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THE MICROBIAL GUT COLONIZATION PROCESS

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SUMMARY

The composition and evolvement of early infant gut microbiota is of interest, due to developmental windows in early life that rely on stimulus from the gut. Many factors have been shown to influence the composition of early gut flora, amongst them antibiotics and caesarean delivery. Caesarean delivery is of special interest since it is associated with an aberrant gut microbiota of long lasting character and since it is increasingly more common in the western world. Furthermore, epidemiological studies indicate that caesarean delivery is associated with an increased risk of subsequent food allergy. However, the underlying mechanism is unknown. Preliminary results from the NoMic study indicate that early gut microbiota composition is diverse. It highlights the importance of studying a large number of samples from babies, and takes into account factors with gut microbiota modifying properties such as caesarean delivery, in order to increase our knowledge of early gut microbiota and its role in health and disease.

INTRODUCTION

Over the last decades an increasing amount of attention has been given to gut microbiota as we have come to understand its crucial role in a number of functions and its possible role in many diseases. The composition and evolvement of early infant gut microbiota is of special interest, due to developmental windows in early life that rely on stimulus from the gut. Specifically, early gut microbiota play a crucial role in the development of tolerance to antigens (*Sudo* et al., 1997; *Mazmanian* et al., 2005), the development of the capillary network of the gut (*Stappenbeck* et al., 2002) and in down-regulation of stress responses (*Sudo* et al., 2004). Furthermore, the colonization process during early infancy is of interest as a determinant for the subsequent adult-like microbiota (*Midtvedt*, 1994; *Hooper* et al., 1999). Knowledge of the composition of normal healthy gut microbiota and understanding the community dynamics that takes place during infancy is a prerequisite for understanding its role in disease. It may also enable us to identify early determinants for the adult ecosystem, providing an opportunity for early intervention (*Dethlefsen* et al., 2007).

The aim of this review is to discuss factors that have been shown to affect early gut microbiota while also epidemiological studies that tie such factors to child health will be presented. Finally an overview will be given of the NoMic study which, amongst others,

aims at characterizing the colonization process in babies who have not been exposed to gut modifying factors and thus may exhibit a more undisturbed colonization process.

The hygiene hypothesis proposes that the common underlying factor for allergic and other immune based diseases is lack of necessary microbial stimuli from the gut (*Guarner* et al., 2006). It is well documented in experimental studies that the commensal intestinal gut microbiota plays a crucial role in the development and maturation of the immune system. The hygiene hypothesis is indirectly supported also by a number of epidemiological studies (*Garn* and *Renz*, 2007). Interestingly, epidemiological studies also show a strong positive association between asthma and type I diabetes. Thus a country with a high prevalence of asthma has been shown to have also a high prevalence of type I diabetes (*Stene* and *Nafstad*, 2001). This supports the assumption that these two diseases may share a common underlying factor. Within an individual, however, there is some support for an opposite relation, e.g. individuals with diabetes type 1 are less likely to suffer from asthma and allergy I (*EURODIAB Substudy 2 Study Group*, 2000; *Caffarelli* et al., 2004). Thus if the hygiene hypothesis is assumed to hold true, individual susceptibility factors, such as genetic predisposition, probably play a role in whether and how lack of microbial stimulus manifests itself as disease in an individual.

Studies indicate that the composition of intestinal microbiota in babies has changed over time in the Western world (*Adlerberth* et al., 2006) and that it differs from infant gut microbiota in developing countries (*Adlerberth* et al., 1991; *Bennet* et al., 1991; *Sepp* et al., 1997). For instance, *E. coli* was a dominating early colonizer, present in most babies within three days after birth in earlier studies (*Mata* and *Urrutia*, 1971; *Gareau* et al., 1959) while more recent studies from Sweden show that a declining proportion of babies is colonized with *E. coli* (*Adlerberth* et al., 1991, 2006; *Nowrouzian* et al., 2003). Furthermore, the turnover rate of different strains is slower in western babies. Also an increase in *Staphylococcus* and a decline in *Bifidobacterium*, which dominated the microbiota of newborns in the middle of the last century, is observed (*Adlerberth* et al., 2006; *Mata* and *Wyatt*, 1971). General hygienic measures, hospital delivery, caesarean delivery and antibiotics may have played a role in these changes.

The effect of caesarean delivery on infant gut microbiota is of special interest, since the incidence of caesarean delivery is steadily increasing. For instance, in Norway only 2% of children were delivered by a caesarean section in the 1970s, 12% in the 1990s and 15% in 2001 and the incidence is still increasing (*Hager* et al., 2006). In other countries even more marked increases have taken place with half of all children now being delivered by a caesarean section (*de Moraes* and *Goldenberg*, 2001). In babies delivered by a caesarean section the input of bacteria from the maternal birth canal is missing. Thus, colonization occurs at a slower pace, partly through hospital acquired microbes. A number of studies have shown that babies delivered by a caesarean section have a delayed and different colonization, with lower colonization rates of *Bifidobacterium*, *Enterobacteriacea* and *Bacteroides* (*Biasucci* et al., 2008; *Bennet* and *Nord*, 1987; *Neut* et al., 1987; *Salminen* et al., 2004; *Grönlund* et al., 1999). Moreover, studies have indicated that the different composition may be of a long-lasting character, still evident at 7 years of age (*Salminen* et al., 2004; *Grönlund*

et al., 1999). Also of interest is a recent study, which reports an association between the mode of delivery and neonatal immune responses. The authors conclude that robust regulatory T-cell suppressive functions were observed in 59% of vaginally delivered babies compared to only 29.4% of caesarean delivered children (*Ly* et al., 2006).

An indirect way of studying whether a delayed or altered microbiota plays a role in child health is thus to study whether caesarean delivery is associated with increased risk of diabetes or allergic diseases. We have studied the associations between caesarean section and egg allergy (*Eggesbø* et al., 2003) and milk allergy/intolerance (*Eggesbø* et al., 2005), in the "Oslo Birth Cohort" which included 2803 families. Allergy to egg and milk were confirmed by a stepwise procedure which included measurement of specific IgE as well as open and double-blind placebo-controlled food challenges. Detailed information on the study sample and diagnostic procedure can be found in the published papers (*Eggesbø* et al., 2003). We did choose to study both parentally reported as well as confirmed reaction to food, since they are associated with different types of bias (misclassification and selection bias), thus consistency between the outcomes would be of importance (*Eggesbø* et al., 2003). We reported an increase of parentally reported adverse reactions to egg, fish and nuts, (but not parentally reported reactions to milk), among children delivered by a caesarean section (*Eggesbø* et al., 2003, 2005). Confirmed reactions to egg, as well as to milk, were also significantly more common among children delivered by a caesarean section. Even more interestingly, we observed that the increased risk of food allergy in caesarean delivered children was primarily confined to children born by allergic mothers (*Eggesbø* et al., 2003, 2005). These results have been published separately for egg and milk previously, and here we report them combined. Among children of non-allergic mothers the percentage of children with confirmed reactions to egg or milk was 0.6% and 0.8% among vaginally and caesarean delivered children, respectively. However, among children of allergic mothers the percentage of children that developed food allergy was 2.2% among vaginally delivered children and 6.2% among caesarean delivered children, e.g. a nearly 3-fold increased risk in food allergy among caesarean delivered children. Adjusting the results for a number of important potential confounding factors did not alter the results (*Eggesbø* et al., 2003, 2005).

Several meta analyses have recently been published which summarizes the finding of epidemiological studies on this topic. Two meta analyses have been published on food allergy and the conclusion of both is that there is support for an association between mode of delivery and food allergy (*Bager* et al., 2008; *Koplin* et al., 2008). Also a meta analysis on the association between caesarean delivery and asthma reaches the same conclusion (*Thavagnanam* et al., 2008). Finally, a meta analysis on the association between caesarean delivery and type 1 diabetes concludes that caesarean delivery is associated with a 20% increased risk of type 1 diabetes (*Cardwell* et al., 2008).

Thus epidemiological studies indicate that there is indeed an association between mode of delivery and subsequent allergy or diabetes. Although confounding by one of the many factors associated with caesarean delivery may not yet be entirely ruled out, these findings could be taken as indirect support for the "microbial deprivation hypothesis". However, if we assume that

the gut microbiota indeed plays a causal role in the association between caesarean delivery and immune related diseases, what properties of gut microbiota are driving this increased risk? Is the increased risk due to a general delay of microbial encounter, or are the aberrant "first arrivers" important, either directly or trough their influence on the colonization process? Or could a mismatch between maternal and infant gut microbiota play a role?

Despite the many unsolved questions and obvious importance of infant gut microbiota, we have limited knowledge of the overall composition of infant gut microbiota and the colonization process, since longitudinal studies targeting the dynamics occurring in this period are rare and most studies are restricted to known inhabitants of the gut (*Palmer* et al., 2007; *Adlerberth* et al., 2007; *Thompson* et al., 2008). To our knowledge only three small longitudinal studies have used an open approach based on clone libraries, which gives the possibility of discovering hitherto unknown microbial constitutions of infant gut microbiota (*Palmer* et al., 2007; *Wang* et al., 2004; *Favier* et al., 2002). A further limitation of previous studies, whether cross sectional or longitudinal, is that they are based in part on babies who have been delivered by a caesarean section, stayed at neonatal intensive care units, received early supplemental feeding or antibiotics; factors which all may have a profound disruptive effect on gut microbiota composition (*Bennet* et al., 1986; *Songjinda* et al., 2005; *El-Mohandes* et al., 1993; *Hällström* et al., 2004; *Benno* et al., 1984; *Harmsen* et al., 2000; *Yoshioka* et al., 1983; *Stark* and *Lee*, 1982; *Grönlund* et al., 2007; *Gueimonde* et al., 2007). Thus these babies may not exhibit an undisturbed colonization process.

The NoMic cohort in Norway, which consists of 524 newborns and their mothers, was established for the purpose of studying closer these issues. Faecal samples were collected from the babies at day 4, 10, 30 and at 4, 12 and 24 months. From the mothers we collected one sample after delivery. Questionnaires were filled out by the mothers at 1, 6, 12, 18 and 24 months and a follow-up is planned at 7 and 12 years. We especially ensured that detailed information on mode of delivery, indications for caesarean section, and antibiotic use during pregnancy and delivery was obtained. Microbes were identified by targeting the gene encoding ribosomal RNA (16S rRNA) (*Rudi* et al., 2007). We used an approach based on clone libraries generated from faecal samples from the study population, thus not limited to known species. We will in this study also examine microbial-dependent metabolic functions (*Midtvedt* et al., 1987; *Midtvedt* et al., 1988). More details on this cohort and the methods used will soon be published (*Eggesbø* et al., 2010).

Our first aim was to characterize the composition and natural evolvement of the gut microbiota during early infancy in Western babies who had not been exposed to a number of factors which interfere with the colonization process. We thus restricted our study samples to term babies, who had not been transferred to an intensive care unit, who were exclusively breastfed the first month of life and thereafter exclusively or partially breastfed, who were not delivered by caesarean section, and who were not exposed to antibiotics, either directly or indirectly via the mother. Interestingly, only 87 out of 524 babies fulfilled these criteria. In short, the overall composition of the infant gut microbiota among our babies corresponds well with previous studies with microbes belonging to four divisions: Firmicutes, Proteobacteria, Bac-

teroidetes and Actinobacteria (*Palmer* et al., 2007). We found that almost all newborns harboured *Staphylococcus,* γ-proteobacteria and *Bifidobacterium* in their guts four days after birth. Interestingly, even in this clearly defined sub-set of babies, we observed distinct sub-clusters of microbiota. We will further seek to identify microbial species or groups that co-evolve in a dependent manner and we will study whether any specific microbial groups can be identified as predictors for the subsequent microbiota (*Eggesbø* et al., 2010). These results will be published elsewhere.

CONCLUSION

Epidemiologic studies indicate that early infant gut microbiota composition plays a role in the development of allergic diseases. The underlying mechanism is unknown. There is a need for more information on nearly all aspects of gut colonization in early infancy. The large variation in infant gut microbiota composition highlights the need to study larger cohorts when aiming at describing gut microbiota composition and to take into account the many factors are involved in the shaping of gut microbiota.

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