^{th} and 12^{th} weeks of life than children without FGID (Table 4). Other modes of infant distress were similar between the groups (Table 4).

However, there was no difference in daily crying profiles during the first three months of life between children with neuropsychiatric disorders by the age of 13 years and those who remained healthy (Table 4, unpublished data).

Table 4. Daily crying profiles at the age of 7 and 12 weeks of children manifesting and not manifesting FGID and ADHD/AS by the age of 13 years (II, unpublished data).

	Healthy	**FGID**	**p-value**	**Healthy**	**ADHD/AS**	**p-value**
Crying during the 7th week of life (min/day)	n=41	n=10		n=47	n=5	
Fussing	56 (50)	80 (48)	0.20	61 (50)	57 (55)	0.86
Other cry	31 (27)	33 (21)	0.82	32 (26)	30 (30)	0.87
Colic-type cry	10 (13)	31 (26)	0.01	14 (18)	15 (20)	0.93
Total distress	97 (70)	143 (81)	0.09	107(73)	101 (87)	0.87
Crying during the 12th week of life (min/day)	n=39	n=10		n=45	n=5	
Fussing	39 (37)	30 (20)	0.48	36 (34)	39 (40)	0.90
Other cry	23 (30)	26 (20)	0.74	24 (29)	9 (18)	0.24
Colic-type cry	4 (8)	14 (22)	0.08	7 (13)	2 (4)	0.43
Total distress	66 (47)	70 (49)	0.80	68 (46)	50 (50)	0.42

Results are given as mean (SD) minutes per day.

5.4 The effect of the probiotic and prebiotic supplementation on preterm infant crying (III)

During the first two months of life 29% of the Preterm study population (n=94) were defined as excessive criers. The proportion of criers was significantly lower in the pro- and prebiotic groups than in the placebo group (Figure 7).

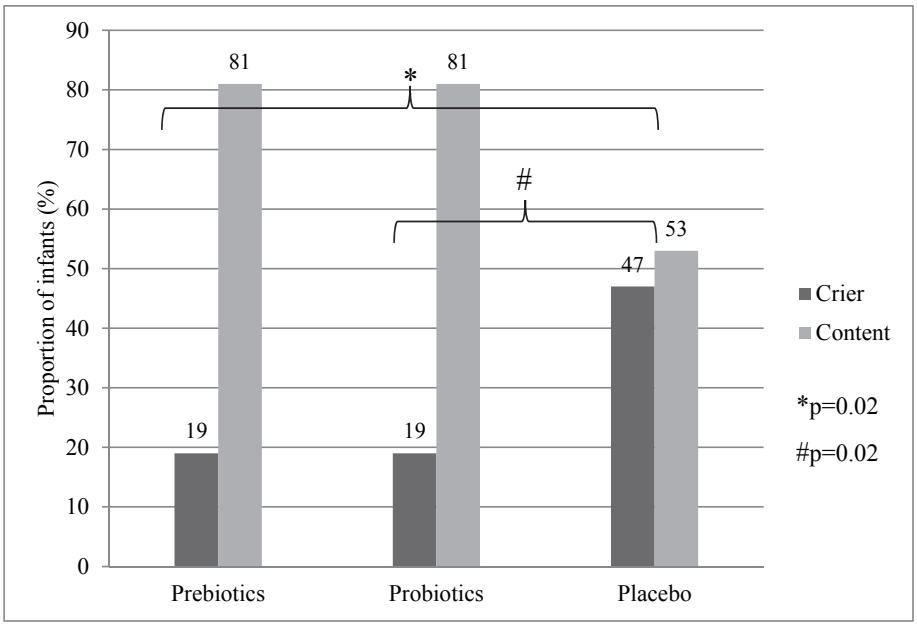


Figure 7. The proportion (%) of infants classified as excessive crier (n=27) or content (n=67) during the first two months of life in the Preterm study (III).

5.5 The impact of the probiotic and prebiotic interventions on gut microbiota composition in preterm infants (III)

The proportion of *Clostridium histolyticum*-type bacteria to total bacterial counts at the age of one month (Figure 8) was the only significant difference in the Preterm study (III) found between the three intervention groups in the gut microbiota analyses by FISH and qPCR. This proportion was highest in the placebo group and lowest in the probiotic group (Figure 8).

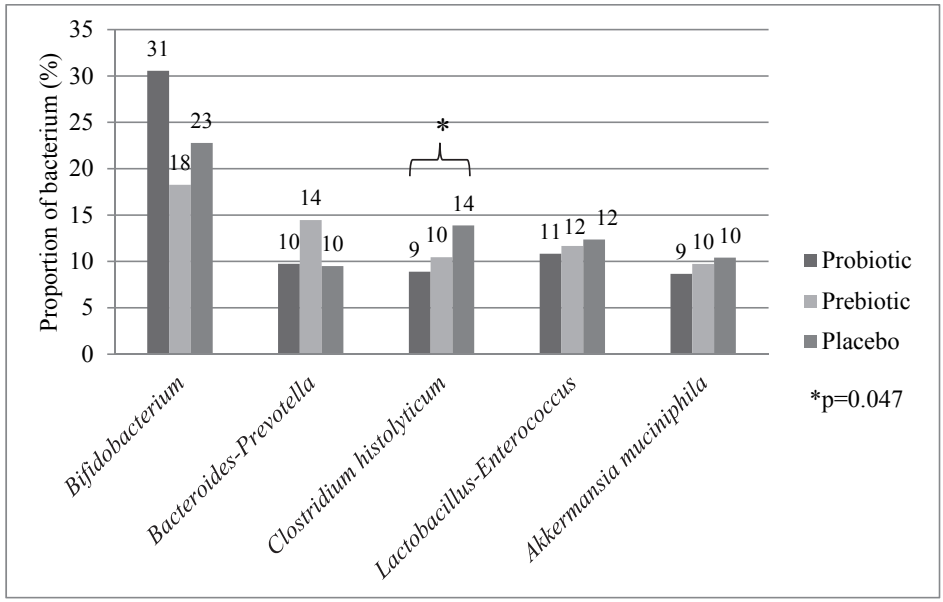
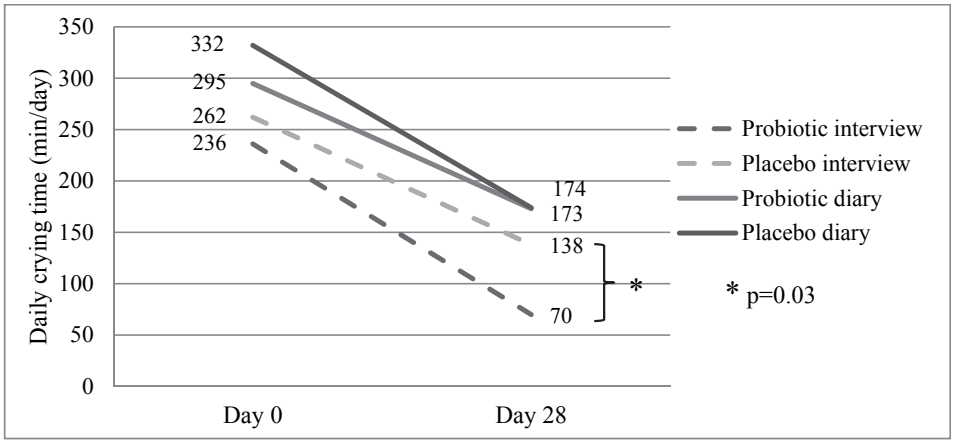


Figure 8. The mean proportion (%) of different bacterium counts to total bacterial count in stool samples in the Preterm study among pro-, prebiotic and placebo groups at the age of one month (III).

5.6 The effect of the probiotic intervention on crying in colic infants (IV)

During the Colic study (IV), the mean (SD) daily crying time (min/day) decreased 68% (18) in the infants who received LGG and 49% (30) who received placebo (p=0.0495), as reported by a parental interview. However, based on the diary data the effect of LGG was not significant (Figure 9a). At the end of the study, the mean daily crying time and the number of days the infant cried over three hours per day were significantly lower in the probiotic group compared to placebo according to the interview (Figure 9a and 9b). In addition, the number of responders, i.e. infants whose daily crying decreased over 50%, was significantly higher in the probiotic than in the placebo group according to the interview (87% vs. 47%, respectively, p=0.02).



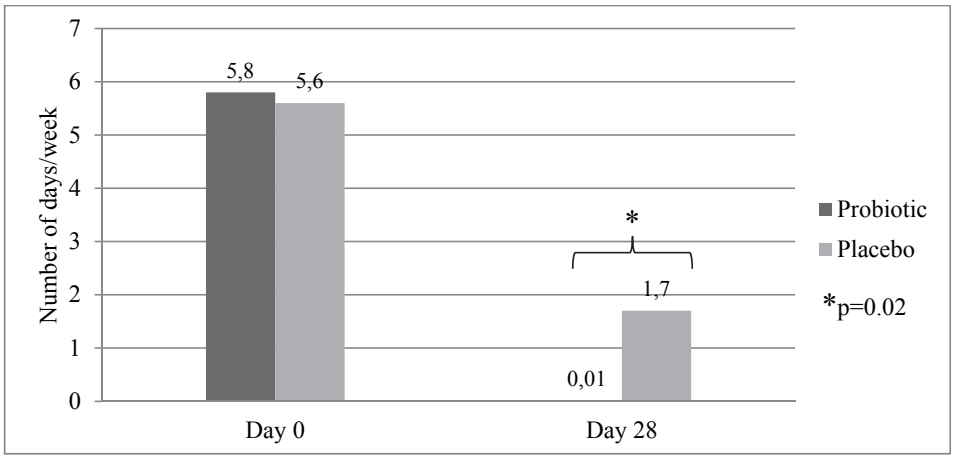


Figure 9a and 9b. Mean daily crying time (based on interview and diary) and number of days the infant cried over three hours (based on interview) during the study period in the probiotic and placebo groups (IV).

5.7 The influence of probiotics on gut microbiota composition among colic infants (IV)

In the Colic study (IV) there were no statistically significant differences in gut microbiota composition analyzed by qPCR between infants in the LGG and placebo groups at day 0 or day 28. Nevertheless, the detection rate of *B. infantis* tended to be lower in the probiotic than in the placebo group at day 28 (27% vs. 60%, respectively; p=0.07). Furthermore, a large proportion of observations was below the detection limit and therefore within-group changes (any increase/decrease) in the composition of *Bifidobacterium* were also analyzed (Figure 10).

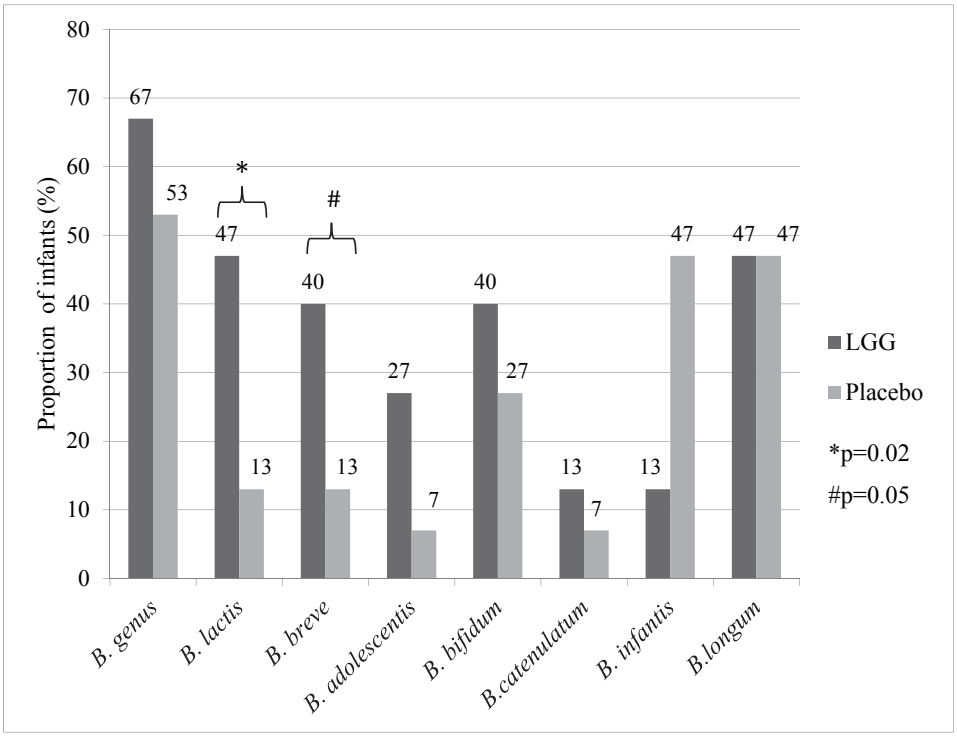


Figure 10. The mean proportion (%) of infants in each study groups in whom the amount of different *Bifidobacterium* species increased during the study period (IV).

5.8 Safety and adverse events in the probiotic and prebiotic interventions (III, IV)

Probiotic intervention in the Crying study (I) has previously been demonstrated to be safe and no side-effect has been related to it (Laitinen, 2005, Rinne, 2006). These findings were extended here to preterm (III) and colic infants (IV). In neither of the studies were there withdrawals attributable to any adverse effects related to the study products or their administration regimen.

The pro- or prebiotic interventions (III, IV) had no influence on the growth of the infants during the study periods. In the Preterm study (III) the mean (SD) weights at the age of one year were similar among the probiotic, prebiotic, and placebo groups (9717g (1415) vs. 9873g (1083) vs. 10107g (1633), respectively p=0.75). Similarly, in the Colic study (IV) mean weight gains were comparable between the probiotic and placebo groups during the study period (867g (326) vs. 941g (308), respectively; p=0.54).

The effect of the interventions on gastrointestinal function (stool consistency, frequency and regurgitation) was also analyzed as an adverse event in the Preterm (III) and the Colic studies (IV). In the Preterm study (III) the frequency of infants with more than three stools per day tended to be higher in the prebiotic than in the probiotic and placebo groups at the age of one month (71% vs. 50% vs. 57%, respectively; p=0.08), but there was no difference among the groups in consistency of stool. During the four-week follow-up period in the Colic study (IV) the number of daily regurgitations reported by the parents increased more often in infants receiving placebo than probiotic (73% vs. 27%, p=0.04). However, stool frequency was comparable between the groups (data not shown).

6 DISCUSSION

6.1 Gut microbiota composition as a possible cause of crying and colic

Colic crying coincides timely with maturational processes such as immunological maturation and the compositional development of the gut microbiota as well as changes in feeding patterns. These processes take place especially in the gastrointestinal tract in response to antigen challenges by milk and microbial colonization. The gut-associated lymphoid tissue is responsible for maintaining mucosal immune homeostasis. It distinguishes between harmless and pathogenic antigens and invokes immunity to pathogens while sustaining tolerance to innocuous antigens. This developmental simultaneity adds weight to the idea that deviations in gut microbiota composition might be one of the causes behind infant excessive crying. On the other hand, any inflammation such as food allergy or infection-related might alter gut microbiota colonization, and the possibility of an epiphenomenon must thus also be kept in mind, i.e. deviations in the gut microbiota might be cause or consequence of infant colic (Isolauri, 2012).

In any case, the results of the Crying (I) and the Colic study (IV) support earlier findings indicating that crying infants have decreased numbers of *Lactobacilli* and *Bifidobacteria* compared to healthy controls (Savino, 2004; de Weerth, 2013b). However, the Crying study (I) was first to analyze the gut microbiota composition among infants across the entire spectrum of crying, not only colic and non-colic infants. These findings support the previous results regardless of whether the child fulfills the criteria of colic or not. Consequently, colic crying would rather constitute an arbitrary upper limit of normal crying than a separate clinical entity. Similarities in crying behavior in infants with and without colic further supports this conception (Brazelton, 1962; Hunziker, 1986; Barr, 1992; St James-Roberts, 2012).

Contrary to previous results, the Preterm study (III) demonstrated that the number of *Lactobacillus–Enterococcus* group was higher in excessive criers than content infants. Both high amounts of *Lactobacillus* and *Enterococcus* have been related to prematurity and are thought to be the first colonizers of the intestine (Arboleya, 2012). Consequently, this finding might indicate that excessively crying preterm infants might evince a less mature gut microbiota colonization process than contented controls. Moreover, preterm infants have several risk factors associated with perturbation of the development of the gut microbiota, for example increased numbers of cesarean sections, more common use of peripartal

antibiotics, greater frequency of formula-feeding and a reduced amount of skin-to-skin contact compared to term infants (Berrington, 2012). However, according to the results here, these confounding factors did not influence the gut microbiota composition, since they were similar between excessive criers and content preterms. Furthermore, in line with a previous work, the Preterm study (II) demonstrated that excessively crying preterm infants tended to have a higher proportion of *Clostridium histolyticum*-type of bacteria than contented infants. However, an interesting earlier finding is that one week's treatment with metronidazole, an effective antibiotic against *Clostridium difficile*, has not proven more effective than placebo in the treatment of colic symptoms further supporting the idea that small-bowel bacterial overgrowth or *Clostridium difficile* infection is not a significant cause of infant colic in term infants (Hochman, 2005).

Furthermore, individual specific *Bifidobacterium* species and even different strains exert distinct effects on immunity and disease risks such as allergies (He, 2002; Young, 2004). Our findings here suggest that content preterm infants have higher amounts of *Bifidobacterium infantis* than excessive criers. This species has been found to strengthen the gut-barrier defenses and has been associated with the appropriate development of immune tolerance (Ewaschuk, 2008; Chichlowski, 2012). Similarly *Bifidobacterium breve,* the colonization of which is associated with reduced abdominal gas load, tended to be decreased in term colic infants when compared to healthy controls (IV), (Kitajima, 1997). However, in contrast to the results of the Colic study (IV), those of the Crying study (I) indicated that *Bifidobacterium breve* is associated with increased amounts of daily crying and fussing. Although the study populations were different and the Crying study cohort consisted of infants from allergy-risk families, this finding emphasizes the complexity of the infant gut microbiota composition during its development, and the intricacy of interpreting of what is normal colonization. This also constitutes a reminder that we are far from understanding exactly how the gut microbiota development differs between colic and healthy infants.

The present series (I), together with a recent Dutch study, would indicate that gut microbiota deviations in crying infants already exist during the first weeks of life, before the peak period of crying (de Weerth, 2013b). This supports the hypothesis that deviations in gut microbiota might be the cause of colic, not a consequence. However, in most studies the gut microbiota composition has been evaluated only during the colic period. There is only one longitudinal study monitoring the development of the gut microbiota at the age of 3-4 months. This study found no significant difference in the gut microbiota composition between

the colic and control group (de Weerth, 2013b). Nevertheless, in the Crying study (I) the development of the gut microbiota was also analyzed at the age of 6 months and the findings suggest that microbiota alterations persist even though the amount of crying decreases. It is thus intriguing to speculate whether these alterations are permanent or whether they disappear over time.

In addition to the finding in the Crying study (II) that colic crying may precede the development of FGID later in life, a search for associations between infant crying and the subsequent development of FGID is justified on the basis of a possible common etiology, aberrant gut microbiota composition (Francavilla 2010; Simren, 2013). Parallel to colic infants, patients with FGID have proven to have decreased numbers of *Lactobacillus* and *Bifidobacterium*, and increased numbers of *Escherichia coli* and *Clostridium,* supporting the conception that infant colic might be associated with the later development of FGID (Simren, 2013). These microbiota alterations in FGID patients are thought to activate mucosal innate immune responses, increasing epithelial permeability and activating sensory pathways leading to dysregulation of the enteric nervous system (Simren, 2013). It is conceivable that similar mechanism of action could be involved in colic crying.

In conclusion, there is accumulating evidence to indicate that the gut microbiota of colic infants is different from that of healthy infants, but it is too early to state whether these gut microbiota deviations are the cause of colic or simply an epiphenomenon. Well-controlled and -powered longitudinal colic studies including gut microbiota analysis before and after colic period could give an answer to this question.

6.2 Potential mechanisms connecting gut microbiota and crying

The early gut colonization process begins *in utero* and proceeds in a stepwise manner during birth and infancy (Rautava, 2012b). This process is substantially influenced by a number of factors, including maternal gut and vaginal microbiota, maternal health status, diet and probiotic use during pregnancy, prenatal and postnatal antibiotic use, mode of delivery, type of feeding, and environmental factors in hospital and at home (Rautava, 2012b). Consequently, an aberration in any of these components might hold the answer to gut microbiota alterations underlying colic crying.

Prenatal stress has been shown to modulate the intestinal physiology by increasing gut permeability as well as influencing the microbiota composition (Santos, 2000; Bailey, 2004). Furthermore, maternal stress during pregnancy has

been found to be associated with the development of colic, suggesting possible causality between perinatal stress, gut microbiota and colic (Miller, 1993; Rautava, 1993; Akman, 2006). However, adverse environmental circumstances during birth, for example labor onset and analgesia, use of syntocinon to accelerate labor, fetal heart rate during labor, resuscitation, antenatal complications or Apgar scores have not proven to be associated with infant crying or colic (St James-Roberts, 2005).

Probiotic and antibiotic use as well as diet during pregnancy influence the composition of maternal gut microbiota, which in turn is one of the major factors influencing gut microbiota colonization in an infant (Rautava, 2012a; Rautava, 2012b). In the Colic study (IV) the use of probiotics during pregnancy tended to be higher in the placebo group (47%) than in the probiotic group (20%), which might have strengthened the true placebo effect. In the Crying study (I) 47% of the mothers received probiotics during the last month of pregnancy according to the study protocol. However, the probiotic supplementation was not associated with the total daily crying time in this study. Earlier colic studies have not reported the use of probiotics during pregnancy (Savino, 2007b; Savino, 2010; de Weerth, 2013b), and this should be taken into account in future studies. However, this may nowadays be challenging at least in Finland, where many dairy products and juices contain probiotics and pregnant women might consume them without being aware that they are using probiotics.

The effect of the maternal diet during pregnancy on the development of crying has not so far been widely explored, nor was this aspect studied in this series. One earlier study has found that a low maternal serum vitamin B12 concentration during the first trimester of pregnancy is associated with excessive infant crying (Goedhart, 2011). The authors assumed that the possible mechanism behind this theory could involve the methionine-homocysteine metabolism, which is essential for neurodevelopment, or the maturation of the sleep-wake rhythm, because B12 vitamin is also involved in melatonin synthesis (Goedhart, 2011). Maternal nutrition and health status during pregnancy constitute the first step influencing the colonization process in the infant gut microbiota. Maternal diet further influences the composition of breast-milk, which constitutes the second step in this continuous colonization process.

Breastfeeding has a major role in the gut microbiota colonization process in early life (Harmsen, 2000). *Bifidobacteria* have been reported to dominate the gut microbiota of breast-fed infants, while the microbiota in formula-fed infants is more diverse (Harmsen, 2000; Rautava, 2012b). These deviations might be due to the unique composition of maternal milk or possible direct effects of microbes

in breast-milk (Rautava, 2012b; de Weerth, 2013a). Maternal diet and health status influence the composition of breast-milk, and the diet of the breast-feeding mother is associated with colic crying, as previously discussed (Iacovou, 2012; Rautava, 2012b). In the last analysis, breast-feeding *per se* has not been shown to provide a protective effect on the development of infantile colic, and the incidence of infant colic is similar among formula- and breast-fed infants (Lucassen, 2001; Clifford, 2002b; Yalcin 2010), although breast-feeding has proven to provide important maturational stimuli to the developing immune system (Kainonen, 2013). These results are also supported by those of the Preterm study (III), since there was no significant difference in the total duration of breast-feeding between excessive criers and contented infants.

Infants born by cesarean section have lower microbial diversity, lower numbers of *Bifidobacteria*, *Lactobacilli* and *Bacteroides* as well as higher numbers of *Clostridia* (Penders, 2006). These gut microbiota alterations are in line with the composition of the gut microbiota in colic infants, suggesting a possible linkage between mode of delivery and colic. However, according to the results of the present and some earlier studies, excessive crying or colic presents with similar frequency among infants born by vaginal delivery or cesarean section, and thus the mode of delivery independently may not play a key role as a cause of infant colic (Mentula, 2008; Rhoads, 2009; Yalcin, 2010).

Finally, the environment sets its own pattern on the gut microbiota colonization process. First-born infants have slightly lower numbers of *Bifidobacteria* and are earlier colonized with *Clostridia* than infants with older siblings (Penders, 2006; Adlerberth, 2007). Nevertheless again, the prevalence of colic has been shown to be similar among infants with and without older siblings (Illingworth, 1954; St James-Roberts, 1991; Yalcin, 2010).

As a conclusion, many of the separate factors such as mode of delivery and type of feeding which might interrupt the stepwise process of colonization in the colic infant have already been widely studied, and there is evidence to indicate that these factors, at least separately, may not be associated with the development of colic. However, the effect of maternal health status, diet, the use of probiotics and antibiotics during pregnancy or their joint effect on gut microbiota development in the colic infant has not been widely investigated and should be addressed in the future (Figure 11).

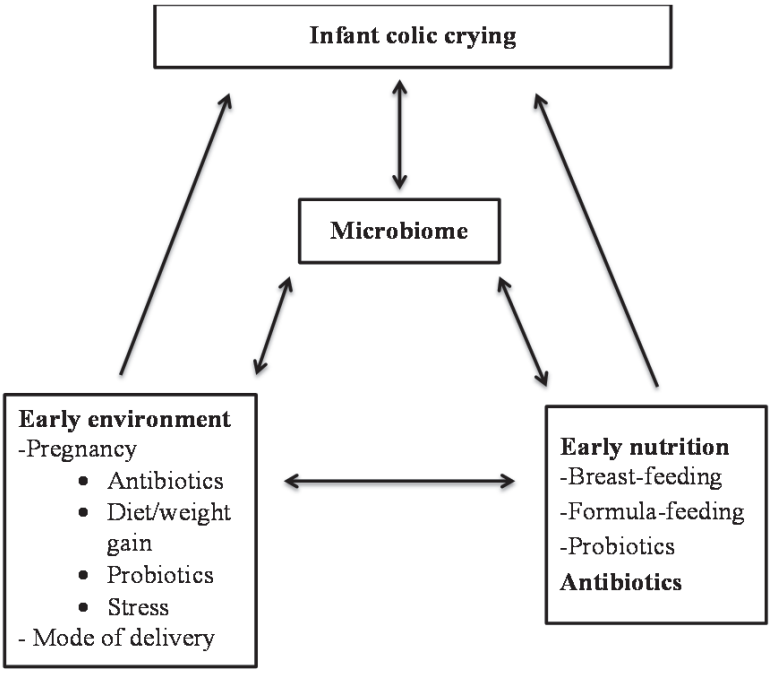


Figure 11. The possible key determinants influencing gut microbiota and colic crying.

6.3 The management of infant crying and colic

Current treatment methods for infant colic are various and controversial, including behavioral, nutritional and pharmacological approaches. With the self-limiting nature of colic many types of therapies seem effective in single cases, but obviously for the reason that the precise pathogenesis of colic remains elusive, no evidence-based form of therapy thus far exists. The following section focuses mainly on treatment options involving the gastrointestinal tract.

All families with an excessively crying infant should be recognized and ensured referral to health care professionals to exclude potential serious underlying conditions (Freedman, 2009). If history and physical examination raise clear suspicion of colic crying, families should be offered information on colic and support during this stressful period of life (Freedman, 2009; St James-Roberts 2012), since maternal emotional distress, anxiety and even child abuse might ensue without an appropriate measure of support (Miller, 1993; Räihä, 1996; Akman, 2006; Lee 2007).

Since some breast-fed colic infants seem to benefit from a maternal low-allergen diet, some formula-fed infants, again, from the use of hydrolyzed formulas, these treatment options might be tried out, especially in severe colic cases (Iacovou,

2012). Also the nature of dietary interventions as a low-risk and drug-free approach supports their use. However, strict hypoallergenic diets are difficult for mothers to follow, they may expose mothers to low intake of protein, calcium and vitamin D and therefore their use should be evaluated case-specifically and discussed with parents.

There are abundant pharmacological treatment options such as anticholinergic and homeopathic medications, as well as herbal extracts marketed as treatment for colic. However, none of these can thus far be recommended as an effective and safe treatment (Danielsson, 1985; Weizman, 1993; Metcalf, 1994; Alexandrovich, 2003; Savino, 2007b; Arikan, 2008). For example, up to 16% of parents of colic infants have tried simethicone, although there is accumulating evidence that it is not more effective in reducing crying than placebo (Danielsson, 1985; Metcalf, 1994; Headley, 2007; Savino, 2007b). However, the most important question concerns the safety profile, appropriate dosage as well as nutritional and immunomodulatory aspects of these various pharmacological and herbal approaches and they need to be clarified in randomized placebo-controlled trials before they can be recommended as treatment options for infant colic. It should be kept in mind that the anticholinergic medication dicyclomine hydrochloride, an effective treatment for infantile colic, had to be withdrawn from the market, because of serious adverse effects related to a minority of treated infants (Hwang, 1985; Randall, 1986). Similarly, in a retrospective case-control study Gali-col Baby, a homeopathic agent indicated for infant colic, was associated with an apparent life-threatening event (Aviner, 2010).

On the other hand, repeated findings that the administration of sweet treats during painful procedures reduces crying safely have led researchers to study the effect of glucose and sucrose on infant colic (Stevens, 2010). Three published randomized controlled trials have shown that glucose or sucrose solutions might reduce the symptoms of infant colic (Markestad, 1997; Akcam, 2006; Arikan 2008). However, none of these studies was without methodological limitations and the results should be repeated before any reliable conclusion can be drawn.

6.3.1 Is there a role for probiotics and prebiotics as a treatment option for colic?

One of the most promising new advances in the management of infant colic is the modulation of gut microbiota by means of probiotics, especially *Lactobacillus reuteri* (Sung, 2013; Anabrees, 2013). Hitherto, six randomized controlled trials

(RCT), including the Colic study (IV), have investigated the effects of probiotics in treating infant colic (Savino, 2007b; Mentula, 2008; Dupont, 2010; Savino, 2010; Szajewska, 2013). However, some other RCTs, have reported the preventive effect of probiotics on infant colic or crying in general or as a secondary outcome in term (Saavedra, 2004; Rinne, 2006; Vendt, 2006; Weizman, 2006; Chouraqui, 2008; Kukkonen 2008; Vlieger, 2009; Rozé, 2012) and preterm infants (Indrio, 2008). Only three of these studies report as a positive preventive effect of probiotics on crying or colic when compared to placebo (Saavedra, 2004; Indrio 2008; Rozé, 2012).

The first study to support the effectiveness of probiotic in treatment of infant colic was an open randomized controlled trial of 83 breast-fed colic infants who received probiotic *L. reuteri (*ATCC 55730) or simethicone for 28 days during the first three months of life (Savino, 2007b). All breast-feeding mothers were guided to avoid cow's milk in their diet. The results demonstrated that *L. reuteri* alleviated colicky symptoms within one week of treatment compared with simethicone. However, the study was hampered by methodological limitations, including the lack of blinding, no intention-to-treat analysis as well as absence of a true placebo group, which might have influenced the result (Savino, 2007b). Consequently, this study was replicated with similar results three years later with a different strain, *L. reuteri* (DSM 17938), in a double-blind placebo-controlled manner with 46 colicky infants. Besides reducing the crying amount during the study period, the study revealed that the treatment group had increased numbers of *Lactobacilli* and decreased numbers of *E. coli* and ammonia in fecal samples when compared to placebo, but there was no difference in stooling frequency or incidence of constipation between the groups (Savino, 2010). The fecal samples of the same study population were subsequently analyzed by a pyrosequencing technique. The analyses demonstrated, however, that the probiotic treatment did not alter the global composition of the gut microbiota (Roos, 2013). To confirm the clinical results of this latter study a separate trial with a different group and country repeated the study protocol with a larger effect size (n=80) and with exclusively or predominantly breast-fed infants, whose mothers followed a normal diet (Szajewska, 2013). The results confirmed the previous findings of *L. reuteri* reducing the symptoms of infantile colic in both respects; the daily crying time and the number of responders (>50% crying time reduction) to treatment. On the other hand, there was no difference between the study groups in the number of infants who fulfilled the colic criteria at the end of the study period. Furthermore, the placebo groups had fairly high response rates (63%) to treatment, suggesting that the true effect of active treatment is likely to be slightly lower and that the interpretation of results should be made with caution

(Szajewska, 2013). Apart from assessing the improvement of colic symptoms the latter study extended the previous findings by analyzing family quality of life, showing that this was significantly improved in the probiotic group when compared to placebo. To conclude, two recent meta-analyses of these three trials showed that *L. reuteri* reduce the daily crying time in colic infants (Sung, 2013; Anabrees, 2013).

The results of the Colic study (IV) supported the findings of these three earlier intervention studies with positive results (Savino, 2007b; Savino, 2010; Szajewska, 2013). However, the study design differed in some respects from earlier set-ups. Firstly, this was the first study to demonstrate the effects of LGG on treatment for infant colic crying, since previous works investigated the effects of *L. reuteri*. Secondly, the enrolled infants were younger than 6 weeks of age in order to time the probiotic intervention during the maximum crying time, while earlier studies enrolled infants up to 16 or 20 weeks of age (Savino, 2010; Szajewska, 2013). Thirdly, all infants here received behavioral counseling and lactation support as well as cow's milk elimination diet in tandem with the probiotic intervention to eliminate the action of these other potentially effective treatment strategies as confounding factors.

On the other hand, two double-blind placebo controlled trials using different mixtures of probiotics (a mixture of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bbi99, *Propionibacterium freudenreichii ssp. Shermanii* JS and a mixture of *L. rhamnosus*, *B. infantis*) for the treatment of infantile colic report no significant decrease in daily crying time in the probiotic group when compared to placebo (Mentula, 2008; Dupont, 2010). The limited number of patients together with different probiotic strains from previous studies might be factors contributing to the negative results of these studies. In line with these negative results the Crying study (I) showed that the LGG intervention did not influence infant total distress during the first months of life. As the study population did not consist of colic infants, the results might also suggest that probiotics are effective in reducing the crying amount only among colic infants, not as a preventive strategy. Such a conception is also supported by the negative results of six earlier preventive studies (Rinne, 2006; Vendt, 2006; Weizman, 2006; Chouraqui, 2008; Kukkonen 2008; Vlieger, 2009).

In contrast to the above-mentioned hypothesis, but in line with results obtained by Indrio and colleagues (Indrio, 2008), the Preterm study (III) reported positive effects of pro- and prebiotic intervention on excessive crying in preterm infants. In addition to different probiotic species and study population, the timing of the intervention was different from previous set-ups (Savino, 2007b; Savino, 2010;

Szajewska, 2013), as it started during the first days of life, before the peak period of crying, not after the problem had risen. Thus, this is the first RCT to demonstrate that pro- and prebiotics might have a preventive role in colic in preterm infants. However, the results cannot be generalized to all infants, since the gut microbiota colonization process in preterm infants differs substantially from that in term infants and thus preterm infants may constitute a risk group for excessive crying (Maunu, 2006; Arboleya, 2012; Milidou, 2013).

Furthermore, this is also the first study to report the effects of prebiotic supplementation on infant crying, since there are no double-blind placebo-controlled prebiotic studies with a primary outcome of infant crying or colic, although some studies have evaluated crying, colic, fussiness or irritability as a secondary outcome. None of these studies have shown that prebiotics reduce infant crying or fussiness compared to placebo (Moro, 2002; Moro, 2006; Nakamura, 2009; Scalabrin, 2012). In contrast, one of the studies reported that infants receiving prebiotic evinced more fussing than those receiving placebo, but without difference in the incidence of colic between the groups (Ziegler, 2007). However, only two of these earlier studies used the same prebiotic mixture (PDX/GOS) as the Preterm study (III), albeit with different dosage and timing of the intervention (Nakamura, 2009; Scalabrin, 2012).

As a conclusion, the role of prebiotics as an efficient preventive or treatment strategy in colic remains unclear. However, the use of probiotics, especially *L. reuteri* and possibly LGG, might offer a safe and effective treatment option for infant colic crying, although it is too early to recommend their routine use (Sung, 2013; Anabrees, 2013). It is also intriguing to speculate whether early administration of probiotics to colic infants could have a preventive role against the later development of FGID, since probiotics have also been demonstrated to relieve the symptoms of FGID by a mechanism of action as yet unknown (Simren, 2013).

6.3.2 Possible theories on the mechanism of action probiotics and prebiotics in reducing infant crying

While the mechanisms of action of pro- and prebiotics have been only relatively recently understood and are still unknown at the molecular level, they mediate their beneficial effects in a number of ways which can be separated into three different levels; they interfere with the growth or survival of the gut microbiota and direct a metabolic effect by supplying enzymatic activities; they enhance the mucosal barrier function, the mucosal immune system and the enteric nervous

system; and beyond the gut impact also affect the systemic immune system (Rijkers, 2010; Jeurink, 2013; Oozeer, 2013) (Figure 12).

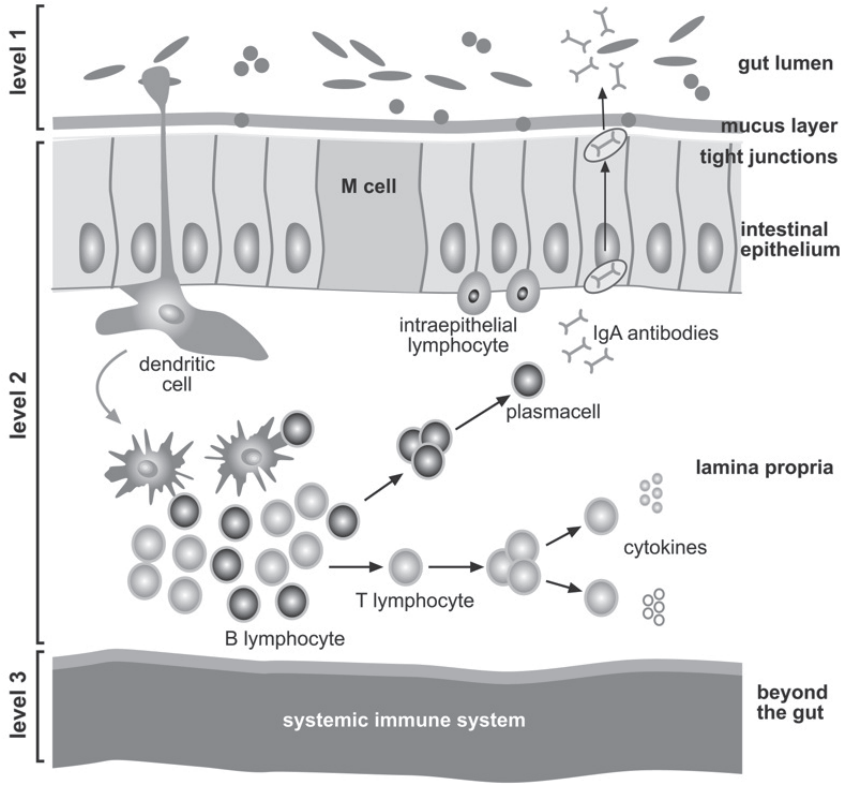


Figure 12. The three levels of the mechanism of action of probiotics. Modified from Rijkers, 2010 and Luoto, 2010.

The mechanism of action of probiotics on excessive infant crying is not yet established. Only two previous intervention studies have evaluated the direct effect of probiotics on the gut microbiota composition (Mentula, 2008; Savino, 2010), although the former intervention had no impact on colic crying. The latter study showed a significant decrease in the number of *Escherichia coli* and an increase in the number of *Lactobacilli* by FISH during the study period in the probiotic group when compared to placebo (Savino, 2010).

The coliform bacteria are thought to be gas-forming and consequently to increase the intra-abdominal air load as well as aerophagia and pain, symptoms related to infantile colic (Mentula, 2008; Savino, 2009). However, this cause-relationship remains vague and needs to be clarified. Savino and associates moved on to analyze the protective mechanism of *Lactobacillus* species against gas-forming

coliforms by isolating coliforms from colicky infants and analyzing the antimicrobial effect of *Lactobacillus* strains against these by culture methods (Savino, 2011a). The results of the study showed that only two out of 27 *Lactobacillus* species (*L. delbrueckii* DSM 20074 and *L. plantarum* MB 456) possessed an antimicrobial effect against six species of gas-forming coliforms isolated from colicky infants (Savino, 2011a).

In contrast to earlier findings, in this present series of studies the LGG intervention seemed to influence the proportion of *Clostridium histolyticum*-type bacteria (III) and the proportion of *Bifidobacterium lactis* (IV). LGG may lessen excessive crying through a reduction of *Clostridium histolyticum* colonization via different mechanisms such as competitive exclusion (Ji, 2012; Nylund, 2013). High numbers of *Clostridium* species might be associated with degradation of antigen-specific IgA, which impairs the gut barrier function (Bakker-Zierikzee, 2006). On the other hand, LGG and *B. lactis* have been shown to normalize increased intestinal permeability, and to increase antigen-specific IgA responses and fecal IgA, all reflecting to gut barrier function (Kaila, 1992; Isolauri, 1993; Mohan, 2008). In summary, according to these and earlier results, the effects of probiotics on crying exclusively through direct gut microbiota modification are inconsistent and moderate and thus other mechanisms of action are presumably also involved.

One possible mechanism of action of probiotics on infant colic is regulation between pro- and anti-inflammatory cytokines, since colic crying has been associated with increased amounts of calprotectin, a gut inflammatory marker (Rhoads, 2009). The results of the Colic study (IV) do not support this earlier finding, because the mean values and the range of calprotectin in both intervention groups were within the limits of normal for this age group (Kapel, 2010). The potential gut inflammation in infant colic might be induced not only by aberrant gut microbiota composition, but also by sensitization to specific food allergens (Arikan, 2008). Probiotics might help in breaking down antigens towards more tolerogenic structures, and assist in oral tolerance induction and a reduction in clinical symptoms (Pessi, 1998). Especially in preterm infants, inadequate gut barrier function and deviating immune responses might cause an increased risk of oral tolerance failure and hence further excessive irritability and crying (Sudo, 1997).

The Preterm study (III) showed that pro- and prebiotic supplementations were equally effective in reducing excessive crying among preterm infants. However, there was no significant difference in gut microbiota composition between the pre- and probiotic groups or prebiotic and placebo groups, suggesting that the

mechanism of action of prebiotics may not directly involve investigated gut microbiota composition. The study showed that the stool frequency was higher in the prebiotic than in the probiotic or placebo group, similarly to previous reports (Ziegler, 2007; Costabile, 2012; Ribeiro, 2012; Scalabrin, 2012). These findings might indicate that frequent defection may reduce abdominal distention and gas formation, and thus be one of the potential mechanisms whereby prebiotics lessen excessive infant crying. Furthermore, prebiotics have been shown to modulate the electrical activity and gastric emptying in preterm infants, and this could thus be another mechanism of action of prebiotics in improving intestinal tolerance and reducing excessive crying (Indrio, 2009).

In conclusion, the mechanism of action of pro- and prebiotics in reducing excessive crying remains unknown. However, the potential mechanisms of probiotics include interference with the growth or survival of the gut microbiota and enhancement of the mucosal barrier function and mucosal immune system. However, these mechanism need to be clarified before any further assumptions can be made. Moreover, the possible influence of probiotics beyond the gut to impact on the systemic immune system and other cell organ systems warrants further elucidation in the future.

7 SUMMARY AND CONCLUSIONS

7.1 Main findings

The results of the present series suggest that deviations in gut microbiota composition, especially in the numbers of *Bifidobacteria* and *Lactobacillus*, are associated with infant crying. Along with earlier data these findings support an intriguing theory of aberrant gut microbiota composition as a cause of infant colic crying, although documentation of causality would necessitate well-powered intervention studies from independent study groups. In addition to the as yet unknown cause of colic crying, one daunting question pertains to the long-term sequelae of colic, since there are reports of the later development of allergic disorders as well as behavior problems (Kalliomäki, 2001; Wolke, 2002; Savino, 2005b). The present findings suggest that infants at high risk for allergies with colic-type crying might have more functional gastrointestinal disorders during adolescence than children without previous colic-type crying.

Furthermore, one other aim of this series was to evaluate the potential effects of pro- and prebiotic supplementation during the first months of life on preterm infants' crying. Both pro- and prebiotic intervention during the first two months of life equally reduced the frequency of excessive crying in the preterm population. Similarly, probiotic intervention in tandem with behavioral counseling and a cow's milk elimination diet during the colic period reduced the daily crying amount in colic infants when compared to placebo according to parental interview data. In both studies the probiotic intervention had a moderate influence on gut microbiota composition. Although the exact mechanisms of action of probiotics in connection with crying remain unknown, over and above direct interference with the growth or survival of the gut microbiota, they may act through enhancement of the mucosal barrier function and the mucosal immune system.

In conclusion, medical examination should be offered to all colic infants whose families have contacted the health-care system, as well as parental behavioral support. In addition, a cow's milk elimination diet might be tried in severe colic cases. In any case, while it is too soon to recommend the routine use of any probiotics in treating colic, they may provide a health benefit safely to these distressed infants or even function as potential preventive agents. Discussions regarding both of these interventions are to be recommended for parents of colic infants.

7.2 Future lines for research

Firstly, among the most important issues for future studies is the appropriate definition of colic, since thus far dozens of different definitions are to be found in the literature, leading to the inclusion of very dissimilar groups of crying infants (Reijneveld, 2001). All the studies should use the same criteria, preferably concerning both duration of crying and parental distress, to ensure that results can be compared with each other (Reijneveld, 2001). For instance the criterion used in the Colic Study (IV) is a good example how colic should be defined in the future studies. On the other hand, probably due to the exact criterion, the recruitment of the study population in the Colic study (IV) was more laborious and time consuming than expected. Another reason for the slow recruitment procedure might be that the incidence of colic has decreased in Finland and therefore it would be interesting to update these data in the future.

Secondly, different methods such as distinct cry diaries, parental interviews, questionnaires and recall have been used to measure infants' daily crying. Thus far, cry diaries such as Barr's Baby Day Diary have been considered "the gold standard" in evaluating daily crying time (Barr, 1988). However, the Colic study (IV) included two different means of evaluating daily crying; a validated, detailed cry diary and a parental interview on the amount of daily crying (Barr, 1988). The diary was assumed to give more objective data, while the interview may be affected by parents' subjective experience of infant crying, reflecting the relevance of crying to the parents. The inclusion of this perspective opens up a new aspect in addition to diary data. According to the results of the study the parental perception is not only reliable, but also clinically more significant; it is, after all, the parents' perception of colic which leads to referrals to professionals. Thus, future studies should take account of the parental experience of crying, not only the diary data.

A third important point of view is the age of the infant during the study period. Since colic crying begins to decrease after the age of 6 weeks, all interventions studies should be timed during the maximum crying time (Brazelton, 1962). Excessive crying continuing beyond this age might be of distinct etiology (St James-Roberts, 2012). However, in relation to gut microbiota development, it would be interesting to explore in greater detail the gut microbiota development before and after the colic period, for example from birth up to early adulthood. Furthermore, achievement of these goals with the gut microbiota requires uniform markers to objectively measure the composition of the gut microbiota. In most studies the composition of the intestinal microbiota is extrapolated from

fecal samples, although it may not reflect its specific features in the upper gastrointestinal tract (Mentula, 2005).

Fourthly, in spite of decades of research into colic crying many open questions remain. The results of this series of studies may especially stimulate researchers' interest to explore the factors possibly impacting on the colonization process in the colic infant's gut microbiota, which have not yet been widely studied, for example maternal health status and diet and the use of probiotics and antibiotics during pregnancy. Moreover, as breast milk is the optimal nutrition for infants and strict hypoallergenic diets are hard for mothers to follow, it would be interesting to further explore possible triggers and blockers of colic in breast milk and their possible mechanisms of action. Furthermore, since the size of the study populations in both the Crying and the Colic studies (II, IV) was limited, the study protocols should be repeated with larger sample size to confirm the findings before any firm conclusions can be drawn. Moreover, the exact mechanism of action of probiotics in ameliorating the colic symptoms needs to be clarified in the future.

Finally, one further interesting field for future study is the long-term consequences of colic crying. The Crying study (II) showed that colic-type crying may precede other FGID, especially abdominal migraine. Interestingly this result is supported by the finding that colic is associated with the later development of migraine (Romanello, 2013). Consequently, the association between colic, abdominal migraine and migraine and especially the possible mechanisms linking these disorders offers an interesting focus for future research.

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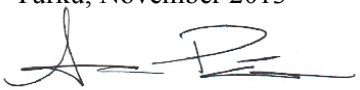

Anna Pärtty

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