MICROBIAL ECOLOGY
in Health and Disease

GUT in FOCUS
Symposium NOBEL FORUM, Karolinska Institutet

February 2nd 2015

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MICROBIAL ECOLOGY in Health and Disease

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GUT IN FOCUS: EDITORIAL

We all need friends

The idea that the intestinal content can cause a number of physical and psychological ailments is very old (1). Remedies to clean the bowel were used in ancient Egypt, probably inspired by the peculiar behaviour of the ibis bird, as stated by Plinius (23–79 A.D.): 'The bird which is called the ibis and which is a native of Egypt, by means of its hooked beak, laves the inside of his body by introducing water into the channel, by which it is especially necessary for health that the residuous food should be discharged' (2). Charles-Joseph Bouchard (1837–1915) wrote a book about 'Auto-intoxication' in 1894 (3), and the Royal Society of Medicine discussed the role of 'Alimentary toxæmia' at a symposium in 1913 (4). Although these thoughts gradually fell into disrepute (5), new molecular methods to investigate the microbial ecology of the gut have been developed during the past decades (6), enabling scientists to revisit and renew old concepts (7).

The human body contains about 10 times more microbes than human cells, and the gut harbours around 1,000 different bacterial species. Regarding its size and metabolic activity, the 'microbe organ' is comparable to the liver. Communication between microbiota and their hosts has been denoted as 'inter-kingdom signalling' (8), and constitute a promising research area. The gut microbial flora performs several protective, structural, and metabolic functions, and may be incriminated in a number of disorders and diseases (9). However, our understanding of the complex gastrointestinal ecosystem is in its infancy, and the 'microbe organ' is still largely a *terra incognita*.

The papers included in the present thematic cluster of *Microbial Ecology in Health and Disease* constitute a unique collection of ongoing cutting-edge research projects. Such efforts to elucidate the role of the gut microbiome are to be congratulated. Hopefully, the results will have a major impact. The thematic cluster may also serve to illustrate an emerging understanding within gastrointestinal biology: The importance of microbes that are *not* there. Such microbial deprivation may be difficult to spot. Nevertheless, the implications and consequences of 'missing microbes' (10) are probably underestimated. In the end, we all need friends.

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GUT IN FOCUS: PROGRAMME

Gut in Focus Symposium February 2nd 2015

Focus on development of intestinal microbiota (IM)

1. Dennis Lang: Challenges to the establishment and maintenance of IM in infants in developing countries.

2. Merete Eggesbø: Factors affecting infant gut microbiota and possible consequences for health.

3. Rochellys Diaz Heijtz: The gut microbiota and developmental programming of the brain.

Focus on specific compounds

4. Jan Raa: b-glucan Immune modulation by non-digestible and non-absorbable b-1,3/1,6-glucan particles.

5. Derrick MacFabe: Enteric short chain fatty acids: Microbial messengers of metabolism, mitochondria and mind: Implications in autism spectrum disorders.

Focus on restoration of IM

6. Johan Bakken: Feces transplantation – US recommendations & experience.

7. Torbjörn Norén: Feces transplantation – EU recommendations.

8. Experience with cultivated microbiota transplant (CMT)

a. Elisabeth Norin: On-going treatment of CDI-patients in Sweden.

b. Kjetil Kjelstad Garborg: ACHIM as first line treatment for difficile infection.

c. Peter Benno: FMT a new option for IBS patients: case reports.

9. Tore Midtvedt: Summary and concluding remarks.

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GUT IN FOCUS: INTRODUCTORY REMARKS

Introducing the thematic cluster

The one-day symposium Gut in Focus, which was held at the Karolinska Institutet on February 2nd, 2015, focused on certain aspects related to the gut, by far the largest organ of the body. When I started studying medicine more than 60 years ago, I heard two statements about the gut: (1) *The gut has a brain, if not a mind, of its own* and (2) *Bacteria constitute up to 50 per cent of feces' mass and may be of importance for its texture.* Together, these two statements did not make sense to me. Nine years later, I came to the Karolinska Institutet as a bacteriologist, working in the team around Nobel Laureate Professor Sune Bergström and also Professor Bengt E Gustafsson and his germfree animals. Both of them were eagerly studying biochemical similarities and differences between germfree and conventional animals. It became my job to find microbe(s) that could close the gap.

The functional importance of gut microbes was obvious for these two pioneers, and today, it is generally accepted that 'Man + Microbes = Superorganism', and there is increasing consensus that 'Gut + Microbiota = Superorgan'.

In our first Gut in Focus symposium in early 2012, the focus was on 'The Gut and the Brain'. The 2015 symposium focused on the gut itself. The first session dealt with the *establishment* of a gut microbiota, and the second session with selected *functional* aspects. The third session was devoted to *restoration* of the gut. Most of the contributions are published in this Special Issue of *Microbial Ecology in Health and Disease.* As usual, access is free!

Tore Midtvedt
Editor-in-Chief

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GUT IN FOCUS: EXTENDED ABSTRACT

Opportunities to assess factors contributing to the development of the intestinal microbiota in infants living in developing countries

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Recent evidence suggests that establishment of a healthy gut microbiota shortly after birth is important to achieve optimal growth and development of children. Being born into a resource-poor environment presents challenges to the establishment of a healthy gut microbial flora in the newborn. Among these challenges are births that occur at home, traditional pre-lacteal feeding of newborns leading to failure to initiate lactation, poor sanitation and water quality, early environmental exposure to, and infection with, enteric or other pathogens, suboptimal breast feeding duration and intensity, deficiencies in weaning and childhood diets contributing to micro- and macro-nutrient deficiencies, and the frequent use of antibiotics. These factors should be considered in the design and implementation of preventive and therapeutic interventions aimed at improving the health and development of these children.

Keywords: *probiotics*; *gut microbiota*; *environmental enteropathy*; *enteric infections*; *under-nutrition*; *child development*; *developing countries*

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It is now recognized that the human microbiota (microbial flora) and its collective genetic content (microbiome) play an important role in determining health status. The gut microbiota has been described as a 'microbial metabolic organ' because of its co-evolution with humans (1), its communication with other human organs including the brain (2), and because it contributes to health by metabolizing otherwise indigestible components of the diet (e.g. polysaccharides to short chain fatty acids) and synthesizing essential amino acids and vitamins and other bioactive compounds, by shaping a balance between pathogens and commensals, and by influencing the development of the innate and adaptive immune systems. Perturbation of the microbiota by infection with enteropathogens, biologic or chemical toxins, antibiotics, altered diet, and other environmental factors may give rise to dysfunction and diseases in the host. Recognizing the important role that the microbiota contribute to health has led to interventions aimed at restoring a healthy gut microbiota to prevent or treat disease. Fermented food-associated probiotics, fecal transplants from healthy individuals, and diets rich in the foods (prebiotics) that support a diverse and balanced intestinal microbiota are being evaluated as treatments for intestinal disorders such as *Clostridium difficile* colitis (3), inflammatory bowel disease (4), irritable bowel syndrome (5), diarrhea (6, 7), ulcerative colitis, and others (8).

Environmental enteropathy (EE), more recently referred to as environmental enteric dysfunction (EED) (9), is an ill-defined and difficult to diagnose intestinal pathology characterized by gut and systemic inflammation, altered villus architecture, alterations in gut barrier function, and absorptive capacity. It has been postulated that EED may develop when individuals live in a fecally contaminated environment where they are frequently exposed to enteric pathogens. It has also been postulated that EED may have a number of negative consequences, particularly in children under the age of two, including contributing to undernutrition, decreased growth velocity, stunting, depressed immune response to orally delivered vaccines, and impairments in cognitive development (10, 11). Unfortunately, these symptoms are slow to develop and thus difficult to recognize in their early stages. Improved methods for early diagnostics are essential. Current efforts

†See Appendix for list of investigators in the MAL-ED Network.

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are underway to identify improved biomarkers of gut inflamation, permeability, and decreased absorptive capacity that would identify children at risk of developing EED and its sequelae before they occur (12).

Approaches to prevent and treat EED in children are being considered (13, 14). These include improvement in water quality and sanitation (15), promotion of optimal breast feeding, improving the quality and diversity of diets, micronutrient supplementation of pregnant women and newborns, appropriate use of antibiotics, vaccination against enteric pathogens, and the use of pro- and prebiotics. The key question is – do we yet know enough to intervene effectively? It is likely that combinations of approaches, possibly varying from place to place based on the unique characteristics of the site, may have to be applied simultaneously to achieve maximal results. This forecast is based upon the fact the gut microbiota represents a variable dynamic human 'metabolic organ', which changes its structure and function from the time it is established shortly after birth. Factors such as age, diet, infectious diseases, medications, living environment, and many other variables may affect its composition (16, 17).

Subramanian et al. (16) describe a definable postnatal developmental program of assembly ('maturation') of the intestinal microbiota in children in Dhaka, Bangladesh, during the first two years of life while age-matched children from the same community with various forms of undernutrition exhibited relative microbiota immaturity. Treatment of children with severe acute undernutrition with ready-to-use therapeutic foods had only a temporary positive effect on the maturity of the microbiota. Either prolonged administration with existing therapeutic foods or new types of interventions may be needed to confer a lasting effect on the gut microbiota and prevent or reduce undernutrition and its persistent sequelae (stunting, cognitive deficits, and reduced immune response to certain vaccines). Kolling et al. (17) describe changes to the composition of the gut microbiota and the concurrence of pathogens that occur as humans age.

Plans and progress

The Etiology, Risk Factors and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development Project (MAL-ED) was started in 2009 to investigate the role of enteric infections, nutritional intake, and other environmental exposure variables on child development. It was designed as an observational, longitudinal, birth cohort study that followed approximately 200 children from birth to two years of age at each of the eight sites by the use of a harmonized common protocol and data collection forms. All participants consented, and then enrolled on a staggered schedule (10–12 per month over two years) to capture seasonal effects. Twice weekly home surveillance collected data on environmental factors, illness indicators (emphasis was placed on enteric infections), gut function biomarkers, nutrition, and anthropometry. Diarrheal stool samples were collected once during each episode and again at 14 days in the case of prolonged diarrhea episodes; normal, non-diarrheal surveillance stool was collected once per month for the duration of the study. All stool samples were analyzed to identify bacteria, viruses, and parasites known to produce enteric diseases. Study variables were examined for their contribution to child growth, immune response, and cognitive development. A more detailed overview of the MAL-ED project and the methodologies employed has been published (18). The study is being conducted at field sites in Iquitos, Peru (PEL); Fortaleza, Brazil (BRF); Venda, South Africa (SAV); Haydom, Tanzania (TZH); Vellore, India (INV); Naushero-Feroze, Pakistan (PKN); Bhaktapur, Nepal (NEB); and Dhaka, Bangladesh (BGD). These sites were known to have experienced high burdens of enteric diseases and growth deficits (stunting).

One of the study hypotheses of MAL-ED is that frequent enteric infection leads to EED, which could contribute to deficits in physical growth, cognitive development, and immune responses to expanded program on immunization (EPI) scheduled vaccines. As seen in Fig. 1, we observed that variable degrees of stunting (length for age Z score of at least two standard deviations below the World Health Organization (WHO) standard growth chart) did develop in MAL-ED cohort children during the first two years of life. Rates ranged from a few percent in BRF, to 20–40% in six of the other sites, to $>70\%$ in TZH. Even among those children who did not become stunted, most grew at a slower rate than would be expected under optimal conditions (data not shown).

The MAL-ED sites are representative of communities where effective sanitation is in short supply. Our study has revealed environmental conditions characteristic of such environments that could interfere with the establishment and maintenance of a healthy gut microbiota during the first two formative years and perhaps throughout early development. Among these factors are the high percentage of childbirths occurring at home in three of our sites; suboptimal breastfeeding (BF) and weaning diets; early exposure to, establishment of, and infection with enteric pathogens; and frequent use of antibiotics. These factors may conspire to create an environment that makes the use of probiotics to prevent or treat disease more difficult than in developed countries.

Childbirth at home

In five of the eight MAL-ED sites, there are relatively few home deliveries (Fig. 2). Most study sites are in close proximity to a medical facility (hospital or clinic) where improved delivery practices were available and where delivery by Cesarean section is uncommon.

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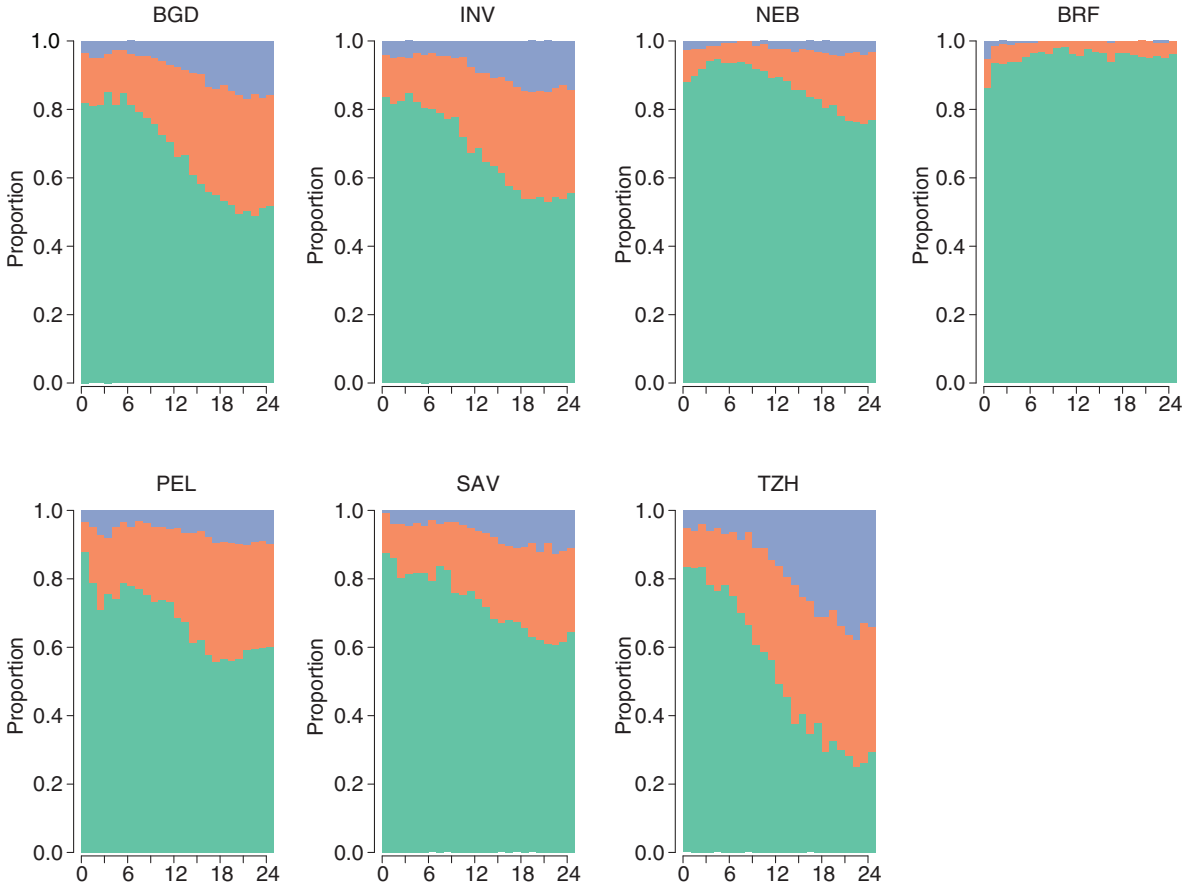

Fig. 1. Proportion of children stunted during the first two years at MAL-ED sites. Each child was measured every month for the first two years. Green – proportion of children not stunted (> -2 LAZ), Orange – proportion of children stunted (< -2, > -3 LAZ), Blue – proportion of children severely stunted (< -3 LAZ) at seven MAL-ED sites. Data pertaining to Pakistan are not available.

From a microbiologic perspective, vaginal delivery in an uncontaminated environment is preferred because it allows the microorganisms transferred from the mother's anal, vaginal, and skin microbiota to serve as the inoculum that seeds the gut ecosystem. However, at three study sites a significant percentage of births occurred at home [BGD (31%), PKN (59%), and TZH (50%)]. Home delivery in a contaminated environment has an assumed inherent risk of introducing pathogenic microbes to the infant's microbiota during or shortly after birth.

Suboptimal BF and weaning diets

The WHO recommends that the child be offered colostrum immediately after birth followed by six months of exclusive BF. None of the MAL-ED study sites achieved this goal. Figure 3 depicts survival curves for exclusive BF at each of the eight sites. The PKN site had the lowest rate (50% of children were no longer exclusively BF at 15 days of age) while BGD had the highest rate (50% still exclusively BF at about 110 days). However, additional data reveals that, while exclusive BF may be less than ideal, many children continue to receive predominant or partial BF for much longer. At BGD, more than 80% of the children were still receiving some breast milk in their diet at two years of age, while in INV, PKN, SAV, TZH, and PEL, less than 20% were. The introduction of other liquids and solids before six months of age increases the likelihood of pathogen exposure. Pre-lacteal feeding and early introduction of liquids or solids is the cultural norm in some of the MAL-ED sites and contribute to the rapid decline in exclusive BF observed in this study.

Methods we employed for dietary and micronutrient assessments have been described (19). Standardized dietary diversity indices were used to compare variation in diets across the sites during the first eight months of life. A varied diet is achieved by consuming four or more of seven different food groups during a 24 h recall period. All sites, with the exception of BRF, failed to reach a desirable diet diversity score. In addition, an adequate weaning and infant diet is needed to obtain required vitamins and micronutrients. The most common food groups consumed at many of the sites were grains, beans,

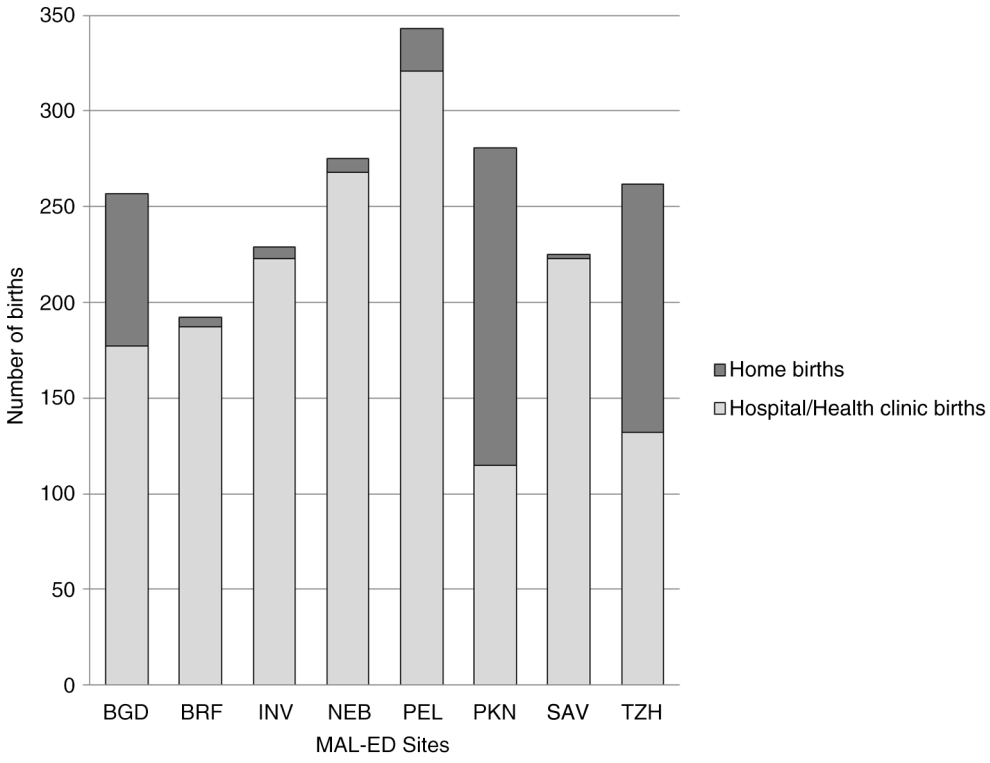

Fig. 2. The number of births occurring at home and at a medical facility in each of the MAL-ED sites.

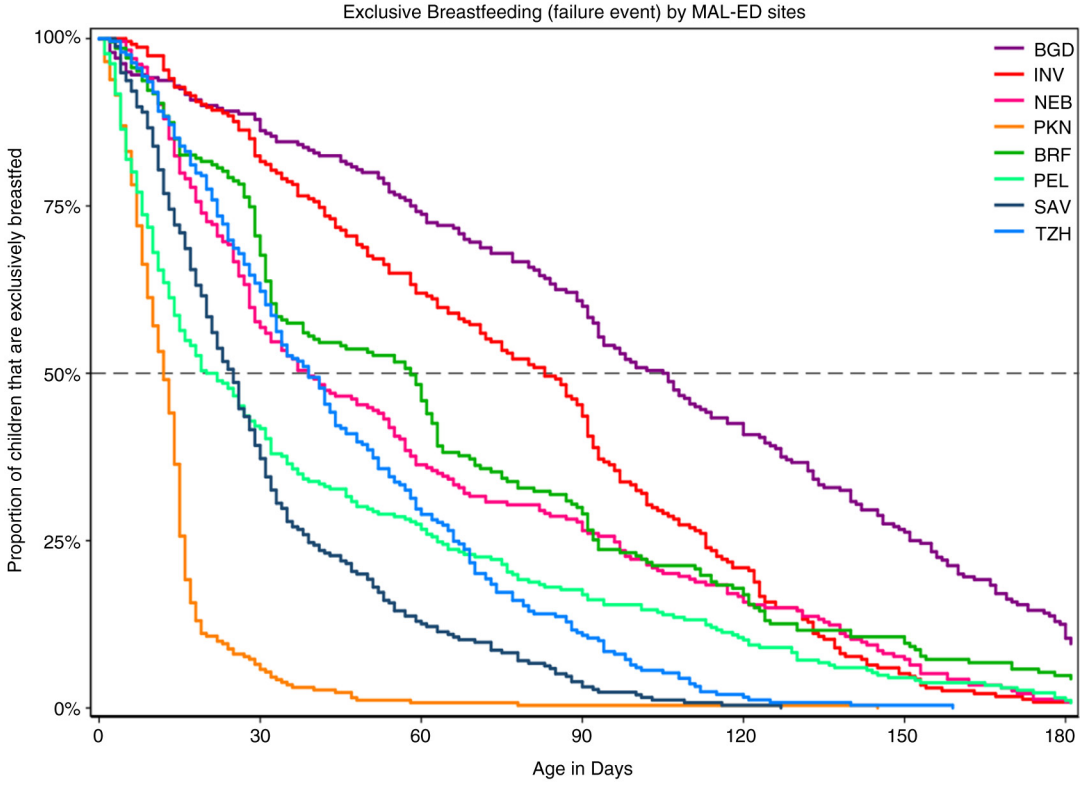

Fig. 3. Decrease in exclusive breastfeeding at MAL-ED Sites. Survival curves of exclusive breastfeeding are shown for each of the MAL-ED sites. Exclusive breastfeeding is defined as only having received colostrum and breast milk until such time as other liquids such as water, tea, solids are given.

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and dairy. Micronutrient deficiencies were observed in the MAL-ED cohort children. As an example, Table 1 shows the levels of anemia and zinc deficiency present at 7, 15, and 24 months of age (the times of blood draws) at each site.

Early exposure to, establishment of, and infection with enteric pathogens

At MAL-ED sites, newborn children are exposed to potential enteric pathogens early and often. As seen in Fig. 4a, in PKN, PEL, BGD, and TZH, about 50% of the children had potential pathogens (PP) present in normal stool at approximately one month of age. In INV, SAV, BRF, and NEB, that level was reached in two to three months. Virtually all infants had been colonized by PP at least once by the time they reached nine months of age. Most often, the presence of PP is not recognized because they produce no obvious symptoms. They were identified in this study because complete microbiologic analyses were conducted on these 'asymptomatic' normal stool samples that were collected once a month during the 24 months of active surveillance. That analysis detected enteric bacteria, parasites, and viruses and is described more completely in Ref. (20).

The first normal stool samples were collected in all cohort children one month after their birth. The most common PP identified in these samples are shown in Table 2. The most frequently identified pathogen at all sites was enteroaggregative *Escherichia coli* [EAEC, range: 8.4% (PEL) to 37.3% (PKN). *Campylobacter* was the second most common pathogen in six sites, and enterotoxigenic *E. coli* (ETEC) was in the top five pathogens at six sites (BGD, PKN, BRF, PEL, SAV, and TZH). Other common pathogens in these samples were *Cryptosporidium* and astrovirus. For a complete description of the microbiological findings from this study, see Platts-Mills et al. (Lancet, Global Health, in press).

While presence of enteric pathogens occurred very early at all sites, the age when the first diarrhea episodes occur in these children is quite varied (Fig. 4b). PKN is notable, in that diarrhea occurs at about the same time as the first detection of PP. At the other sites, the age at which 50% of the children have experienced their first diarrhea episode varies from about four months (BGD, INV, NEB, and PEL) to six months (TZH), 15 months (SAV), and 22 months (BRF). In these sites, particularly in SAV and BRF, many children never experience diarrheal symptoms despite the fact that they carry PP, often multiple pathogens simultaneously, during the first two years of their lives.

The high burden of PP does not appear to decrease as the children get older. As can be seen in Fig. 5, at least one enteric pathogen (bacteria, virus, or parasite) can be detected by culture, ELISA, PCR (for E. coli pathotypes), RT-PCR (for norovirus) or microscopy in 30–60% of non-diarrheal stool obtained from children at one month of age. This infectious burden increases throughout the observation period of the study and peaks at about 60% of normal stool in SAV, 70% in NEB, and 80–90% in the other six sites by the time the children are one year old. The frequency of isolation of pathogens from diarrhea stool is only slightly higher (peaks at 70–100% in all sites, data not shown). As noted above, many normal and diarrheal stools contain multiple pathogens simultaneously – as many as eight have been detected in normal stool (data not shown).

Use of antibiotics

The use of antibiotics to treat diarrhea and respiratory illness is high in many MAL-ED sites. As shown in Fig. 6a, the percentage of time during the first two years of life that children are treated with antibiotics for any reason

Table 1. Anemia and zinc deficiencies at 7, 15, and 24 months at MAL-ED sites

	Anemic[a]						Zinc deficient[b]					
	Number tested, % deficient						Number tested, % deficient					
	7		15		24		7		15		24	
BGD	202	49%	196	42%	175	27%	206	24%	195	19%	175	3%
PKN	261	72%	239	88%	223	83%	252	79%	238	69%	174	60%
INV	206	58%	228	56%	226	43%	221	51%	227	73%	224	88%
NEB	226	69%	220	50%	120	29%	221	33%	218	13%	118	23%
BRF	166	44%	150	40%	134	25%	147	4%	139	4%	81	2%
PEL	261	65%	227	51%	133	28%	233	2%	211	4%	104	0%
SAV	202	47%	227	53%	191	42%	30	7%	87	1%	44	7%
TZH	184	42%	197	40%	185	25%	132	29%	157	30%	150	27%

[a]Hb $<$110 g/L.
[b]Zn $<$9.9 mmol/L.

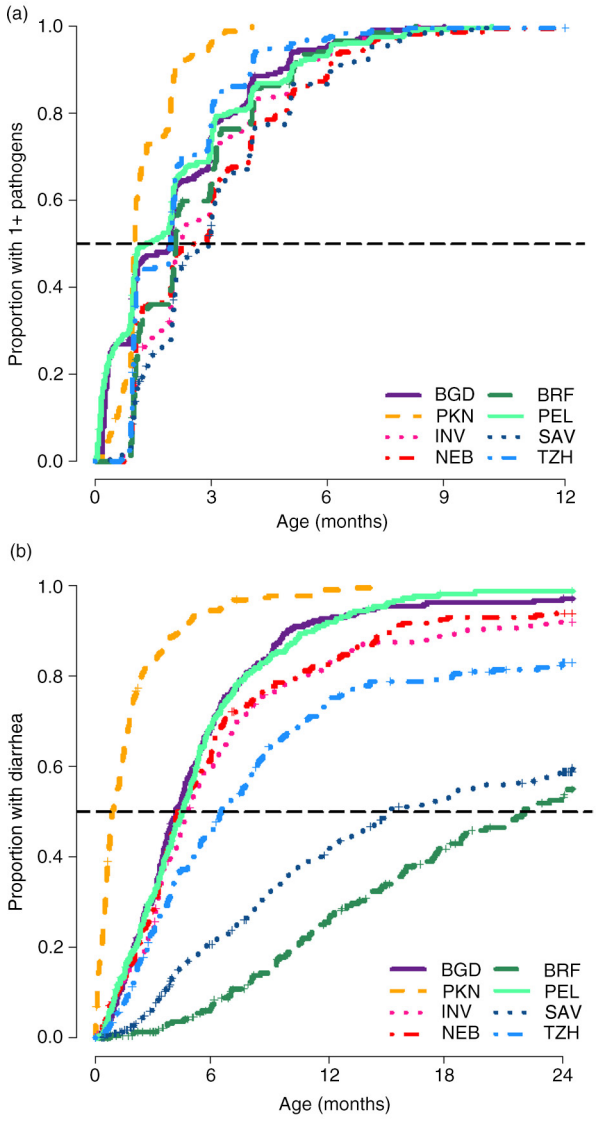

Fig. 4. (a) Proportion of cohort children at each MAL-ED site that have been infected with at least one enteric pathogen. (b) Proportion of cohort children at each MAL-ED site that has experienced at least one diarrhea episode.

ranges from a high of 16.9% in PKN to a low of 1.3% in BRF. Figure 6b shows that the number of diarrhea episodes for which antibiotics are given, also varies widely. In BGD, 60% of episodes are treated while in BRF only 11% are treated. Other sites fall between these extremes. Figure 6b also shows the average number of different antibiotics that are prescribed per episode of diarrhea by site. PKN used the highest number (average 0.9, range 0–8) while BRF used the lowest number of antibiotics (average 0.1, range 0–2). The most frequently prescribed class of antibiotic is metronidazole in PKN, NEB, and TZH; macrolides in BGD and PEL, cephalosporins in INV, penicillins in SAV, and sulfonamides in BRF.

Discussion

The MAL-ED study has identified factors that may contribute to the development of malnutrition and subsequent negative impact on a child's physical growth and cognitive development. These same factors may also hinder the use of probiotics as a way to establish or restore a beneficial microbiota to combat childhood malnutrition and to prevent or treat EED and its postulated negative effects. The study has revealed the need for renewed effort to educate mothers about the benefits of exclusive BF. In addition to providing the optimal early diet and defense against early diarrheal diseases, BF has been shown to have lasting positive effects on intelligence and educational achievement later in life (21), thus reinforcing the critical role that it plays in the establishment of a healthy microbiota and subsequent child development. None of the sites have achieved the WHO recommendation of exclusive BF for the first six months of life.

Diet has also been shown to affect the composition of the gut microbiota of children (22). In addition to providing the nutrients necessary for healthy growth and development, the weaning and infant diet should be thought of as contributing to the composition of a beneficial microbiota. The degree to which dietary supplements can be provided to support a healthy microbiota will be an important consideration in the design and implementation of trials aimed at testing the effectiveness of probiotic treatments.

While the use of antibiotics is justified in cases of serious bacterial infections, the use of narrow spectrum antibiotics could be used judiciously to target specific pathogen sensitivity and to minimize perturbations in the development of the microbiota that may have long-lived effects. The use of probiotics to treat EED or other intestinal disease should take into account the status of antibiotic use in the individual patient so as not to jeopardize the effectiveness of the probiotic microorganisms. The frequency at which antibiotics are used to treat enteric or respiratory infections in these settings will present a challenge to the effective implementation of probiotic therapies, especially if they will be used for prolonged periods of time. As shown recently by Rogawski et al., treatment of diarrhea in young children in India with antibiotics actually shortens the time to the next diarrheal episode by an average of eight weeks (23).

Petri et al. showed that the number of pathogens detected in either normal or diarrhea stool was about seven times higher in Bangladesh than in Virginia, USA, during the first year of life (13). This disproportionately heavy infectious burden borne by children in the developing world goes largely unrecognized as it occurs mostly in the absence of diarrheal symptoms. The questions remain as to the effects of these 'silent' infections on gut physiology and functions, including those associated with EED such as inflammation, leaky gut, and decreased absorptive capacity; what are the effects on the composition of the early microbiota?; should these pathogens be

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Table 2. Top five pathogens detected in non-diarrheal stools for one-month-old children in MAL-ED

	Sample size	1st pathogen (%)	2nd pathogen (%)	3rd pathogen (%)	4th pathogen (%)	5th pathogen (%)
BCD	241	EAEC (11.6)	Campylobacter (8.3)	Cryptosporidium (5.8)	ETEC (2.9)	Astrovirus (2.5)
PKN	185	EAEC (37.3)	Campylobacter (17.3)	Aeromonas (6.5)	ETEC (5.9)	Cryptosporidium (4.3)
INV	162	EAEC (17.3)	Campylobacter (3.7)	EPEC (1.9)	Astrovirus (1.2)	Rotavirus (1.2)
NEB	183	EAEC (17.5)	Campylobacter (7.7)	Astrovirus (4.9)	Cryptosporidium (4.9)	Atypical EPEC (2.2)
BRF	94	EAEC (27.7)	Cryptosporidium (12.8)	EIEC (9.6)	ETEC (7.4)	Atypical EPEC (6.4)
PEL	250	EAEC (8.4)	Campylobacter (7.6)	Cryptosporidium (6.0)	ETEC (3.2)	E. Histolytica (2.4)
SAV	214	EAEC (9.8)	Campylobacter (8.9)	ETEC (1.9)	Atypical EPEC (1.4)	Rotavirus (0.9)
TZH	239	EAEC (26.8)	Cryptosporidium (8.4)	Campylobacter (6.7)	ETEC (3.8)	Astrovirus (2.9)

Monthly, non-diarrheal stool is defined as a stool collected after 2 diarrhea-free days and preceding 2 diarrhea-free days. Only the first monthly stool is considered.
Table represents complete data only. All microbiology tests must have been performed for each sample to be included in the table.

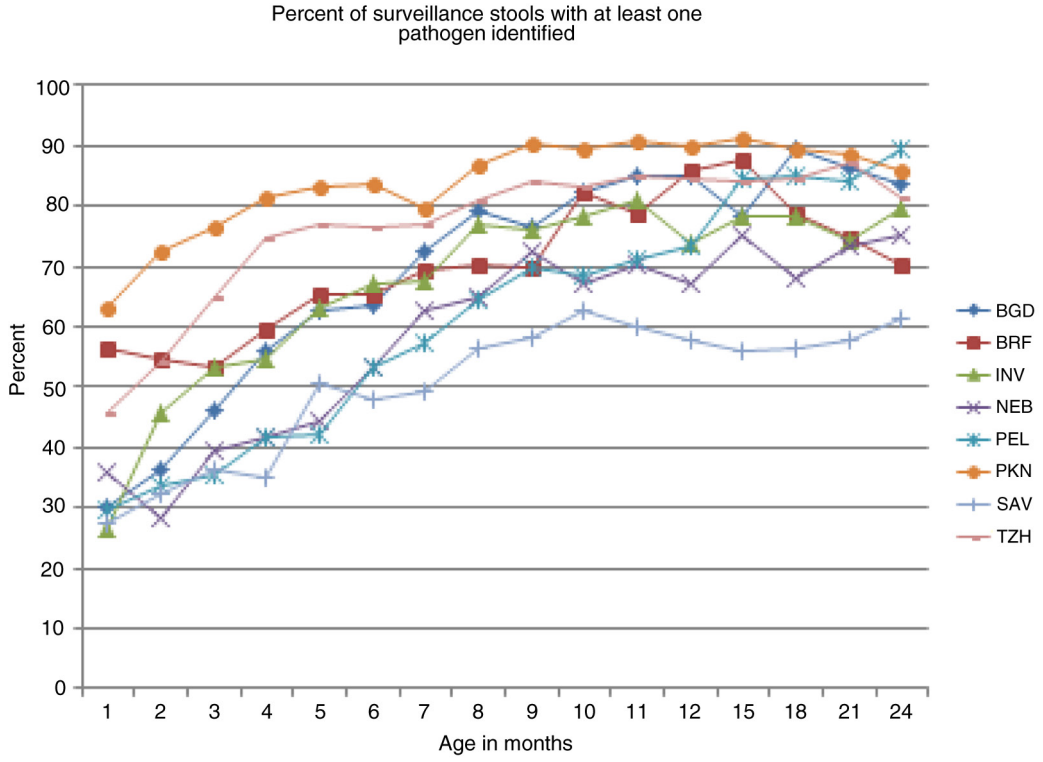

Fig. 5. Percent of normal stool samples containing at least one enteric pathogen. Normal stool samples were collected monthly and assayed for all enteric pathogens including bacteria, viruses, and parasites studied in MAL-ED. In the case of norovirus a subset of 10% of subjects were randomly selected from each site to have their normal stool samples assayed. The results for each site are shown as different colors indicated in the legend at the right of the figure.

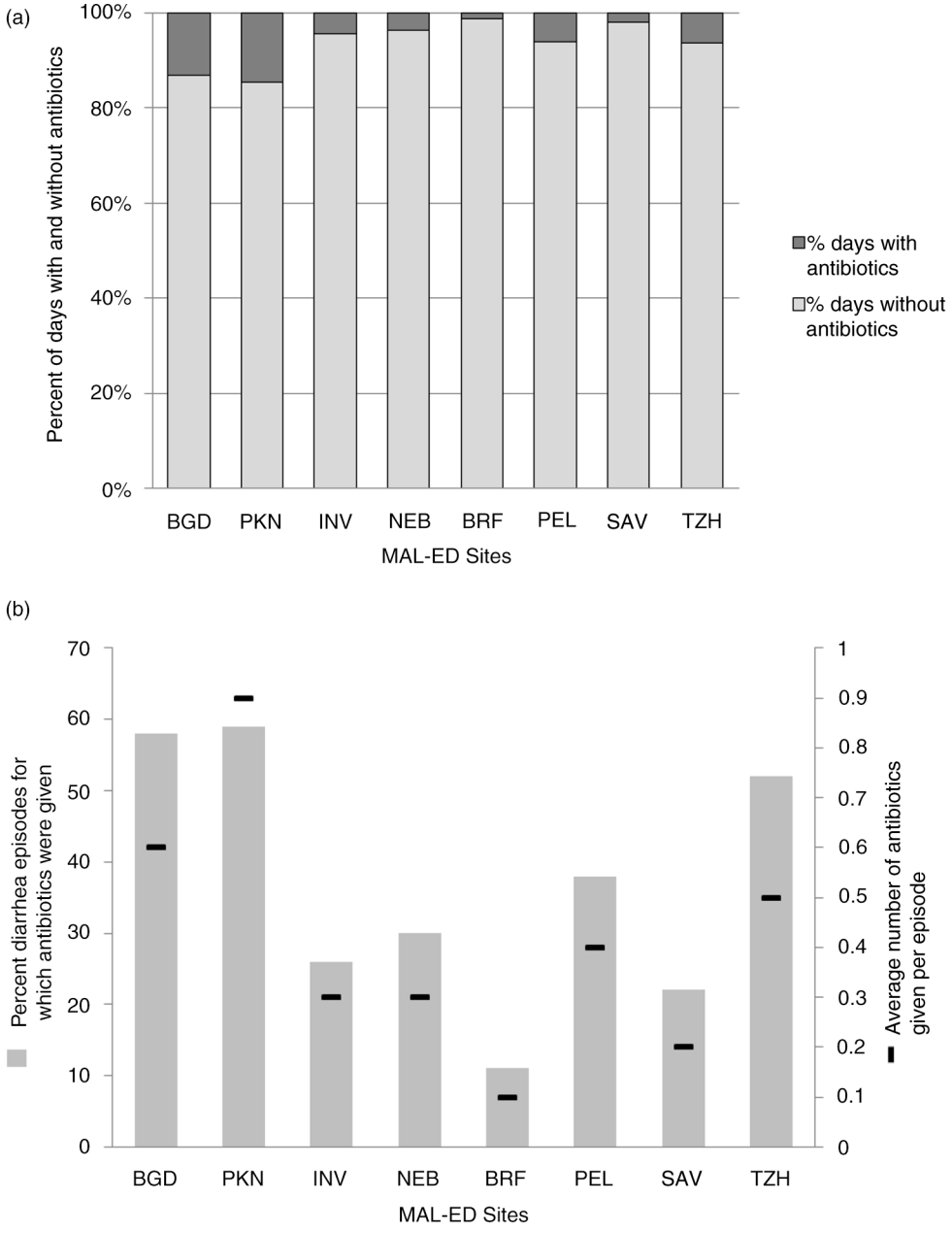

Fig. 6. (a) Percentage of days during the first two years of life that cohort children at each MAL-ED site received or did not receive antibiotics. (b) Treatment of diarrhea episodes with antibiotics at each MAL-ED site. Gray bars represent the percent of diarrhea episodes for which antibiotics were given. Black hash marks indicate the average number of antibiotics given.

considered as part of the 'normal' microbiota in areas of high fecal contamination?

It will be important to consider the relative extent of each these complicating factors at the sites where interventions will be tested. The MAL-ED data demonstrate the heterogeneity that exists between the sites. This heterogeneity will have to be considered in the design of a combination of interventions to target the specific conditions existing at each site.

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Conflict of interest and funding

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GUT IN FOCUS: EXTENDED ABSTRACT

Factors affecting infant gut microbiota and possible consequences for health

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From once being employed as an extreme measure, to save a fetus on the death of the mother, cesarean section is used more commonly now as a mode of delivery for entirely non-medical reasons (1). A sharp increase in cesarean delivery has been noted in most Western countries during the past decades; in the United States, one-third of all babies is now being delivered by cesarean section (2); in Norway, there has been a seven-fold increase in cesarean rates between the 1970s and 2001 (from 2 to 15%) and the incidence is still increasing (3). Focus so far has mainly been on the short-term health consequences of cesarean delivery, but the long-term effects may be even more important since cesarean delivery may disrupt gut microbiota during early infancy (4). Even if the gut microbiota is normalized with time, the early disruptions may have long-term effects due to the presence of developmental windows that rely on microbial stimulus from the gut, involving development of diverse functions, such as food tolerance, behavior, stress responses, and metabolism (5–8). Given the importance of early gut microbiota composition, it is important to gain knowledge on natural composition and factors altering it (9). However, a major limitation of previous studies is that they are based on infants who have been subject to factors which can have a profound disruptive effect on the natural colonization process (10–12).

In this review we are reporting on previous findings from a Norwegian cohort study. NoMIC is a prospective birth cohort established for the purpose of studying the colonization of infant gut microbiota and subsequent health (13, 14). Participating mothers were recruited at the maternity ward of a county hospital (Sykehuset Østfold) between 2002 and 2005. For every preterm-birth mother enrolled, two mothers of consecutively born term infants were recruited. The children are now aged between 9 and 12 years and have been invited for a clinical examination this year.

Gut microbiota composition was determined using 23 probes targeting 16S rRNA specifically developed for this study (13) as well as using 16S rRNA Illumina amplicon sequencing of feces, at Day 4 and 10, Month 1 and 4, and Year 1 and 2 (15). Extensive information on the use of antibiotics, mode of delivery, maternal diet, birth outcomes, and so on, is available through linkage to pregnancy records, medical birth registry, and repeat questionnaires to the mothers.

We examined the progression of gut microbiota from birth until 2 years. We identified children not subjected to medical interventions (vaginally delivered; term infants; not exposed to antibiotics directly nor through breast milk; breastfed for at least 4 months, exclusively in the first month; no antibiotics to the mother during the last trimester) to describe the gut microbiota composition in children as 'naturally as possible'.

Then, we calculated the change in weight from birth until 6 months of age to study whether early life gut composition was associated with early growth (14). A child's growth is expected to follow the percentile according to its birth weight. Mothers extracted information on weight from their 'baby health visit' cards, we used the weight closest to 6 months and the World Health Organization's weight-for-age growth curves.

The results we observed, and which are noted below, have already been published for most parts. A marked progressive change in microbial phyla composition with age was observed. The most marked change was observed in the phylum of *Proteobacteria* (15). Mode of delivery and antibiotics were among the determinants of decreasing diversity over time (15). Among term infants, only 85 out of 362 (23%) had not been subjected to measures that may alter gut microbiota (13). Even in newborns not subjected to cesarean delivery or antibiotics, *Staphylococcus* was the most prevalent microbial group detected at 4 days but no longer at 4 months. *Escherichia coli* was present in 70% of infants at 4 days and was increasing toward 4 months.

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At 4 months, different Bifidobacterial groups dominated in these breastfed infants (13). Absence of the probe coding for *E. coli* during the first month of life was associated with rapid growth during early life while the presence of *Bacteroides spp* at one month was associated with reduced growth, in males (14).

Discussion

Factors that may alter gut microbiota are surprisingly commonly applied (according to the findings in the NoMIC study) as only 23% of term babies had not been subjected to any such measure. Compared to the past century, *E. coli* is no longer a ubiquitous microbe in newborns. Previously, it was reported as being present in all newborn infants within 4 h after delivery, whereas studies in Sweden have shown that it is steadily less prevalent, especially among cesarean-delivered infants (16–18). We confirm these findings even in newborns not subjected to interventions. Moreover, our study shows that the absence of *E. coli* may be tied to adverse child health outcomes, since its absence was tied to rapid growth which is an early marker of the risk of obesity in later life. We hypothesize that *E. coli* may play an important role in very early life, maybe due to cascading events set in motion by the very early colonizers.

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GUT IN FOCUS: EXTENDED ABSTRACT

Immune modulation by non-digestible and non-absorbable beta-1,3/1,6-glucan

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Chemistry of beta-1,3/1,6-glucans

Beta-glucans comprise a very diverse group of polysaccharides (even paper) in which glucose molecules – as the only building block – are linked together by beta-linkages. Only very few beta-glucan structures, notably beta-1,3/1,6-glucans, are bioactive in the sense that they interact with receptors on immune cells and elicit specific biological responses.

Beta-1,3/1,6-glucans are branched chains of glucose molecules connected by beta-1,3-glycosidic bonds, and in which the branching points are beta-1,6 linkages, as shown in the schematic diagram for the beta-glucan found in yeast.

This net-like molecular structure constitutes the inner layer of the cell wall of baker's yeast, providing mechanical strength to the cells. In live cells, the beta-1,3/1,6-glucan structure is attached to a surface layer of complex proteoglycans (mainly proteomannans) and chitin.

Many published studies on biological effects of 'beta-glucans' have been carried out with poorly defined products and created misconceptions regarding mode of action of beta-1,3/1,6-glucans. A reference product like Zymosan contains, for instance, mannose-rich proteoglycans attached to the beta-1,3/1,6-glucan structure. Since these mannose-proteins are very potent allergens and antigens, whereas the pure beta-1,3/1,6-glucan component counteracts allergy and does not elicit any antibody production against itself, it is difficult to interpret experimental results on biological effects of Zymosan. Poor chemical description holds true also for extracts from mycelial fungi used in experimental studies. The beta-1,3/1,6-glucan component in crude fungal extracts has therefore incorrectly been held responsible for allergenic and pro-inflammatory effects of such extracts and of molds.

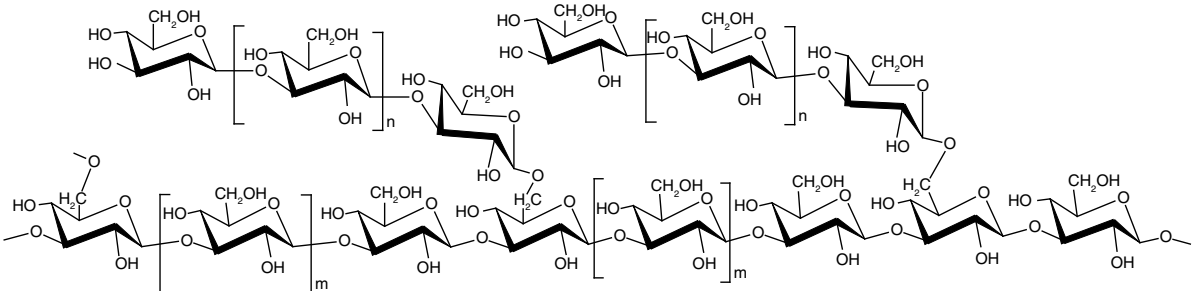
The ability of beta-1,3/1,6-glucans to activate innate immune cells depends on its branched structure. A beta-1,3-glucan without any side chains (branches) does not activate macrophages. Products with only one single glucose molecule in the 'side chain', as in most mushroom beta-1,3/1,6-glucans (e.g. lentinan), have lower macrophage activating activity than yeast cell-wall beta-1,3/1,6-glucan.

In addition to chain length, the frequency of side chains is essential for immune-stimulating and immune-modulating ability of beta-1,3/1,6-glucans (1, 2). These molecular structures may change or be destroyed during extraction, hence affecting the biological activity of the extracted product. The materials section of papers on biological effects of 'beta-glucans' should therefore be studied carefully.

Biological effects of beta-1,3/1,6-glucans

More than 30 years ago, Seljelid and co-workers screened a large number of glucans and glycans (polysaccharides containing also other sugars than glucose) for their macrophage-activating ability *in vitro*, and found that a particulate beta-1,3/1,6-glucan prepared from baker's yeast was the most active (3).

In the mid-1980s, it was first discovered in a practical field experiment that the same preparation Seljelid (3) had tested, enhanced resistance of salmon to infectious



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disease (vibriosis), even when it was incorporated in the feed at a low inclusion level (0.1% on dry weight basis). The discovery that a non-digestible feed component (fish or warm-blooded animals do not produce beta-1,3- or beta-1,6-glucanases) enhanced disease resistance was not in line with textbook teaching at the time. The discovery had to be scrutinized in a comprehensive research program before it could be considered trustworthy. But before scientific studies had fully confirmed and widened the scope of the initial field observation, the feed industry took the lead and introduced the same beta-1,3/1,6-glucan product (MacroGard) as an immune enhancer in animal feeds – worldwide.

During the last 25 years, this yeast beta-1,3/1,6-glucan preparation has been introduced as a feed additive to improve health and performance of farmed shrimp, fish, pigs, chicken, laying hens and calves, and of horses and pet animals. References (4–13) are a short 'short list' of experimental studies and reviews supporting the rationale of such use.

Mode of action of beta-1,3/1,6-glucans

The beta-1,3/1,6-glucan interacts with specific receptors (dectin-1, TLR 2/6, CR3) on white blood cells in the innate immune system (14–17), such as macrophages, neutrophils, granulocytes, natural killer cells, dendritic cells, and corresponding cells found in tissue surfaces.

Mucosal/oral administration of beta-1,3/1,6-glucans initiate biochemical processes leading to enhanced infection defense (4–13), enhanced antibody production against mucosal antigens (18), enhanced efficacy of injected monoclonal cancer antibodies (19), faster regeneration of physically damaged tissues (20), enhanced healing of (diabetic) wounds (21), reduced toxicity of bacterial endotoxin (22), and reduced gut infections (23, 24).

It is difficult to find one unifying, mechanistic model of mode of action 1,3/1,6-glucan at the cellular level explaining all of these effects. It is not clear to what extent the substance is taken up from the gut when it exerts its effects. Small amounts of beta-1,3/1,6-glucan may be taken up into endothelial cells, and the quantity in other tissues has always been found to be extremely low (25). It seems therefore most likely that the primary action of beta-1,3/1,6-glucan is in the gut epithelia and that systemic effects are secondary results of this interaction.

Anti-inflammatory effects of beta-1,3/1,6-glucans

Numerous farm reports have consistently referred to observations that orally administrated particulate beta-1,3/1,6-glucans have anti-inflammatory effects in animals, in addition to enhancing infection defense, in particular in settings where there is a high infection load. Commercial field trials have, for instance, confirmed that beta-1,3/1,6-glucan used as supplement to feeds reduces gastroenteritis problems in chicken and improve their general performance and increase egg yield in laying hens.

These positive effects may be the result of enhanced activity of intestinal immune cells (26), improved intestinal barrier function (27), stimulated formation of intestinal immune cells (23, 28), and reduced LPS-induced toxicity (22).

Already in the 1980s, the group of Rolf Seljelid (29) showed that injection of a pure beta-1,3/1,6-glucan derivative into mice rendered the animals very resistant to injected *Escherichia coli* and to endotoxemia. Later studies have shown that a highly purified and soluble version of the particulate beta-1,3/1,6-glucan used in animal feeds protects against lipopolysaccharide (LPS)-induced shock in rats (22). These studies demonstrated that the protective effect of orally administered beta-1,3/1,6-glucan was better than that of injected product. The serum concentration of beta-1,3/1,6-glucan was very low (3 ng/ml) in animals given the product orally and less than 1/40 of the concentration in animals given it by injection. In the experimental model study, rats were given beta-1,3/1,6-glucan (20 mg/kg body weight/day) for 14 days orally before they were subjected to endotoxemia by intravenous infusion of *Escherichia coli* LPS (6 mg LPS/kg). Rats pretreated with oral beta-1,3/1,6-glucan recovered significantly faster from LPS-induced blood pressure collapse than rats in the control group and faster than in rats pretreated by injected beta-1,3/1,6-glucan. Oral pre-treatment also significantly attenuated LPS-induced rise in plasma creatinine, aspartate aminotransferase and alanine aminotransferase, indicating protection also against LPS-induced renal and hepatic injury.

A US-patent from 2009 (23) describes how the same beta-1,3/1,6-glucan product (as in 21) can be used as an oral treatment and prevention of inflammatory diseases in the intestinal tract. A pure mushroom beta-1,3/1,6-glucan (lentinan) has later been shown to have corresponding effects (24), even at the same low concentration. Both groups have shown that oral administration of beta-1,3/1,6-glucan exhibits intestinal anti-inflammatory activity, and they suggest that beta-1,3/1,6-glucan may be effective for the treatment of gut inflammation, including inflammatory bowel disease (IBD).

Beta-1,3/1,6-glucans counteract not only LPS-induced inflammations but also the inflammation elicited by influenza virus (18). Nasal administration of particulate beta-1,3/1,6-glucan prior to intra-nasal infection by the virus, remarkably reduced disease score resulting from the cytokine storm elicited by the influenza virus.

The ability of beta-1,3/1,6-glucan to suppress inflammatory response has been tested also in humans scheduled for coronary artery bypass grafting (20). Pretreatment for 5 days with oral particulate beta-1,3/1,6-glucan caused significantly lowered creatine kinase isozyme

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and cardiac troponin levels the first day post operation, and it was concluded that beta-1,3/1,6-glucan pretreatment is safe and *may protect against ischemia reperfusion injury following CABG.*

Beta-1,3/1,6-glucan and gut microbiota

The biological effects beta-1,3/1,6-glucans are usually explained within the framework of conventional immunology, as the result of the interaction between beta-1,3/1,6-glucan and specific receptors on epithelial surfaces (24). However, there are effects which may involve the gut microbiota, such as suppressive effects of pure beta-1,3/1,6-glucan on asthma and allergy symptoms.

Beta-1,3/1,6-glucan molecules will pass non-digested through the small intestine, but become the substrate for microbes in the colon. The amount of pure beta-1,3/1,6-glucan exerting significant biological effects is low, however, and it is therefore unlikely that it will be a significant energy substrate for microbes living under anaerobic conditions in the colon. Beta-1,3/1,6-glucan may nevertheless interact indirectly with the gut microbiota by affecting intestinal barrier function and LPS toxicity, and by enhancing the production and secretion of components such as lysozyme, antimicrobial peptides and IgA.

Immune modulating, pure beta-1,3/1,6-glucans exert their effects at very low concentration in the diet, and should therefore not be compared with the 'fiber effects' of dietary cereal beta-glucans. The latter are different in chemical composition and they affect gut functions and gut microbiota at much higher concentrations.

There are unreported observations on the effects of pure immune modulating beta-1,3/1,6-glucans on the composition of the gut microbiome of warm-blooded animals, but such studies have not been given priority in scientific follow-up studies – yet. But within the fish farming sector it is different (30).

Perspectives

Many studies with poorly described, crude beta-glucan preparations have contributed to confusion, misconceptions, and controversies regarding biological effects of chemically well-defined beta-1,3/1,6-glucans. Beta-1,3/1,6-glucans are already attractive pharmaceutical products and product candidates for a number of clinical indications.

Due to their ability to enhance infection defense mechanisms and simultaneously down-regulate inflammations, beta-1,3/1,6-glucan is very promising as an alternative to the mainstream use of immunosuppressive drugs to treat inflammatory diseases, for instance, IBD. New formulations – not necessarily based on beta-1,3/1,6-glucans alone – will certainly be developed and used prophylactically and possibly also therapeutically to reduce the need for antibiotics in human and veterinary medicine.

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GUT IN FOCUS: EXTENDED ABSTRACT

Enteric short-chain fatty acids: microbial messengers of metabolism, mitochondria, and mind: implications in autism spectrum disorders

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Clinical observations suggest that gut and dietary factors transiently worsen and, in some cases, appear to improve behavioral symptoms in a subset of persons with autism spectrum disorders (ASDs), but the reason for this is unclear. Emerging evidence suggests ASDs are a family of systemic disorders of altered immunity, metabolism, and gene expression. Pre- or perinatal infection, hospitalization, or early antibiotic exposure, which may alter gut microbiota, have been suggested as potential risk factors for ASD. Can a common environmental agent link these disparate findings? This review outlines basic science and clinical evidence that enteric short-chain fatty acids (SCFAs), present in diet and also produced by opportunistic gut bacteria following fermentation of dietary carbohydrates, may be environmental triggers in ASD. Of note, propionic acid, a major SCFA produced by ASD-associated gastrointestinal bacteria (*clostridia*, *bacteroides*, *desulfovibrio*) and also a common food preservative, can produce reversible behavioral, electrographic, neuroinflammatory, metabolic, and epigenetic changes closely resembling those found in ASD when administered to rodents. Major effects of these SCFAs may be through the alteration of mitochondrial function via the citric acid cycle and carnitine metabolism, or the epigenetic modulation of ASD-associated genes, which may be useful clinical biomarkers. It discusses the hypothesis that ASDs are produced by pre- or post-natal alterations in intestinal microbiota in sensitive sub-populations, which may have major implications in ASD cause, diagnosis, prevention, and treatment.

Keywords: *autism spectrum disorder*; *short-chain fatty acids*; *food preservative*; *antibiotic*; *microbiome*; *carnitine*; *gastrointestinal*; *mitochondria*; *oxidative stress*; *glutathione*; *epigenetics*; *neuroinflammation*; *neurexin*; *gap junctions*; *lipids*

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Let food be thy medicine, and medicine be thy food
Everything in excess is opposed to nature
All disease begins in the gut

—Hippocrates

The Prophet said: Stomach is the home (source) of all illness

—Hadith (Islam)

When Jesus climbed out of the boat, a man possessed by an evil spirit came out from a cemetery to meet him. This man lived among the burial caves and could no longer be restrained, even with a chain. Whenever he was put into chains and shackles—as he often was—he snapped the chains from his wrists and smashed the shackles. No one was strong enough to subdue him. Day and night he wandered among the burial caves and in the hills, howling and cutting himself with sharp stones . . .

Then Jesus demanded, 'What is your name?'
And he replied, 'My name is Legion, because there are many of us inside this man'.

—Gospel of St. Mark, New Testament.

Autism spectrum disorders (ASDs) are a family of neurodevelopmental conditions of rapidly increasing incidence. The condition was originally prevalent in approximately 1 in 10,000 when first reported in the middle of the 20th century. At present, ASD occurs in one in 68 persons in the United States, and may be as many as one in 30 in Korea. More prevalent in males (4:1), ASD comprises behavioral symptoms, including communication and social impairments, sensory abnormalities, and restricted and repetitive behavior, often with self-injurious behavior (1). In many children and adults with ASD, comorbidities include restrictive eating, gastrointestinal symptoms, and seizure

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disorder (2, 3). Recent studies have suggested that ASD is not a primary brain disorder, but may be a 'whole body' disorder with broad systemic abnormalities in immune and metabolic function (4–8). Anecdotal reports also suggest these findings may be associated with possible regression after apparently normal development in a subset of children (9). This is particularly evident in some populations that have migrated from underdeveloped countries to more developed ones, such as Somali expatriates (10), which was also discussed in a recent Canadian documentary ('The Autism Enigma', Canadian Broadcasting Corporation, Cogent Benger Productions, 2011). How these disparate findings relate to ASD symptoms or pathogenesis is unclear.

It is becoming apparent that complex interactions between genetic, epigenetic, and environmental factors contribute to the development and expression of ASD. A wide number of genes involved in immune regulation, mitochondrial function, and neural circuit formation have been implicated (11). However, known genetic factors discovered thus far account for 10–20% of ASDs and concordance rates among monozygotic twins are less than 100%, suggesting an important role for environmental risk factors which act on the underlying genetic susceptibilities (12), possibly by altering expression of ASD-implicated genes or functional pathways (6, 13). We have proposed that many of these environmental factors may arise, directly or indirectly, from small molecule metabolites from microbial populations in the gut (6, 7, 14).

Enteric short-chain fatty acids – gut microbiota metabolites in health and disease

There is growing evidence that the diverse populations of microbes, which inhabit the human digestive tract, termed the gut *microbiota* (GM), play a major role in the modulation of diverse host metabolic and immune pathways in both health and disease (15, 16). This microbial ecosystem outnumbers host cells 10 to one and genetic material 100 to one. It behaves as a functional 'organ', playing a major role in gut–brain communication, immune function, metabolism, and even behavior (6, 7, 17–19). Enteric short-chain fatty acids (SCFAs) are a major class of signaling molecules produced from bacterial fermentation of dietary carbohydrates, odd-chain fatty acids, and some proteins (20, 21). The most abundant of these are acetic acid (AA), butyric acid (BA), and propionic acid (PPA) (20). These SCFAs directly affect the host digestive tract through phenotypic alteration of colonic epithelial cells and act as major energy substrates. They can act as tumor suppressor agents, in apoptotic cell death, and have recently been shown to be modulators of the enteric neuroendocrine system. SCFAs are also involved in gene regulation of anti-inflammatory processes both *in vitro* and *in vivo* (22–29).

Being weak organic acids miscible in both aqueous and lipid phase, the majority of SCFAs are absorbed both passively and via active transport by monocarboxylate transporters from the gut, which also transport ketones. Those not metabolized by colonocytes (principally BA) are transported via the portal circulation and metabolized in the liver before reaching the systemic circulation. Traditionally, hepatic clearance was thought to reduce systemic effects of SCFAs on systemic metabolic and regulatory pathways. However, the distal colon, where the majority of GM reside, bypasses the portal circulation enabling systemic access (6, 7, 20).

In addition to production through colonic bacterial fermentation, SCFAs may also be present in diet. Of note, PPA and its chemical derivatives have increasing use in agriculture and the food industry (20). PPA occurs naturally in many foods (i.e. Swiss cheese). It is a major animal silage and food preservative in wheat and dairy products, either as sodium or calcium salt (30, 31) or is produced by adding high fructose corn syrup substrate to propionibacteria cultures which are then inoculated into foods. Inulin propionate has recently been suggested as a weight loss agent (32, 33), and aspartame is known to increase PPA levels in rodent gut flora. Nitropropionic acid, a derivative of PPA produced by many plants and fungi, is a potential contaminant of processed rice and sugar cane, and also produced sometimes in ruminant fermentation. It is a potent mitochondrial toxin, capable of causing neurotoxicity, and its administration in rodents is an acceptable model for Huntington's chorea (34).

There is growing evidence that the systemic effects of SCFAs (especially PPA and BA) on host physiology are underappreciated. This may have been secondary to 1) their production across tissues of large surface area and relative inaccessibility (i.e. small and large intestine), 2) their rapid colonic uptake by monocarboxylate transporters, 3) their ability to intracellularly concentrate, particularly during acidotic states, and 4) their rapid metabolism, all of which make SCFA measurement difficult (6, 20).

SCFAs have a number of direct effects on gastrointestinal physiology. They are known to reduce gastric motility and increase the frequency of contractions, presumably via a reflex that involves direct contact of these SCFAs with the terminal ileum (35). In addition, PPA and BA increases contraction of colonic smooth muscle (36), dilates colonic arteries (37), activates mast cells (38), and increases the release of serotonin from gut enterochromaffin cells (39, 40). Specific free fatty acid G-protein-coupled receptors (GPCRs) have recently been located throughout the enteric nervous and immune (T reg) systems, and offer many opportunities for novel pharmacotherapeutic agents (29).

Most of the emerging literature supports the beneficial effects of SCFAs on weight control, lipid profiles, and colon health, which appear to be dose and tissue specific (see 6, 20 for reviews). In spite of the multiple beneficial effects of SCFA on host gastrointestinal activity, it is important to note that excessive quantities of PPA have

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been reported in acne (41), gingival inflammation (42), irritable bowel syndrome (43), and necrotizing enterocolitis (44). It is also elevated in the neurometabolic condition propionic acidemia (45). In this heterogeneous inborn error of fatty acid metabolism, which may be underreported (46), accumulation of PPA and possibly other SCFAs is associated with developmental delay, seizure and extrapyramidal findings, acidosis, hyperammonianemia, increased oxidative stress, and mitochondrial dysfunction, often accompanied by bouts of gastrointestinal symptoms (45). Furthermore, PPA and related SCFAs have broad effects on nervous system physiology, including activation of specific free fatty acid GPCR, neurotransmitter synthesis and release, intracellular pH/calcium gating, mitochondrial function, lipid metabolism, immune function, gap junction gating, and gene expression (6).

Potential links of SCFAs in autism

Recent evidence suggests potential, but unproven, links between dietary, metabolic, immune, infective, and gastrointestinal factors and ASDs. Although inheritable factors, mostly implicated in synaptic transmission, have been traditionally studied in ASDs (11), the fact that 1) known genetic factors thus far account for only 10–20% of cases, 2) there is less than 100% concordance in identical twins, and 3) there is a growing prevalence in the condition, collectively suggest an important role for environmental factors which act on the underlying genetic sensitivities (12). In particular, C-sections, hospitalization, early infections, and associated antibiotic exposure (47), which are risk factors for ASD, may alter the developing GM (16). Increased mean levels of PPA in stool of ASD children have been shown (48). Given that PPA is a key fermentation product of ASD-associated bacteria (*clostridia*, *bacteroides*, *desulfovibrio*) (49) and modulates many ASD-related biochemical processes, we have proposed that SCFAs represent a group of host GM metabolites that are plausibly linked to ASD and can induce widespread effects on gut, brain, immune and metabolic function, and behavior (6, 7).

Further to this, we (14, 50–55) and others (56–59) have shown that short-term central nervous system intracerebroventricular (ICV) and peripheral administration (intraperitoneal, subcutaneous or oral gavage) of PPA and, to a lesser extent, other SCFAs, at various developmental time periods in rodents, induce broad behavioral and brain effects remarkably consistent with findings in persons with ASD, and even predict potential biomarkers in patients.

ICV infusion of SCFAs in rats induces reversible behavioral and electrographic effects consistent with autism

Repeated (5–14 days) pulsed ICV infusions of buffered SCFAs (0.026, 0.052, 0.26 M, 4 μl, pH buffered to 7.5), approximating the levels found in propionic acidemia patients, elicit a number of reversible behavioral changes reminiscent of ASD. Within 2–30 min post-ICV infusion, PPA and, to a lesser extent, BA and AA-treated rats were found to show reversible repetitive dystonic behaviors, retropulsion, object preference, and behavioral perseveration. These behaviors are absent in rats receiving control compounds (isomolar l-propanol or phosphate-buffered saline vehicle). Behavioral scoring and electrographic recordings also provided evidence of seizure activity involving both cortical (neo/hippocampal) and subcortical (striatal spiking) abnormalities. With repeated SCFA treatment, some animals also showed evidence for 'kindling' of seizures (PPA > BA > AC). These effects were not seen with the control compounds. Additional behavioral effects observed in PPA-, BA-, and AA-treated rats included hyperactivity in an automated open-field test, impairment in social behavior when tested in pairs in a large open-field, impairments in the reversal of spatial learning in the Morris Water Maze, object preference, and enhanced startle response magnitude to an acoustic stimulus. Of particular importance was the finding that rats treated with SCFAs showed very clear impairments in social interaction which were not a function of changes in locomotor activity (Fig. 1). Overall, PPA was the most effective in elicitation of ASD-like behaviors. Thus, enteric SCFA exposure in rodents mimics many behavioral findings in ASD.

Potential mechanisms for these rapidly induced and reversible behaviors are complex, and include SCFA-mediated effects such as enhanced calcium-dependent glutamate, serotonin and dopamine release, inhibition of GABAergic receptors, activation of specific SCFA GPCR, increased glutamate receptor sensitivity, increased catecholamine synthesis, intracellular acidification, mitochondrial dysfunction, and closure of gap junctions (see 6, 7, 14, 50, 51, 60, 61 for reviews).

Regarding the latter, we have postulated that many of the effects of PPA may be due to its ability to reduce intracellular connectivity via the closure of gap junctions (6, 7, 14).

Gap junctions are intercellular channels which allow passage of ions and small molecules. They are composed of protein subunits known as *connexins* and are gated by a number of factors, including dopamine, calcium, and cytokines, all of which are influenced by PPA (14). Gap junctions play a major role in cellular differentiation and, in particular, peripheral nerve, cardiac, uterine, and gastrointestinal function. However, in the CNS, gap junction coupling is vital for the synchronization of neural electrical activity within discrete functional cell groups. Gap junction-mediated coupling is more extensive during early brain development and neuronal migration and is thought to play a major role in brain development. Astrocytes are extensively electrotonically connected by gap junctions, forming a physiological syncytium to uptake and spatially

A – PPA repetitive behavior
B – control rat
C – PPA social
D – control pair social
E – Ethovision pair
F – PPA object fixation

Fig. 1. Behavioral videos of propionic acid infusions in rats (click headings to view videos). Single intracerebroventricular (ICV) infusions (4 μl of 0.26 M solution over 4 min) of propionic acid (PPA), a metabolic end product of autism-associated enteric bacteria, produce bouts of reversible hyperactive and repetitive behavior (A) in adult rats, compared with phosphate-buffered saline (PBS) vehicle infused control rat (B). Rat pairs infused with PPA show markedly reduced social interaction and play behavior (C), compared with pairs of rats infused with PBS vehicle (D), which show typical social behavior. Ethovision behavioral tracking of control and PPA-treated rat pairs (E), showing further evidence of PPA-induced hyperactive, repetitive, and antisocial behavior. PPA-treated rat displays fixation on objects (F) and specific object preferences (i.e. block vs. sphere). PPA-infused rats also show turning, tics, dystonia, and retropulsion and electrographic evidence of complex partial seizures and basal ganglia spiking, consistent with findings in patients with autism spectrum disorders. With permission from MacFabe (6).

buffer calcium, glutamate, and potassium, to stabilize the extracellular CNS microenvironment (62).

Small molecules, many of which are apoptotic factors (calcium, sodium, lysophospholipids, inositol triphosphate), are capable of passing through these glial gap junctions (63, 64). Therefore, closed glial gap junctions may render neurons hyper excitable due to rising extracellular potassium and glutamate (64), while closed neuronal gap junctions would be neuroprotective (63). In turn, this decrease in gap junction coupling may lead to inhibited cortical pruning in development, consistent with the increased neuronal density found in ASD (14). Gap junction communication is involved in neurotransmission in the basal ganglia, prefrontal cortex, nucleus accumbens, and hippocampus: all areas that are implicated in seizure and movement disorders. Intrastriatal injections of gap junction blockers produce stereotypical movements, hyperlocomotion, and disruption of motor sequencing in rodents (65, 66). Furthermore, gap junction knockout mice show abnormal brain development, exaggerated responses to neurotoxic insults, seizure disorder, and abnormal behaviors (67).

Interestingly, gap junction blockers, such as volatile anesthetics, ethanol, oleamide, glycyrrhetinic acid, carbenoxylone, and SCFAs, also inhibit tight junctions in many cellular systems (68, 69), possibly contributing to altered barrier function in the placenta, brain, and GI tract in ASD (70). Given these findings, it seems plausible that PPA-induced alterations to gap junction and tight junction function may have widespread effects on behavior, neural development, and gut and placental function, and may play a role in ASD (6, 7, 14).

Brief systemic exposure of SCFAs at critical neurodevelopmental windows have sex specific enduring behavioral effects consistent with autism

Traditionally, most potential environmental factors implicated in ASD are thought to principally exert their effects at critical pre- and early post-natal neurodevelopmental windows, either alone, or synergistically with other factors (i.e. the 'double-hit hypothesis'), possibly by epigenetic means (71). Further to this, we sought to examine the behavioral effects of early brief exposure to systemic PPA. This SCFA exposure occurred with or without the microbial cell wall product lipopolysaccharide (LPS), a known activator of innate immunity and another acceptable model of ASD (54, 55). Pregnant Long-Evans rats were subcutaneously injected once a day with PPA (500 mg/kg) on gestation days G12–16, LPS (50 μg/kg) on G15–16, or vehicle control on G12–16 or G15–16. Male and female offspring were injected with PPA (500 mg/kg) or vehicle twice a day, every second day from postnatal days (P) 10–18. Physical milestones and reflexes were monitored in early life with prenatal PPA and LPS. Developmental milestones including delays in eye opening, locomotor activity, and anxiety were assessed in adolescence (PND40–42) in the elevated plus maze (EPM) and open-field motor activity. Prenatal and postnatal treatments altered behavior in a sex-specific manner. Prenatal PPA decreased time spent in the center of the open-field in males and females while prenatal and postnatal PPA increased anxiety behavior on the EPM in female rats. Prenatal LPS did not significantly influence those behaviors. Evidence for the double-hit hypothesis was seen as females receiving a double hit of PPA (prenatal and postnatal) displayed increased repetitive behavior in the open-field.

In similar experiments, acoustic startle and pre-pulse inhibition were measured on PND 45, 47, 49, and 51. Prenatal and postnatal treatments altered startle behavior in a sex-specific manner. Prenatal LPS treatment produced hyper-sensitivity to acoustic startle in males, but not females and did not alter pre-pulse inhibition. Subtle alterations in startle responses that disappeared with repeated trials occurred with prenatal PPA and postnatal PPA treatment in both male and female offspring. Prenatal PPA treatment decreased pre-pulse inhibition in females, but not males. Finally, females receiving a double hit of PPA, prenatal and postnatal, showed sensitization to acoustic startle, providing evidence for the double-hit hypothesis. Furthermore, both male and female PPA-treated pups were impaired in a test of their nest seeking response, suggesting impairment in olfactory-mediated neonatal social recognition. As well, adolescent males, born to PPA-treated dams, approached a novel object more than control animals and showed increased levels of locomotor activity compared to prenatal PPA females.

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Prenatal LPS produced subtle impairments in social behavior in adult male and female rats. These findings raise the possibility that brief systemic prenatal exposure to elevated levels of GM products, such as PPA or LPS, can subtly influence neonatal, adolescent, and adult social behavior. Collectively, these findings show early exposure to SCFAs, with or without combined immune stimulation from other gut-derived compounds (LPS), are capable of inducing long term behavior effects consistent with ASD.

Central infusions of SCFAs produce neuroinflammatory and oxidative stress effects consistent with autism, and also activate neuroplastic memory (CREB) and fatty acid transport (monocarboxylate) systems. Twice-daily ICV infusions over 7–14 days of buffered PPA, BA, or AA (0.026, 0.052, 0.26 M, 4 μl, pH 7.5) produce broad brain changes reminiscent of ASD. Neuropathological analysis (hippocampus and external capsule white matter) (Fig. 2) revealed increases in reactive astrocytes (PPA, BA) and activated microglia (PPA), consistent with findings in ASD autopsy cases. In addition, further histochemical studies revealed increased monocarboxylate transporter/phosphorylated cyclic AMP respondent element binding protein (pCREB) immunoreactivity, a key factor in the epigenetic control of memory acquisition, in the absence of gross neuronal loss and apoptotic effects (caspase 3'), indicating broad effects in neuroplasticity and fatty acid metabolism (6, 7, 14, 50, 51, 60, 61).

Analyses of homogenates of brain regions produced evidence of increased oxidative stress and impaired glutathione (GSH) metabolism in discrete regions in PPA-treated animals. Biomarkers of protein and lipid peroxidation, total GSH as well as the activity of the antioxidant enzymes superoxide dismutase, catalase, GSH peroxidase, GSH reductase, and glutathione *S*-transferase (GST) were examined. Some brain regions of PPA-treated animals (neocortex, hippocampus, thalamus, striatum, cerebellum) showed increased lipid and protein oxidation accompanied by decreased total GSH in neocortex. Catalase activity was decreased in most brain regions of PPA-treated animals, suggestive of reduced antioxidant enzymatic activity against broad environmental zenobiotics implicated in ASD (metals, Tylenol administration). Collectively, these findings are consistent with those found in ASD patients (4–7, 14, 50, 60).

Lipid/mitochondrial/acylcarnitine profiles in PPA rodent model are consistent with findings in autism patients

We then wished to determine if there were any alterations in brain lipids associated with the ASD-like behavioral changes observed following intermittent ICV infusions of PPA, the related enteric metabolite BA or PBS vehicle. As in previous studies, both PPA and BA produced significant increases ($p < 0.001$) in locomotor activity (total distance travelled and stereotypy). PPA and to a lesser extent BA infusions decreased the levels of total monounsaturates, total omega-6 fatty acids, total phosphatidylethanolamine plasmalogens, the ratio of omega-6:omega-3 and elevated the levels of total saturates in separated phospholipid species. In addition, total acylcarnitines, total long-chain (C12–24) acylcarnitines, total short-chain (C2–9) acylcarnitines, and the ratio of bound to free carnitine were increased following infusions with PPA and BA.

We applied electrospray ionization mass spectroscopy analysis to determine how brain and blood intact phospholipid species were altered during the induction of ASD-like behaviors in rats following ICV infusions with PPA. Animals were infused daily for 8 days, locomotor activity assessed, and animals were sacrificed during the induced behaviors. PPA infusions increased locomotor activity. Lipid analysis revealed treatment altered 21 brain and 30 blood phospholipid molecular species. Notable alterations were observed in the composition of brain sphingomyelin, diacyl mono and polyunsaturated phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, and plasmalogen phosphatidylcholine and phosphatidylethanolamine molecular species. These alterations suggest that SCFAs are able to cause broad changes in CNS lipid physiology, including membrane fluidity, cell signaling, redox capacity, and mitochondrial/carnitine function, consistent with findings in ASD patients (6, 7, 14, 50, 52, 60, 72).

Mitochondrial dysfunction found in the PPA rodent model predict novel biomarkers in a sub-set of autism patients

A comprehensive review has noted that ASD may occur with genetic and biochemical changes (lactate, pyruvate, carnitine alterations) consistent with mitochondrial disease (73). However, specific genetic mutations to explain mitochondrial disease are rare, suggesting that mitochondrial disease in some ASD patients may be environmentally acquired. As mentioned, our animal model similarly demonstrates many mitochondrial lipid changes associated with ASD, including a unique pattern of elevated short-chain and long-chain acyl-carnitines, suggesting broad alterations in fatty acid metabolism (6, 7, 52). To determine if these mitochondrial-related biomarkers are present in ASD patients, the laboratory results from a large cohort of children with ASD (n = 213) who underwent screening for metabolic disorders, including mitochondrial and fatty acid oxidation disorders, in an autism clinic were reviewed (74). Acyl-carnitine panels were determined to be abnormal if three or more individual acyl-carnitine species were abnormal in the panel by repeated testing. Overall, 17% of individuals with ASD demonstrated consistently abnormal short- and long-chain acyl-carnitine panels consistent with the PPA rodent ASD model. Examination of electron transport

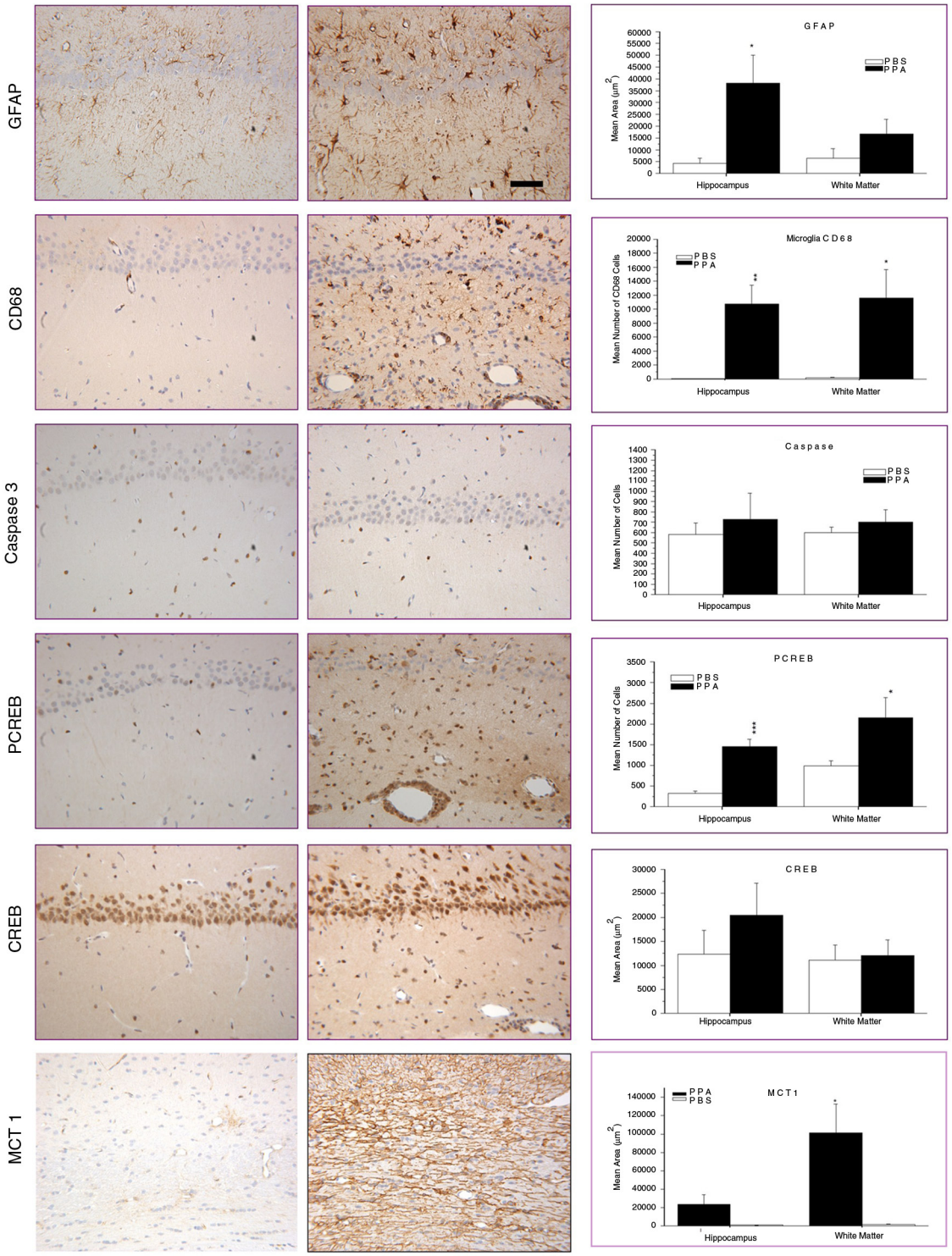

Fig. 2. Neuropathology (avidin–biotin complex immunohistochemistry) and semiquantitative image densitometry of coronal brain sections of dorsal hippocampus (CA2) and external capsule of adult rats with 14-day BID ICV infusions of propionic acid (PPA) or phosphate-buffered saline (PBS). PPA-induced significant reactive astrogliosis (anti-GFAP) and microglial activation (anti-CD68), without apoptotic neuronal cell loss (anti-cleaved caspase 3) in rat hippocampus, similar to finding in autopsy brain from patients with autism. Nuclear translocation of anti-CREB and an increase of anti phosphoCREB immunoreactivity are observed in neural, glial, and endovascular epithelium by PPA treatment, suggestive of gene induction. PPA increases monocarboxylate transporter 1 immunoreactivity, primarily in white matter external capsule, suggestive of alterations in brain short-chain fatty acid transport/metabolism. Black bars indicate PPA-treated animals; white bars indicate PBS (vehicle)-treated animals. Horizontal measurement bar = 100 µ. With permission from MacFabe (6).

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chain function (muscle, fibroblast culture) and histological and electron microscopy examination of muscle suggest that PPA could be interfering with mitochondrial tricarboxylic acid metabolism (Fig. 3.). The function of the fatty acid oxidation pathway in fibroblast cultures and biomarkers for abnormalities in non-mitochondrial fatty acid metabolism were not consistently abnormal across the subgroup of ASD children, suggesting that the fatty acid metabolic abnormalities were secondary to tricarboxylic acid cycle abnormalities. GSH metabolism was abnormal in the ASD subset with acyl-carnitine panel abnormalities, similar to that found in the PPA rodent model (14, 60). These data suggest that there are similar pathological processes between a subset of ASD children and an animal model of ASD with acquired mitochondrial dysfunction. Future studies need to identify additional parallels between the PPA rodent model of ASD and this subset of ASD individuals with this unique pattern of acyl-carnitine and GSH abnormalities. Use of this animal model with ASD patients should lead to better insight into mechanisms behind environmentally induced ASD pathophysiology and should provide guidance for developing preventive and symptomatic treatments (6, 7, 14, 50, 52, 60, 72, 74, 75).

'Common infections, chronic antibiotics, and clostridia colonization contribute to carnitine collapse, colitis, convulsions, and compulsions' – impairment of carnitine metabolism from a variety of causes may be central to autism pathogenesis and regression

Of note, impairments in carnitine metabolism are a common feature in ASD and in our PPA rodent model (6, 52, 74, 76). Although the underlining cause of a relative carnitine deficiency reported in ASD remains unclear, we have noted that diverse neurodevelopmental conditions with gastrointestinal symptoms linked to ASD, such as Reye syndrome (77), valproate toxicity (78), propionic acidemia (79), and mitochondrial disorders (73), often collectively show disruptions in carnitine metabolism. Carnitine plays an underappreciated role in brain physiology and disease (80), particularly in brain astrocyte metabolism and GABAergic metabolism during early post-natal development (81). As carnitine is endogenously synthesized from lysine and methionine, persons with defects in methylation pathways, a common finding in ASD (82), would thus have impaired endogenous carnitine production, including those with an X-linked defect in the 6-N-trimethyllysine dioxygenase (TMLHE) enzyme responsible for the first step in carnitine biosynthesis, a risk factor for autism in males (83). These individuals would thus depend on dietary sources of carnitine, which is critical during periods of rapid development, and thus may be more sensitive to a number of conditions which impair gut carnitine uptake. Carnitine is transported across the gut-blood, blood–brain barriers and reabsorbed in the kidney via the Na^+ dependent organic cation/carnitine transporter 2 ($OCNT_2$) (84). Carnitine transport deficits have been implicated in colitis (85) and also lead to blood–brain barrier impairments, allowing non-neurotropic influenza A virus to enter the CNS, inducing a neonatal encephalopathy (86). Interestingly, long-term administration of common antibiotics (i.e. beta lactams) for routine pediatric infections, in addition to eliciting GM species favoring those which produce PPA, have also been shown

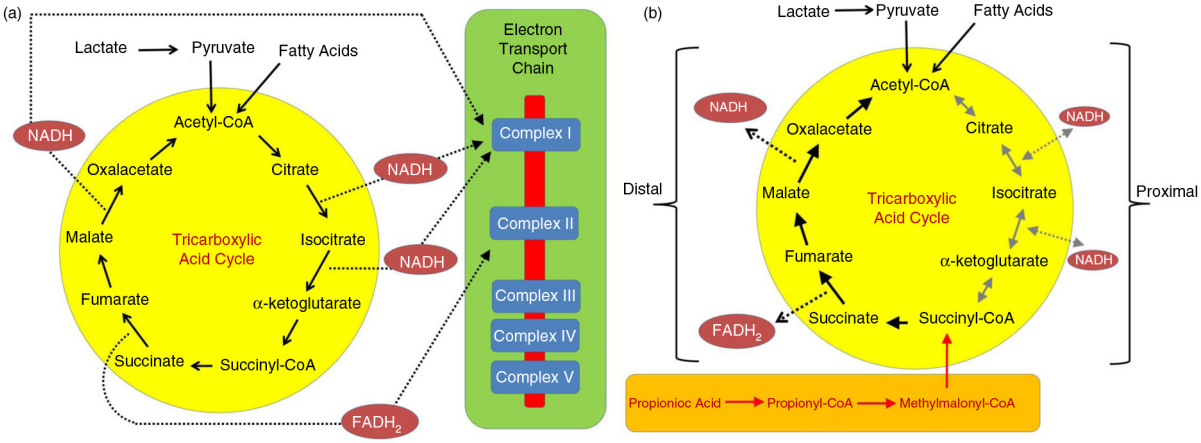

Fig. 3. Effects of the enteric bacterial metabolite propionic acid (PPA) on the tricarboxylic acid cycle during (A) typical metabolism and (B) with high levels of PPA. PPA is metabolized to propionyl-CoA, which inhibits the proximal portion of the tricarboxylic acid cycle and enhances the distal portion of the tricarboxylic acid cycle. Effects of PPA on the citric acid cycle in the PPA rodent model of autism are consistent with those found in a subset of patients with ASD, along with further abnormalities in mitochondrial redox function, phospholipid, and acylcarnitine profiles. $FADH_2$, flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide. With permission from Ref. (74).

to directly inhibit the $OCNT_2$ transporter, including carnitine transport across gut-blood, and blood–brain barriers (84) and thus may elicit a relative systemic carnitine deficiency. Additionally, antibiotics given as forms of pivaloyl esters impair renal tubular carnitine reabsorption, potentially causing an increased urinary loss of carnitine. Such rapid impairments of carnitine metabolism could be significant during critical periods of early post-natal development of brain and gut, considering the reported high incidence of antecedent long-term early antibiotic use in some ASD patients (87–90), coupled with unique enteric PPA producing bacteria and gut carbohydrate malabsorption in regressive ASD (3, 87, 88). We have suggested this offers a potential explanation for autistic regression (6, 50, 74). It also explains the potential benefits of carnitine therapy and also of temporary behavioral improvements in some patients following vancomycin or metronidazole treatment, which transiently eradicates these bacteria and reduces PPA production (87, 88, 90, 91). Furthermore, removal of refined carbohydrates from the diet, which has been suggested as an empiric treatment to improve the behavioral fluctuations and gastrointestinal symptoms in ASD, may act by reducing substrate for these bacteria to produce PPA (14). Feeding of a high carbohydrate diet in rats is known to increase SCFA levels and produce anxiety and aggressive behavior (92). Interestingly, pre-eclamptic mothers, who have an increased risk of having offspring affected by ASD (93), have similar short and long acylcarnitine profiles (94) as those found in the ASD patients and the PPA rodent model. Although the overall relationships remain as yet unproven, we have proposed that the above observations link the decreased carnitine levels in some ASD patients with several genetic and environmental risk factors consistent with regression, gastrointestinal symptomatology, altered GM, lipid biomarkers, some empiric treatments (95), and with experimental findings obtained with the PPA model. Furthermore, oral carnitine and its derivative acetyl-L-carnitine have both neuroprotective (80, 96, 97) and coloprotective properties (98). We feel the use of these compounds as therapeutic agents in neurodevelopmental disorders, including ASD is warranted (14, 52, 73, 99). Furthermore, these observations would also support repeated carnitine/acylcarnitine screening of 'patients at risk'. These would include infants with apparent developmental delay, seizure, and gastrointestinal dysfunction, particularly in the presence of maternal/infant hospital-acquired infection, early hospitalization (i.e. neonatal intensive care unit), or long-term antibiotic use (Table 1). However intriguing, it is important to note at this stage that it is unclear whether these complicated interactions are causative, compensatory, or confounders in ASD (100).

Epigenetic effects of SCFAs – potential links between genetic–environmental interactions in autism

One potential key mechanism where the metabolic products of an altered GM may contribute to ASD pathophysiology is via the alteration of gene expression associated with ASD mutations or ASD-implicated genetic pathways (6, 19, 101). Notably, SCFAs and their derivatives are known modulators of gene expression principally via their histone deacetylase inhibitor (HDACI) activity (102–108).

The rat pheochromocytoma (PC12) cell line is an extensively used *in vitro* cell system to examine molecular biological processes in neurobiology (109). Nankova and colleagues have used the PC12 line to examine the effects of SCFAs and their derivatives (i.e. valropic acid) on gene expression (110–112), particularly examining tyrosine hydroxylase (TH) gene, coding for a key enzyme in the synthesis of catecholamines, which is also implicated in ASD (113, 114). Moreover, CREB, a key factor in neurodevelopment, learning and memory (115), is a key determinant of catecholamine synthesis in PC12 cells, and shows increased CREB immunoreactivity in brains of PPA-treated rats (14). Furthermore, the anti-seizure/mood-stabilizing drug valproic acid, a known prenatal risk factor for ASD and produces an acceptable animal model for the condition, is structurally and pharmacologically similar to PPA, including HDACI properties (116–118) and produces similar effects as BA in PC12 cells (119).

We recently used rat PC12 cells as an *in vitro* system to extend our observations on the epigenetic effects of SCFAs to PPA (1–10 mM incubation over 48 h). Microarray technology was used to compare global changes in gene expression profiles following exposure to the structurally related SCFAs, PPA, and BA.

When PC12 cells were transiently transfected with plasmids having a luciferase reporter gene under the control of the TH promoter, PPA was found to induce reporter gene activity over a wide concentration range. CREB transcription factor was necessary for the transcriptional activation of the TH gene by PPA. At lower concentrations PPA also caused accumulation of TH mRNA and protein, indicative of increased cell capacity to produce catecholamines. PPA and BA induced broad alterations in gene expression including neurotransmitter systems, neuroplasticity and development, neuronal cell adhesion molecules (neurexin 1, neuroligin), inflammation, oxidative stress, lipid metabolism, mitochondrial function, and FMR1 (Fragile X) genes, all of which have been implicated in ASD (13). We are finding similar gene expression in preliminary studies in rats administered SCFA either centrally or in diet (unpublished observations). In conclusion, our data are consistent with a molecular mechanism through which SCFA metabolic

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Table 1. Potential causes and consequences of increased enteric short-chain fatty acid production and/or decreased breakdown and their relation to autism spectrum disorder.

Causes	Consequences of SCFAs
Long term antibiotics for routine infections (maternal/infant) Treatment of maternal β hemolytic strep	Gut dysmotility/inflammation/carbohydrate malabsorbtion/altered gut permeability (tight junction impairment)
Hospitalisation (colonization of nosocomial bacteria) i.e. C-section, neonatal distress	Active uptake of SCFA to CNS (monocarboxylate transporters)
Prenatal drugs (valproate, ethanol)	pH dependent intracellular concentration of SCFAs
Opportunistic infection (*Clostridium* spp., *Desulfovibrio* spp.)	Neurotransmitter synthesis and release (catecholamines, enkephalins) CNS/sympathetic nervous system
Maternal/infant gut dysbiosis	Receptor activity (+NMDA, -GABA) SCFA G protein coupled receptors/ Ca++ infiux
Organic acidemias (propionic/methylmalonic, biotinidase/ holocarboxylase deficiency)	Gap junction closure, altered neurodevelopment, neuroinflammation
(B_{12}/biotin deficiency)	Impaired mitochondrial function/increased oxidative stress
Genetic/acquired impaired carnitine synthesis/ absorption(TMLHE/$OCTN_2$ genes, β- lactam antibiotics)	Reduced glutathione/increased sensitivity to xenobiotics (i.e. acetaminophen)
Mitochondrial disorder/dysfunction (inherited, acquired)	Decreased carnitine/altered lipid metabolism/membrane fiuidity
Colitis (impaired barrier/SCFA metabolism), i.e. celiac disease. Met-receptor tyrosine kinase mutation	Altered gene expression (CREB activation, histone deacetylase inhibition)
Increased refined carbohydrate consumption - substrate for bacterial fermentation	Antisocial/perseverative/anxiety-like behavior, seizure/movement disorder, Restrictive food interests/carbohydrate craving

These findings, which are not mutually exclusive, may contribute to the pathophysiology, behavioral symptoms, and comorbidities of autism. With permission from MacFabe (6).

products of the GM can epigenetically modulate cell function including genes related to ASD pathogenesis, further supporting their role as potential environmental epigenetic contributors to ASD.

Summary – can microbes control the mind? Future directions

In summary, it can be seen that SCFA metabolic products of the GM have remarkable effects on host physiology including brain function and behavior (Fig. 4). Through our translational animal model, *in vitro* and clinical studies, it can be seen that SCFAs (PPA in particular) have unique properties, particularly involving neuroplasticity, memory acquisition, GPCR activation, gut physiology, tissue barrier permeability, oxidative stress, mitochondrial function, carnitine metabolism, and epigenetics, all of which have been implicated in ASD. Furthermore, there are many potential clinical scenarios for genetic–environmental interactions, which are consistent with enhanced exposure to or impaired metabolism of SCFAs in an individual at risk for ASD.

It is important to note that, other than reports of increased PPA in stool samples (120), there are few studies that have systematically examined PPA and its related metabolites in ASD. The short half-life, rapid intracellular concentration of PPA by monocarboxylate transporters, metabolism via the TCA cycle, β-oxidation and incorporation into lipids, coupled with 'difficult' anatomical regions to clinically access (gut, brain) make SCFA measurement problematic, but possible. The evidence of many effects of PPA on diverse biological pathways being consistent with findings with ASD patients is intriguing but largely correlative. Novel translational models (animal models, artificial gut complex microbial colonies) (121) coupled with longitudinal human studies (100) are necessary to correlate these biomarkers to autistic regression or clinical improvement. These studies may also herald novel pharmacological treatments such as special diets, metabolic augmenters (carnitine), G-coupled receptor ligands, fatty acids, probiotics, or even GM repair (121, 122). However, cautious optimism is necessary for these future possible therapies, as we do not yet know long-term effects, or indeed which of these GM and metabolomics changes are contributory, causative, or simply a cofounder in ASD, or indeed any other condition (100).

However, given the acceptance and increased use of PPA in the food industry and agriculture, this does warrant further investigations and awareness of potential risks versus benefits of this SCFA. Of note, PPA's widespread use as a food preservative or weight loss agent, and its increased gut production following aspartame ingestion, may herald some caution, particularly during pregnancy and early postnatal development.

In a broader context, we have speculated that GM, through natural selection, have evolved to use their metabolites to adaptively modulate the physiology and ultimately behavior of the host, to promote survival

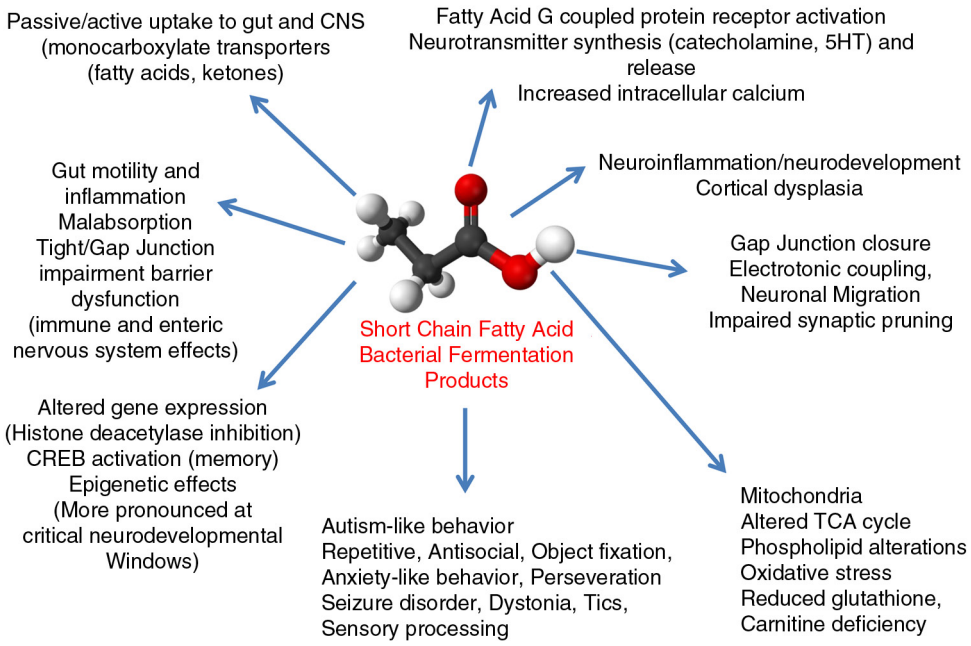

Fig. 4. Broad physiological effects of enteric short-chain fatty acids on host physiology and brain function and behavior. These effects which are dose, tissue and temporally specific may be physiologically adaptive (immune/cellular energy regulation, food seeking, learning and memory, intra species social interaction), but may be pathological with increased production, decreased breakdown or increased early exposure of these bacterial metabolites during key periods of neurodevelopment.

(6, 7). This has been documented in behavioral neurobiology, with such examples as cordyceps fungus producing climbing behavior in ants, and Borna and rabies viruses eliciting salivary transmission and biting behavior in mammals (123, 124). In light of this, the observation of object fixation, restrictive eating of carbohydrates, diarrhea, and fecal smearing in patients with ASDs, which all could theoretically promote organism growth and spread, is intriguing. It is also worth noting that many of the effects of lower doses of SCFAs on gut physiology and immune function, at particular periods during the lifecycle are indeed beneficial to host and ultimately bacterial survival. Finally, it is important to note the ability of SCFAs to elicit anxiety-like, perseverative, repetitive, ritualistic, and antisocial behaviors that are common to many other neuropsychiatric conditions, such as obsessive compulsive, anxiety, attention deficit/hyperactive, mood, and eating disorders, posttraumatic stress disorder, irritable bowel syndrome, pediatric autoimmune neuropsychiatric disorder associated with streptococcal infections (PANDAS), and schizophrenia, where infectious agents have been implicated as contributory (6, 7, 125–127). We have proposed that at least some of these neuropsychiatric conditions may in part represent potentially preventable or treatable metabolic disorders of impaired SCFA metabolism. By analogy, one can recall the tremendous strides which have been made in the management of diabetes, another apparently untreatable metabolic condition, which historically showed broad comorbid effects on multiple body systems including the CNS. Here, patients' lives have been vastly improved subsequent to a better understanding of energy (glucose) metabolism, the exacerbating effect of infection, the role of diet, and the subsequent scientific developments of measurable metabolic biomarkers (glucometers), and pharmacological agents such as insulin and glyburide (7). With the collaborative efforts from experts from diverse scientific disciplines, it is the author's hope that similar strides can be made in ASD. In conclusion, it appears that enteric SCFAs play a major role in host physiology and provide further evidence that GM can modulate brain function and behavior in health and disease conditions, including ASD.

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GUT IN FOCUS: EXTENDED ABSTRACT

Feces transplantation for recurrent *Clostridium difficile* infection: US experience and recommendations

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Patients who have failed to respond to repeated antibiotic treatment for recurrent *Clostridium difficile* infection (RCDI) present a particularly difficult challenge. Recent investigations of patients with RCDI have demonstrated significant disruption of the intestinal microbiome diversity as well as bacterial richness (1). Following the initial report on fecal microbiota transplantation (FMT) published by Eiseman in 1958 (2), instillation of stool collected from a healthy donor into the intestinal tract of patients with RCDI has been used increasingly and with a high degree of success to correct the intestinal dysbiosis brought about by repeated courses of antibiotic treatment. By now, multiple case reports and case series describing the outcome of FMT for more than 1,200 patients from around the Western world have been published. FMT treatment success rates are high and have ranged between 77 and 98% (3); the highest success rates have been observed with instillation of stool via the lower GI tract (4). A prospective randomized controlled trial was recently conducted in Holland (5), demonstrating superior treatment outcomes with FMT when compared to conventional therapy with oral vancomycin. FMT has been universally well accepted by patients and represents a low cost alternative treatment approach to an increasing clinical problem, with unlimited supply of the raw material (human stool). FMT appears to be safe, as no report of severe adverse events directly attributed to the instillation procedure itself has been reported so far (6). However, possible long-term consequences of FMT are unknown, and the US Food and Drug Administration currently considers FMT to be investigational therapy.

Materials and methods

A literature search of the Ovid MEDLINE database was conducted using search terms and synonyms for FMT. The search was limited to articles published in the English language or with an English language abstract between 1958 and December 31, 2014.

Experience with FMT

A total of 66 articles were identified; there were 15 single case reports, 50 case series and one randomized controlled clinical trial for a total case number of 1,212 cases. Prior to 2010 the annual number of reported cases of RCDI treated with FMT was fairly stable and averaged three cases per year. However, during the last 4 years more than 1,100 additional cases have been reported for an average of more than 250 cases per year. The majority of cases have been treated in North America (3, 6), and by the end of 2014 at least 105 practice centers in Canada and the USA were offering FMT for a variety of indications, including RCDI (www.idsociety.org/FMT/). Fecal samples may be instilled via the upper as well as the lower intestinal tract, but the majority of the published cases have had their fecal sample delivered either through a colonoscope or via a retention enema catheter (Table 1).

A recent survey of US infectious disease physicians indicated that patients with RCDI who failed to resolve their infection with standard antibiotic therapy should be considered for FMT after the third infection recurrence (7). The source of human feces for FMT administration has varied from a sample donated by a pre-screened close family member (e.g. spouse or intimate partner) to an unrelated donor (sample provided via a commercial 'stool bank' such as OpenBiome, www.openbiome.org). More recently well-defined suspensions of enteric bacteria have also been utilized with a high degree of success (8, 9). Recently a US consensus group recommended that stool donors should be screened through a detailed interview followed by examination of blood and feces for presence of occult contagious agents before being accepted for FMT donation (10, 11); however, screening practices have varied around the world, and routine screening of stool donors have not been practiced routinely in, e.g. Norway and Sweden (D. Berild, T. Noren; personal communication).

Anecdotal reports strongly suggest that the clinical response to FMT for the treatment of RCDI is superior

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Table 1. Routes of administration of Fecal Microbiota Transplantation (FMT) as reported in 66 published scientific articles between 1958 and December 31, 2014

Feces administration route	Cases	Success	%
Enema/colonoscopy	691	667	96
NGT/NDT/gastroscope	292	234	80
Mixed route: upper GI tract/colonoscopy	162	143	88
Oral capsules	20	14	70
Total	1,212	1,061	88

Mixed route refers to multiple published case series where the fecal sample was administered either via the upper or the lower gastrointestinal tract.

to standard antibiotic therapy (3, 12). The highest success rates (86–100%) have been noted when the fecal sample has been administered into the colon via the colonoscope or as a fecal enema (13–16). Slightly lower success rates (77–94%) have been reported when the fecal sample was administered into the gastric cavity or proximal portion of the small intestinal tract (17, 18). Only one randomized controlled trial has been published to date. Van Nood and coworkers compared vancomycin to FMT for treatment of RCDI. The trial was terminated prior to the planned stop date as interim data analysis demonstrated superiority of FMT over vancomycin (5). Youngster recently reported that human feces may also be successfully administered using pre-screened frozen fecal gel capsules, noting a success-rate of 70% (19).

The practice of administering FMT was unregulated in the United States until February 2013, when the Federal Drug Administration (FDA) classified human stool as a biological drug and imposed restrictions on prescribers of FMT by requiring all providers of FMT to hold an approved new investigational drug (IND) permit (20). The requirement for a valid IND was relaxed in July 2013, when the FDA declared that the agency would use 'enforcement discretion' when FMT was being prescribed as therapy for RCDI, providing that all patients sign an informed consent form that clearly outlines the potential risks of FMT and states that FMT is considered investigational therapy. The efficacy of FMT for gastrointestinal illnesses other than RCDI is currently not well defined, and use of FMT in the United States for treatment of inflammatory bowel disease and other less well-defined indications requires the provider to possess an IND approved by the FDA.

FMT has been tolerated remarkably well, and no significant infectious adverse effects directly attributed to the transfer of human feces from one individual to another have been described to date. Two cases of norovirus gastrointestinal infection was reported in 2012 for two FMT recipients despite asymptomatic stool donors and lack of recent sick contacts (21). The potential for long-term infectious and non-infectious consequences from FMT is currently unknown, but a recent publication reported new-onset obesity in a woman who had been successfully treated for RCDI with stool donated by her obese daughter (22). Physical complications from the FMT instillation procedure (upper gastrointestinal bleeding after nasogastric tube insertion, colon perforation during colonoscopy) has been occasionally reported and may occur with the same frequency as when these procedures are performed for illnesses other than RCDI.

Conclusions

FMT offers a highly effective treatment modality for patients who have failed to resolve their RCDI with standard courses of recommended antibiotic administration. In the United States, individualized FMT may be administered to patients with RCDI by medical providers without an FDA approved IND. However, an IND is required to administer FMT for all other indications. The optimal source of human stool for FMT administration continues to be debated, but stored, frozen stool samples that have been collected from carefully prescreened donors have become increasingly available from commercial biotech companies. Immuno-compromised patients with RCDI have tolerated FMT without reports of increased adverse effects. The recently published European CDI Treatment Guidelines (23) endorses FMT as first line therapy for patients who have had three or more CDI recurrences. The IDSA/SHEA 2010 guidelines (24) are currently being revised and updated, and recommendations for the use of FMT will likely mirror the European recommendations. The potential for long-term infectious and non-infectious unintended adverse effects from FMT are currently unknown. A US stool biorepository bank, which will make it possible to oversee the efficacy and safety of FMT, is currently in the planning stages with the FDA, National Institute of Health (NIH) and the Centers for Disease Control and Prevention (CDC) as the major stakeholders.

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MICROBIAL ECOLOGY in Health and Disease

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GUT IN FOCUS: ABSTRACT

Feces transplantation – EU recommendations

Recurrent *Clostridium difficile* infection (CDI) exhibits a disrupted diverse gut microbiome where recolonization decides the final outcome of this often challenging course of disease. Clinical trial and error treatment with fecal microbiota transplant (FMT) have until recently produced limited amount of scientific data. Lack of a standardized procedure and obscure declaration of product content have made data evaluation difficult. Lately, a randomized controlled study has proven FMT to be superior to standard vancomycin therapy, inspiring several ongoing studies. The European guidelines published by the European Society of Clinical Microbiology and Infectious Diseases (1) has evidenced that FMT is now an acceptable mode of treatment. This discussion will highlight the current position of FMT in treatment, the slowly emerging consensus on different procedures in practice, as well as the concerns that are still prevalent.

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MICROBIAL ECOLOGY in Health and Disease

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GUT IN FOCUS: EXTENDED ABSTRACT

Experience with cultivated microbiota transplant: ongoing treatment of *Clostridium difficile* patients in Sweden

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Bacteria should no longer be seen as pure enemies. Nowadays, there are enough evidences to place microorganisms as key elements in human homeostasis. It is known that a balanced human intestinal microbiota constitutes an important protection against the establishment and overgrowth of pathogenic microorganisms, e.g. *Clostridium difficile* (CD) causing intestinal disorder in the host. The intestinal microbiota has a pivotal role in the maintenance of health of the human beings, especially in the defence against pathogenic microorganisms.

The degree of diarrhoea defines the choice of therapy – most often different antibiotics. However, the risk for recurrent infections is about 35%, which sometimes occur several months after the termination of therapy. These patients are recommended a new antibiotic treatment. After the first recurrence is the risk for a new recurrence up to 65%. The chance of a cure is reduced with the number of recurrent infections.

Faecal microbiota transplantation (FMT), also known as stool transplantation, is a procedure aimed at restoring an altered intestinal microbiota balance by administration of stool microorganisms from a single healthy donor – often from a close relative, or alternatively several donors donating faeces to a 'faeces bank'. The donor(s) and the faeces must undergo a rigorous screening process to determine that no pathogens are transmitted (1). These tests are both time consuming and very expensive. Despite initial scepticism, FMT is today regarded as an alternative for patients with recurrent CD infection in several countries. Regulatory authorities in the EU and the USA have, however, not given formal approval for FMT. But due to high efficacy against CD infections, FMT is more or less accepted on a temporary basis as an experimental treatment. There is a worldwide growing interest for using FMT as a treatment of other diseases as well, including *Clostridium difficile* infection (CDI), inflammatory bowel disease (IBD), and irritable bowel syndrome (IBS). Today, there are groups worldwide who are introducing FMT as a treatment regime, faeces obtained from healthy donor(s) into diseased individuals using different approaches, both for selection of donors and treatment mode either administered by naso-duodenal sond or as an enema.

In our group, we have established an anaerobically cultivated human intestinal microbiota approximately 20 years ago (2–4). The cultured microbiota originates from one faecal sample from one single healthy donor on a Western diet (2). The microbiota has regularly been recultivated on a rich bacteriological medium under anaerobe conditions and carefully checked for the absence of pathogen organisms as *Salmonella, Shigella, Yersinia*, *Clostridium difficile*, *Cryptosporidium, Cyclospora, Isospora*, cysts and worm eggs, HIV, hepatitis A, B and C, and *Treponema pallidum*. This ready-to-use anaerobe culture is kept frozen in tubes at -80°C until use.

In some previous studies, the anaerobic cultured microbiota has been installed into a number of CDI patients during the years – cultured microbiota transplant (CMT) – with very good results (2–4). Now there is an ongoing clinical study, where a group of antibiotic-associated diarrhoea (AAD) patients, both male and female at different ages, are included. Inclusion criteria are one or several metronidazole treatments followed by vancomycin 125 mg four times daily during at least 10 days without recovery. These patients are randomised to either CMT or continuation on vancomycin 125 mg four times daily for at least 10 days, followed by periods of 7, 30 and 90 days control – the responsible scientist phones the patients. In the CMT group, 6 out of 11 patients recovered after one CMT treatment, three patients recovered after two CMT treatments, and one patient after three CMT treatments. One patient was withdrawn for personal reasons. In the

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vancomycin group, two patients out of 10 recovered after the vancomycin treatment, but four patients did not and two patients were withdrawn for personal reasons. Of these four patients, one patient recovered after one CMT treatment and two patients after two CMT treatments, and the last patient, so far, has recovered after two CMT treatments, but the follow-up at day 90 still remains. In this study, there are also possibilities to compare cost effective issue between CMT and antibiotic and hospitalisation treatments.

Despite the increasing number of studies where an unbalanced microbiota is suggested to cause intestinal disturbances, there are no standard protocols. Alterations in the microbiota composition and function are obviously involved in the pathogenesis of a variety of Western world diseases/disturbances, and CDI is one of the most common health care-associated infections in the world. However, CDI, IBD and IBS, obesity, and diabetes are also discussed when research regarding intestinal microbiota ecological balance is actual. Based on the assumption that a disturbed microbiota might cause intestinal disturbances/diarrhoea, it seems logical that a CMT treatment should prove to be both health promoting and cost effective, and in line with this, this current study supports the role of transplanting a balanced anaerobic gut ecosystem into patients with intestinal disturbances – a resetting of the intestinal ecosystem.

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MICROBIAL ECOLOGY in Health and Disease

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GUT IN FOCUS: EXTENDED ABSTRACT

Anaerobically cultivated human intestinal microbiota as first-line treatment for *Clostridium difficile* infection

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Clostridium difficile infection (CDI), most often resulting from an antibiotic-induced disturbance of the healthy intestinal microbiota, is an increasing health problem (1–3). Antibiotics such as metronidazole or vancomycin are well-established and effective treatment options for first occurrences of CDI (4, 5) (so-called primary), but up to one third of patients experience treatment failure or recurrent disease within a few weeks (6–8). Repeated courses of antibiotics may be effective, but multiple recurrences are common (9). Recurrent CDI may result from re-infection with the same or a different *C. difficile* strain. Up to 50% of recurrences may be re-infections with strains different from the primary infection reinforcing the notion that appropriate colonization is disturbed after antibiotic treatment (10).

Fecal microbiota transplant (FMT) has been shown to be effective and safe in recurrent CDI in multiple uncontrolled studies (11), and recently, a randomized controlled trial (RCT) showed FMT to be superior to high-dose vancomycin for recurrent CDI (12). Theoretically, FMT as first-line treatment can prevent the vicious cycle of both types of re-infections (same or different strain) by rapidly restoring a favorable colonic microbial environment, and thus leaving the patient less susceptible to either kind of CDI recurrence. Reduced need for resistance-driving antibiotics and reduced proliferation of other resistant pathogens are possible advantageous spin-off effects of FMT as the primary treatment of CDI.

This is a description of the design of an ongoing RCT to compare the effect of intestinal microbiota transplantation with the effect of standard metronidazole treatment in primary CDI.

Based on the high recurrence rate of CDI after treatment with antibiotics and the convincing results of FMT for recurrent CDI, we hypothesize that intestinal microbiota transplantation can be beneficial also in the treatment of primary CDI (13).

The aim of this study is to investigate whether an anaerobically cultivated human intestinal microbiota (ACHIM) is more effective than metronidazole in inducing a durable cure for primary CDI (ClinicalTrials.gov identifier NCT02301000).

Material and methods

A continuously re-cultivated human intestinal microbiota, obtained from a healthy donor more than 15 years ago, has been shown to be an effective cure for recurrent CDI (14). This ACHIM, which has been extensively analyzed for pathogenic elements, will be used in the current trial, obviating the need for donor screening.

The trial is designed as a multicenter, single-blinded RCT.

Hospitalized patients with a first episode of CDI will be recruited and randomized 1:1 to a rectal instillation of ACHIM or to a 10-day course of metronidazole 500 mg t.i.d.

A first episode of CDI is defined as diarrhea and a positive stool test for *C. difficile* toxin A or B without evidence of recent CDI.

Patients are asked to report the number of daily bowel movements for 4 days after the initiation of CDI treatment. The number of daily bowel movements will also be recorded on day 7, 14, 21, 35, and 70. The primary end point is resolution of diarrhea and no evidence of recurrent CDI within 70 days after treatment initiation. A blinded study investigator will assess the primary end point. A pilot study including 40 patients (20 in each study arm) will be analyzed to guide the final sample size.

Results

The trial started in November 2014, and no results are yet available.

Conclusion

The effect of intestinal microbiota therapy for primary CDI is currently unknown. The current trial aims to document the effect of an ACHIM in primary CDI.

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MICROBIAL ECOLOGY in Health and Disease

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GUT IN FOCUS: EXTENDED ABSTRACT

Is irritable bowel syndrome a dysbiotic bowel syndrome?

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Irritable bowel syndrome (IBS) is a clinical entity that affects up to 20% of the population (1). The etiology and underlying pathophysiology is likely multifactorial, and the condition is often leading to low quality of life. It has been suggested that the intestinal microbiota plays a significant role in IBS (2). Herein we present a case of IBS who fulfills the Rome III criteria for IBS with diarrhea (3).

A 32-year-old woman, nonsmoker, presented with frequent watery diarrhea without blood up to 20 times per day, for nearly 2 years. Four months earlier, she had a 10-day course of flucloxacillin (500 mg, three times daily) against a bacterial skin infection. She was admitted to a gastroenterological inpatient ward for rehydration and investigation. A complete infectious work-up was negative. Colonoscopy and gastroscopy including histology were normal. Blood count and chemistry were also normal.

Bile acid–related diarrhea and bacterial overgrowth were excluded as a cause for the symptoms. Rifaximin treatment was without any effect. Despite symptomatic treatment with codeine and amitriptyline, her symptoms persisted and she required halftime sick leave from work. She requested and received three transplants with an anaerobic cultivated intestinal microbiota during 1 month. The microbiota was infused through a gastroscope in the descending part of the duodenum. A slow and remarkable improvement was noted within weeks, and she experienced one or two solid stools per day without medicines.

Four months later, she was treated with antibiotics for 10 days because of endometritis. Diarrhea returned, and she required codeine again. Two more transplants were given during 2 weeks. Prompt improvement followed, and the medicine was stopped. One year later, the patient was free from symptoms and took no drugs.

In this presented case, it is tempting to assume that the symptoms were caused by alteration of the intestinal flora (dysbios) possibly influenced by antibiotic treatment. The positive outcome and follow-up indicates a true treatment effect and underlines the possibility of relapse and re-treatment with microbiota transplant.

Studies suggest that dysbiosis plays a role in other conditions such as metabolic syndrome, autoimmunity, asthma, atherosclerosis, diabetes mellitus, and inflammatory bowel disease (4–6).

We suggest the introduction of the term dysbiotic bowel syndrome as a new term coined to describe the effects and consequences of a disturbed gastrointestinal microbiota leading to symptoms.

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GUT IN FOCUS: CONCLUDING REMARKS

What did we learn today?

We learnt that the development of the intestinal microbiota (IM) is extremely complex and a giant puzzle to piece together from adults and children in both developing and developed countries, or more experimentally. Each piece may or may not have future consequences for the IM. However, we are now at the end of the beginning of finishing just one microbiological puzzle. Additionally, we have to show how the functional puzzle can be illuminated. The burning question is: will we ever succeed? The answer is simple: we will, if we to go time point by time point, species by species, function by function. And when we have a clearer picture of the continuous host/microbiota and microbiota/microbiota cross-talks going on all through childhood, we will be able to interact prophylactically and therapeutically

We learnt that – when focusing on specific microbe-related metabolites – there are consequences for the host functions. Irrespective of the size of the molecules, the interactions are several. The IM and the host clearly interact.

We learnt that, when focusing on restoration of the IM dysbiosis either by transplantation of fresh feces or cultivated human IM, there are pitfalls that need to be addressed. However, there is hope we will eventually find a way to successfully restore the IM.

Summing up, we learnt that there are complex interactions constantly going on between the IM and its host – in health as well as in disease – and also that we are now beginning to understand how to improve and optimize this interaction. More research is required, however. You are welcome to submit your papers to *Microbial Ecology in Health & Disease.*

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