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| Title | Understanding mode of action can drive the translational pipeline towards more reliable health benefits for probiotics |
| Authors | Kleerebezem, Michiel;Binda, Sylvie;Bron, Peter A.;Gross, Gabriele;Hill, Colin;van Hylckama Vlieg, Johan E. T.;Lebeer, Sarah;Satokari, Reetta;Ouwehand, Arthur C. |
| Publication date | 2018-10-05 |
| Original Citation | Kleerebezem, M., Binda, S., Bron, P.A., Gross, G., Hill, C., van Hylckama Vlieg, J.E., Lebeer, S., Satokari, R. and Ouwehand, A.C., 2019. Understanding mode of action can drive the translational pipeline towards more reliable health benefits for probiotics. Current opinion in biotechnology, 56, (5pp). DOI:10.1016/j.copbio.2018.09.007 |
| Type of publication | Article (peer-reviewed) |
| Link to publisher's version | https://www.sciencedirect.com/science/article/pii/S0958166918300910 - 10.1016/j.copbio.2018.09.007 |
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| Download date | 2024-03-02 06:49:39 |
| Item downloaded from | https://hdl.handle.net/10468/8645 |

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Current Opinion in Biotechnology

# Understanding mode of action can drive the translational pipeline towards more reliable health benefits for probiotics

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The different levels of knowledge described in a translational pipeline (the connection of molecular mechanisms with preclinical physiological and human health effects) are not complete for many probiotics. At present, we are not in a position to fully understand the mechanistic basis of many well established probiotic health benefits which, in turn, limits our ability to use mechanisms to predict which probiotics are likely to be effective in any given population. Here we suggest that this concept of a translation pipeline connecting mechanistic insights to probiotic efficacy can support the selection and production of improved probiotic products. Such a conceptual pipeline would also provide a framework for the design of clinical trials to convincingly demonstrate the benefit of probiotics to human health in well-defined subpopulations.

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**Current Opinion in Biotechnology** 2019, **56**:55–60

This review comes from a themed issue on **Food biotechnology**

Edited by **Herwig Bachmann** and **Ana Rute Neves**

For a complete overview see the Issue and the Editorial

Available online 5th October 2018

**https://doi.org/10.1016/j.copbio.2018.09.007**



## Introduction

More than a century ago, Nobel-laureate Eli Metchnikoff hypothesized that lactic acid bacteria can delay the deterioration of health during aging due to their ability to produce lactic acid and inhibit protein-fermenting intestinal microbes. This was the beginning of the probiotic concept, which is nowadays defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' [1,2•]. A large variety of products containing probiotics are consumed by millions of people on a daily basis, and probiotics have an impressive safety record. As of 2018, almost 2000 clinical studies have reported on a variety of health benefits of probiotics, including a recent landmark study that showed that a probiotic/prebiotic mix resulted in a 40% reduction of neonatal sepsis and death among infants in rural India [3••]. Meta-analyses support clinical benefits of the consumption of probiotics in specific populations that are at risk to develop a disease (Boxes 1 and 2 ). For many other health benefits no generalized conclusions are possible because, although individual studies have reported beneficial effects in a variety of (intestinal) conditions [4], these may be restricted to specific strains or specific subpopulations [5]. In parallel, remarkable advances have been made in understanding the wide array of molecular mechanisms by which probiotic organisms can interact with host cells [6], or how they can persist in [7•] and/or impact on the resident colonic microbiota [8,9]. However, reliable translation of these mechanistic insights into measurable clinical effects remains highly challenging.

Here we present a conceptual translational pipeline (Figure 1) that connects molecular mechanisms of bacterial interactions with the host, to changes in host physiology, and the corresponding health benefits in human applications. We employ this pipeline to evaluate how understanding molecular interactions can assist the prediction of physiological responses in preclinical models, with the ultimate ambition of translating these findings to beneficial outcomes in humans. Inversely, we use the pipeline concept to illustrate the importance of deciphering the physiological changes in the host and the underlying molecular interaction mechanisms involved in established probiotic health benefits. Such knowledge could drive the development of optimized probiotic products for those health benefits.

**Box 1 Probiotics in AAD**

Antibiotic associated diarrhoea (AAD) occurs in 5–39% of hospitalized patients. A commonly reported AAD pathogen is *Clostridium difficile*, but *Candida albicans*, *Clostridium perfringens*, *Staphylococcus aureus* and *Klebsiella oxytoca* are also frequently observed [27]. Most bacteria induce diarrhoea by the production of toxins [27,28], whereas the yeast *C. albicans* can cause invasive candidiasis [29]. However, these five pathogens together do not explain more than 30–40% of all AAD cases, implying that other factors are involved.

Reducing the incidence or duration of AAD by consumption of probiotics during the antibiotic treatment is one of the best-established benefits of probiotics. Various probiotic products can reduce relative AAD risk by more than 40%, while *C. difficile* associated diarrhoea has been reported to be reduced by up to 60% with some probiotics [15,16]. This finding suggests that many probiotics share some 'core properties' which can ameliorate AAD [2•]. The *in vitro* investigation of pathogen inhibitory capacities of probiotic lactobacilli and bifidobacteria in many cases depends on their capacity to produce lactate and acetate and acidify their environment [30,31], which is consistent with a generic mechanism of action in AAD. However, more specific pathogen inhibition has been reported for some probiotics and could involve the production of antimicrobial peptides that inhibit enteric pathogens [32,33]. Antibiotic treatment disrupts the intestinal microbiota and could compromise its homeostatic interactions with the host mucosa. Probiotics were also reported to influence AAD risk by improving the resilience of the faecal microbiota [34•], potentially through stimulation of specific (lactate- and/or acetate-utilizing) members of the endogenous microbiota [35]. Finally, most of the AAD associated pathogens disturb the intestinal barrier, an effect that could be compensated by probiotic stimulation of barrier integrity and/or repair [36,37].

**Box 2 Probiotics in NEC**

Necrotizing enterocolitis (NEC) is an inflammatory necrosis of the gut of premature infants and symptoms include feeding intolerance, bloated and sensitive abdomen, and bloody diarrhoea. NEC also often leads to gastrointestinal perforations. It is a major cause of mortality (estimated to be 20–50%) in neonatal intensive care units throughout the world [38]. NEC is influenced by multiple factors, including gestational prematurity, host genetics, enteral feeding, mucosal injury, bacterial translocation, and inflammatory responses. Although the involvement of intestinal bacteria with the onset of NEC is not entirely clear, increased levels of pathobionts (e.g. Enterobacteriacae) often precedes the NEC diagnosis [39].

Multiple meta-analyses have evaluated the effect of probiotics in NEC [40] and most have reached the conclusion that probiotic treatment decreases the risk of NEC and mortality in premature infants. A number of different probiotics appear to be effective, suggesting a more generalized mechanism of action [2•]. Nevertheless, *Bifidobacterium* probiotics appeared more effective than *Lactobacillus* probiotics, and combination products (multiple species and strains) appeared more effective than a single strain [17]. The higher efficacy of bifidobacteria probiotics could relate to their capacity to utilize human milk oligosaccharides [41–43] and/or their capacity to complement lactase limitation [12••], which could contribute to resolution of feeding intolerance. Despite these positive effects, there is no clinical consensus for the prophylactic use of probiotics as standard care in pre-term infants. Several concerns have been raised concerning the non-uniformity of probiotic products tested, the consistent availability of effective products, and their potential interaction with feeding regimes. These clinical concerns are fuelled by the perceived safety risk of administering bacteria to a preterm infant with a known intestinal barrier defect.

Mechanistic studies on the role of probiotics in NEC largely depend on animal models [44] or on *in vitro* cell culture systems. Probiotics have been proposed to favourably affect intestinal colonization and thereby reduce the risk of NEC, including the inhibition of Enterobacteriacae, although the outcomes of studies in pigs have been inconsistent [45,46]. Alternative mechanisms could include stimulation of mucosal integrity and immune system function, which could reduce intestinal permeability. For example, pilin expressing *Lactobacillus rhamnosus* GG was shown to suppress TLR3, TLR4, and TIRAP-expression and inflammatory responses in a foetal intestinal epithelial cell line, while not affecting tolerance associated markers [47]. *Bifidobacterium longum* subsp. *infantis* secretes a small glycan or glycolipid (5–10 kDa) that prevents epithelial inflammatory responses by downregulating TLR4 and inflammatory signalling in various foetal cell culture models [48•]. Despite these proposed mechanisms, there is no clarity on their roles in probiotic benefits achieved in human NEC.

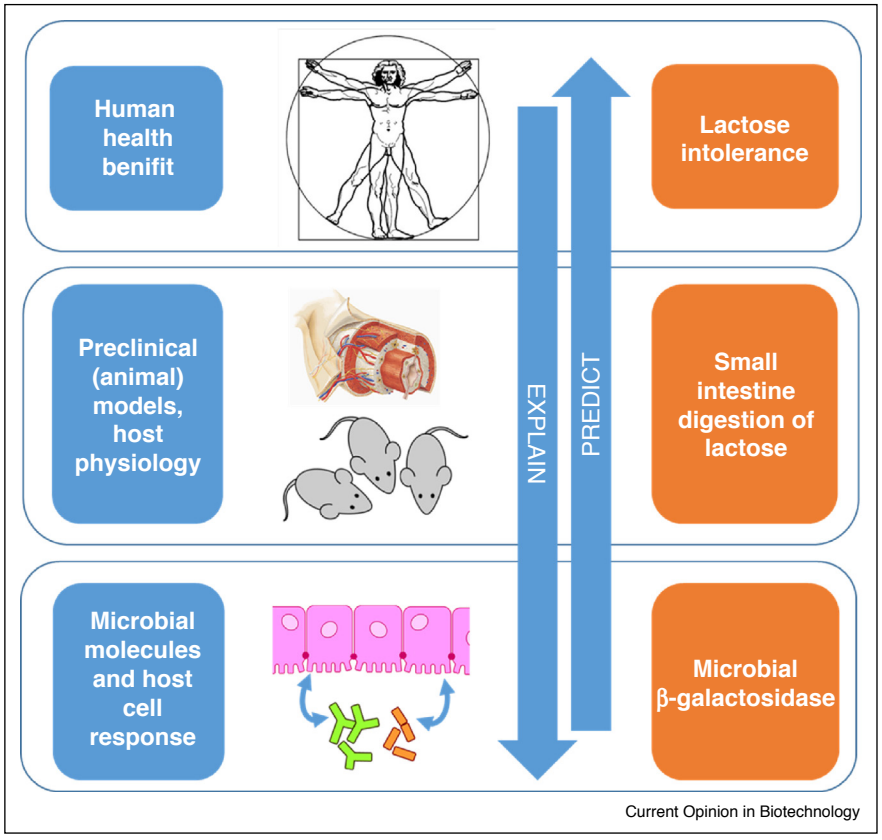
## Lactose maldigestion and yoghurt cultures

Although originally not intended as a health promoting product, it is remarkable that the proven health benefit of yogurt cultures in lactose maldigestion is supported by understanding of the molecular mechanism involved. Lactose maldigestion results from a genetic disposition or acquired deficiency in the enzyme lactase, required for hydrolysing lactose to glucose and galactose in the small intestine of humans. If lactose reaches the colon it is rapidly fermented by the microbiota, leading to gas formation and symptoms that include bloating, diarrhoea, flatulence, and vomiting. However, consumption of fermented milk products, especially yogurt, containing high levels of lactose is commonly tolerated in individuals suffering from lactose maldigestion. This apparently contradictory observation can be explained by the presence of the lactase-like enzyme β-galactosidase in the yoghurt bacteria *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. This bacterial enzyme can compensate for the lack of lactase, thereby preventing the fermentation of lactose in the large intestine and the corresponding lactose maldigestion symptoms [10,11,12••,13]. This example links a discrete bacterial activity (β-galactosidase) to a precise impact on physiology (digestion of dietary lactose in the small intestine) and a health benefit. Interestingly, the effect can in part be recapitulated by ingestion of lactase tablets, further validating this mechanistic interpretation. This mechanistic knowledge allows the selection of yoghurt cultures with enhanced β-galactosidase delivery capacity, which could strengthen the lactose intolerance alleviating capacity of yoghurt produced with such strains, thereby illustrating the translational pipeline concept.

## Exploring the translational pipeline concept for the explanation and prediction of probiotic effects

According to meta-analyses, the mitigation of antibiotic associated diarrhoea (AAD; Box 1) and necrotizing enterocolitis (NEC; Box 2) are among the best-documented clinical benefits of probiotics. The efficacy of a wide range of probiotic strains suggests that they may have

**Figure 1**




Schematic representation of the three layered translational pipeline that illustrates the connection of mechanistic knowledge of the probiotic-host interactions and the molecules and structures involved, with the consequences of these interactions on host physiology and/or the demonstration of health benefits in preclinical (animal) models (i.e. insight in the probiotic mode of action), and ultimately connecting to clinical studies that demonstrate a health benefit in the target human (sub)population. The pipeline concept implies that knowledge of mechanisms of probiotic-host interactions at molecular level can be employed to predict health benefits that these bacteria may elicit in selected human populations, while inversely it can be employed to explain observed clinical effects in humans by linking to underlying modes of probiotic action. The connections between the different layers of knowledge within the pipeline in the compelling example of the role of yoghurt bacteria in preventing lactose maldigestion associated symptoms by *in situ* delivery of the bacterial β-galactosidase that supports small intestinal digestion of lactose is shown on the right.

shared core properties [2•,14], which can positively influence host health in these conditions. However, the molecular basis of these core properties remains not fully understood [2•,14] and there is no convincing mechanistic explanation for probiotics in AAD or NEC (see Boxes 1 and 2). Several plausible mechanisms have been investigated which may contribute to the health benefits observed, but in terms of our translational pipeline this is an obvious gap hampering the development of improved therapies. It could be argued that this is not important since the positive effects of probiotic interventions are reliably observed. However, some AAD and NEC studies did not, or only marginally, reveal a positive effect for probiotics [15–17]. A better understanding of the relevant 'core probiotic properties' could help us to design a more rational strategy to select and produce reliably effective probiotics. This could be very valuable if we are to overcome the understandable clinical hesitation in deploying probiotics in the premature infant population that suffers from NEC.

In other instances, precise mechanisms of probiotic interaction with host cells have been described and specific probiotic effector molecules have been identified that can elicit specific responses [6,18]. For some of these effector molecules their capacity to elicit physiologically relevant effects in preclinical (animal) models has been evidenced successfully. However, as has been found with almost all translational efforts across medicine, the predictive power of how mechanistic knowledge may convert to reliable clinical benefits in human populations remains limited.

For some health benefits, it is not realistic to expect that clinical effects can be scientifically proven in human populations. As an example, the role of bacteriocin production in the ability of the probiotic *Lactobacillus*

*salivarius* UCC118 to reduce *Listeria monocytogenes* infection *in vitro* and in mice has been proven very elegantly [19], but validation in humans could never be ethically performed.

## Is the translation of mechanistic understanding truly failing?

We can question whether laboratory established molecular mechanisms do not translate to corresponding responses in humans, or whether such responses do occur but do not lead to a health benefit. As an example, it is more than a decade ago that it was discovered that the major secreted proteins (P40 and P75) of *Lactobacillus rhamnosus* GG can protect mice against chemically induced colitis. This effect depends on the capacity of these proteins to modulate epidermal growth factor receptor (EGF-R) activity, which leads to inhibition of apoptosis and promotion of growth [20]. Recently, *L. rhamnosus* GG was shown to promote epithelial wound healing in skin and gingival human epithelial cell lines, a process strongly controlled by EGF-R, and most likely involving these major secreted proteins [21,22]. Remarkably, the duodenal tissue transcriptome responses in healthy human volunteers upon *L. rhamnosus* GG consumption revealed activation of 'wound healing' pathways, illustrating the legitimate molecular translation of the EGF-R modulation by this probiotic from *in vitro* cell lines to *in vivo* mucosal tissue [23]. This example highlights that challenges in translation may not be due to lack of conservation of the molecular responses between *in vitro* model systems and human mucosal tissues, but that other factors like interindividual variability of the physiological relevance of these responses may prevent the demonstration of corresponding health benefits in human subjects.

## Concluding remarks

Translation of mechanistic understanding to reliable clinical effects in human subjects is fraught with difficulty. Human individuality and the highly distinct molecular make-up of mucosal tissue was proposed as a key confounder in the translation of molecular mechanisms towards beneficial and perceivable physiological effects in human subjects [23,24]. Moreover, randomized controlled trials (RCTs) are considered by most regulatory bodies as a *sine qua non* for demonstrating probiotic efficacy. Many probiotic RCTs observe limited effect sizes that may at least in part be due to the presence of non-responders. Notably, the validity of RCT studies depends on specific presuppositions intrinsic to the RCT regimen, which may not be valid for probiotic interventions [25•]. Alternative clinical study designs (n-of-1 or adaptation trial design [25•]) may be needed to demonstrate probiotic efficacy in specific subpopulations, and with appropriate molecular read-outs can enable the connection between mechanism of action and individualized health effects.

Determining the mechanism of action of specific probiotics is both scientifically satisfying and clinically important. Filling in the gaps in our understanding of the different layers of knowledge within the translational pipeline concept will help us to deliver appropriate and effective probiotic products to targeted populations. Regulatory bodies also request such understanding for approval of health claims. For example, the European Food Safety Association (EFSA) defines a mechanism of action as a biologically plausible sequence of events that lead to an observed effect, which is supported by robust experimental observations and mechanistic information [26]. However, we may not always require the unequivocal identification of specific probiotic effector molecules, and in some cases (e.g. AAD and NEC) it may be sufficient to define core properties of probiotic products that can be linked to the desired effect. These core properties could relate to metabolites or structures that many probiotics produce that may influence host responses [2•,14]. In other cases that involve species or strain-specific effects, translation to reliable health benefits can be driven by insights into the probiotic mode of action and the effector molecules involved, but may require stratification of subpopulations that would benefit from a particular probiotic product. Therefore, while it may not be necessary to complete the translational pipeline for well-established benefits, understanding mechanism of action of probiotics is critical for the (i) selection of more effective probiotic strains; (ii) optimization of probiotic product manufacturing and quality assurance, (iii) improved design of probiotic formulation, and (iv) support the design of effective clinical trials with the best chance of realizing benefits to human health. In general, the monitoring of the production and bioavailability of known probiotic core properties and/or specific effector molecules during strain selection and product manufacturing and formulation would result in improved probiotic-product quality criteria relative to the number of viable cells that is currently used.

## Acknowledgements

This work was conducted by an expert group of the European branch of the International Life Sciences Institute, ILSI Europe. The expert group carried out the work, i.e. collecting/analysing data/information and writing the scientific paper. The authors would like to thank Dr Gabriella Bergonzelli (Nestlé), who was a member of this expert group, for her active contribution to this work. ILSI Europe facilitated scientific meetings and coordinated the overall project management and administrative tasks relating to the completion of this work. In particular, the authors would like to thank Dr. Tobias Recker and Dr. Jacqueline Whyte for their support. For further information about ILSI Europe, please email info@ilsieurope.be or call +3227710014.

## Funding

The expert group and resulting publication was supported by ILSI Europe Probiotics Task Force. Industry members of this task force are listed on the ILSI Europe website at http://ilsi.eu/task-forces/nutrition/probiotics/ . Experts were not paid for the time spent on this work; however, the non-industry members within the expert group were offered support for travel and accommodation costs from the Probiotics Task Force to attend meetings to discuss the manuscript, and a small compensatory sum (honorarium) with the option to decline.

## Conflict of interest statement

The research reported is the result of a scientific evaluation in line with ILSI Europe's framework to provide a precompetitive setting for public-private partnership (PPP). The expert group carried out the work separate to other activities of the Probiotics Task Force. The opinions expressed herein and the conclusions of this publication are those of the authors and do not necessarily represent the views of ILSI Europe nor those of its member companies. Dr. Sylvie Binda works full time for Danone. Dr. Gabriele Gross works full time for Reckitt Benckiser/Mead Johnson Nutrition. Dr. Arthur Ouwehand works full time for DuPont Nutrition. Dr. Johan van Hylckama works full time for Chr Hansen.

## References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf.

2. • Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S *et al.*: **Expert consensus document. The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic**. *Nat Rev Gastroenterol Hepatol* 2014, **11**:506-514.

Important conceptual paper, that recognizes that certain health benefits depend on core properties that are conserved in different probiotics, while other benefits may be more species or strain specific. See also reference 14 for an expanded description of these core properties.

3. •• Panigrahi P, Parida S, Nanda NC, Satpathy R, Pradhan L, Chandel DS, Baccaglini L, Mohapatra A, Mohapatra SS, Misra PR *et al.*: **A randomized synbiotic trial to prevent sepsis among infants in rural India**. *Nature* 2017, **548**:407-412.

Landmark study that describes the remarkable protection by a synbiotic product against sepsis and death (40% reduction) in a large infant cohort (4556 infants) in rural India, although the study did not disentangle the relative contribution of the probiotic*Lactobacillus plantarum* and the fructooligosaccharides to the observed effect.

4. Ritchie ML, Romanuk TN: **A meta-analysis of probiotic efficacy for gastrointestinal diseases**. *PLoS One* 2012, **7**:e34938.

5. Parker EA, Roy T, D'Adamo CR, Wieland LS: **Probiotics and gastrointestinal conditions: an overview of evidence from the Cochrane collaboration**. *Nutrition* 2018, **45**:125-134 e111.

6. Lebeer S, Bron PA, Marco ML, Van Pijkeren JP, O'Connell Motherway M, Hill C, Pot B, Roos S, Klaenhammer T: **Identification of probiotic effector molecules: present state and future perspectives**. *Curr Opin Biotechnol* 2018, **49**:217-223.

7. • Maldonado-Gomez MX, Martinez I, Bottacini F, O'Callaghan A, Ventura M, van Sinderen D, Hillmann B, Vangay P, Knights D, Hutkins RW *et al.*: **Stable engraftment of *Bifidobacterium longum* AH1206 in the Human gut depends on individualized features of the resident microbiome**. *Cell Host Microbe* 2016, **20**:515-526.

This study demonstrates that the engraftment of a*Bifidobacterium longum* strain can be predicted on basis of metagenomic characterization of the intestinal microbiome of the individual to which the strain is administered, thereby illustrating how molecular stratification can be employed to predict efficacy, in this case of colonization.

8. Derrien M, van Hylckama Vlieg JE: **Fate, activity, and impact of ingested bacteria within the human gut microbiota**. *Trends Microbiol* 2015, **23**:354-366.

9. Kristensen NB, Bryrup T, Allin KH, Nielsen T, Hansen TH, Pedersen O: **Alterations in fecal microbiota composition by probiotic supplementation in healthy adults: a systematic review of randomized controlled trials**. *Genome Med* 2016, **8**:52.

10. Marteau P, Flourie B, Pochart P, Chastang C, Desjeux JF, Rambaud JC: **Effect of the microbial lactase (EC 3.2.1.23) activity in yoghurt on the intestinal absorption of lactose: an in vivo study in lactase-deficient humans**. *Br J Nutr* 1990, **64**:71-79.

11. EFSA Panel on Dietetic Products NaA: **Scientific opinion on the substantiation of health claims related to live yoghurt cultures and improved lactose digestion (ID 1143, 2976) pursuant to Article 13(1) of Regulation (EC) No 1924/2006**. *EFSA J* 2010, **8**:1763-1781.

12. •• Bonder MJ, Kurilshikov A, Tigchelaar EF, Mujagic Z, Imhann F, Vila AV, Deelen P, Vatanen T, Schirmer M, Smeekens SP *et al.*: **The effect of host genetics on the gut microbiome**. *Nat Genet* 2016, **48**:1407-1412.

This study not only confirmed the association between the abundance of*Bifidobacterium* in the intestinal microbiome and SNPs in the hypolactasia associated LCT locus of the human genome, but also found evidence of interaction between host genetics and diet in regulating microbiome composition.

13. Blekhman R, Goodrich JK, Huang K, Sun Q, Bukowski R, Bell JT, Spector TD, Keinan A, Ley RE, Gevers D *et al.*: **Host genetic variation impacts microbiome composition across human body sites**. *Genome Biol* 2015, **16**:191.

14. Sanders ME, Benson A, Lebeer S, Merenstein DJ, Klaenhammer TR: **Shared mechanisms among probiotic taxa: implications for general probiotic claims**. *Curr Opin Biotechnol* 2018, **49**:207-216.

15. Hempel S, Newberry SJ, Maher AR, Wang Z, Miles JN, Shanman R, Johnsen B, Shekelle PG: **Probiotics for the prevention and treatment of antibiotic-associated diarrhea: a systematic review and meta-analysis**. *JAMA* 2012, **307**:1959-1969.

16. Lau CS, Chamberlain RS: **Probiotics are effective at preventing *Clostridium difficile*-associated diarrhea: a systematic review and meta-analysis**. *Int J Gen Med* 2016, **9**:27-37.

17. Aceti A, Gori D, Barone G, Callegari ML, Di Mauro A, Fantini MP, Indrio F, Maggio L, Meneghin F, Morelli L *et al.*: **Probiotics for prevention of necrotizing enterocolitis in preterm infants: systematic review and meta-analysis**. *Ital J Pediatr* 2015, **41**:89.

18. Lee IC, Tomita S, Kleerebezem M, Bron PA: **The quest for probiotic effector molecules–unraveling strain specificity at the molecular level**. *Pharmacol Res* 2013, **69**:61-74.

19. Corr SC, Li Y, Riedel CU, O'Toole PW, Hill C, Gahan CG: **Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118**. *Proc Natl Acad Sci U S A* 2007, **104**:7617-7621.

20. Yan F, Polk DB: **Characterization of a probiotic-derived soluble protein which reveals a mechanism of preventive and treatment effects of probiotics on intestinal inflammatory diseases**. *Gut Microbes* 2012, **3**:25-28.

21. Fernandez-Gutierrez MM, Roosjen PPJ, Ultee E, Agelink M, Vervoort JJM, Keijser B, Wells JM, Kleerebezem M: ***Streptococcus salivarius* MS-oral-D6 promotes gingival re-epithelialization in vitro through a secreted serine protease**. *Sci Rep* 2017, **7**:11100.

22. Mohammedsaeed W, Cruickshank S, McBain AJ, O'Neill CA: ***Lactobacillus rhamnosus* GG lysate increases re-epithelialization of keratinocyte scratch assays by promoting migration**. *Sci Rep* 2015, **5**:16147.

23. van Baarlen P, Troost F, van der Meer C, Hooiveld G, Boekschoten M, Brummer RJ, Kleerebezem M: **Human mucosal in vivo transcriptome responses to three lactobacilli indicate how probiotics may modulate human cellular pathways**. *Proc Natl Acad Sci U S A* 2011, **108(Suppl. 1)**:4562-4569.

24. Bron PA, van Baarlen P, Kleerebezem M: **Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa**. *Nat Rev Microbiol* 2011, **10**:66-78.

25. Zeilstra D, Younes JA, Brummer RJ, Kleerebezem M:
• **Fundamental limitations of the RCT method in nutritional research: the example of probiotics**. *Adv Nutr* 2018, **9**:561-571 http://dx.doi.org/10.1093/advances/nmy046.
This study systematically analyzes the presuppositions that are intrinsic to the Randomized Controlled Trial (RCT) design and provides arguments why such clinical trial design may therefore be unsuitable for proving the efficacy of probiotics, as well as other nutritional inteventions.

26. Hardy A, Benford D, Halldorsson T, Jeger MJ, Knutsen HK, More S, Naegeli H, Noteborn H, Ockleford C, Ricci A *et al.*: **Guidance on the assessment of the biological relevance of data in scientific assessments**. *EFSA J* 2017, **15**.

27. Larcombe S, Hutton ML, Lyras D: **Involvement of bacteria other than *Clostridium difficile* in antibiotic-associated diarrhoea**. *Trends Microbiol* 2016, **24**:463-476.

28. Rineh A, Kelso MJ, Vatansever F, Tegos GP, Hamblin MR: ***Clostridium difficile* infection: molecular pathogenesis and novel therapeutics**. *Expert Rev Anti Infect Ther* 2014, **12**:131-150.

29. Dadar M, Tiwari R, Karthik K, Chakraborty S, Shahali Y, Dhama K: ***Candida albicans* - biology, molecular characterization, pathogenicity, and advances in diagnosis and control - an update**. *Microb Pathog* 2018, **117**:128-138.

30. Delcaru C, Alexandru I, Podgoreanu P, Cristea VC, Bleotu C, Chifiriuc MC, Bezirtzoglou E, Lazar V: **Antagonistic activities of some *Bifidobacterium* sp. strains isolated from resident infant gastrointestinal microbiota on gram-negative enteric pathogens**. *Anaerobe* 2016, **39**:39-44.

31. Schoster A, Kokotovic B, Permin A, Pedersen PD, Dal Bello F, Guardabassi L: **In vitro inhibition of *Clostridium difficile* and *Clostridium perfringens* by commercial probiotic strains**. *Anaerobe* 2013, **20**:36-41.

32. Mills S, Ross RP, Hill C: **Bacteriocins and bacteriophage; a narrow-minded approach to food and gut microbiology**. *FEMS Microbiol Rev* 2017, **41**:S129-S153.

33. Surendran Nair M, Amalaradjou MA, Venkitanarayanan K: **Antivirulence properties of probiotics in combating microbial pathogenesis**. *Adv Appl Microbiol* 2017, **98**:1-29.

34. Engelbrektson A, Korzenik JR, Pittler A, Sanders ME,
• Klaenhammer TR, Leyer G, Kitts CL: **Probiotics to minimize the disruption of faecal microbiota in healthy subjects undergoing antibiotic therapy**. *J Med Microbiol* 2009, **58**:663-670.
This study describes the possibility of the improved resilience of the gut microbiome to recover after antibiotic treatment, a concept of probiotic functionality that is important to consider in this context.

35. Moens F, Verce M, De Vuyst L: **Lactate- and acetate-based cross-feeding interactions between selected strains of lactobacilli, bifidobacteria and colon bacteria in the presence of inulin-type fructans**. *Int J Food Microbiol* 2017, **241**:225-236.

36. Bron PA, Kleerebezem M, Brummer RJ, Cani PD, Mercenier A, MacDonald TT, Garcia-Rodenas CL, Wells JM: **Can probiotics modulate human disease by impacting intestinal barrier function?** *Br J Nutr* 2017, **117**:93-107.

37. Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, Tobe T, Clarke JM, Topping DL, Suzuki T *et al.*: **Bifidobacteria can protect from enteropathogenic infection through production of acetate**. *Nature* 2011, **469**:543-547.

38. Neu J, Walker WA: **Necrotizing enterocolitis**. *N Engl J Med* 2011, **364**:255-264.

39. Elgin TG, Kern SL, McElroy SJ: **Development of the neonatal intestinal microbiome and its association with necrotizing enterocolitis**. *Clin Ther* 2016, **38**:706-715.

40. Underwood MA: **Impact of probiotics on necrotizing enterocolitis**. *Semin Perinatol* 2017, **41**:41-51.

41. Underwood MA, Davis JCC, Kalanetra KM, Gehlot S, Patole S, Tancredi DJ, Mills DA, Lebrilla CB, Simmer K: **Digestion of human milk oligosaccharides by *Bifidobacterium breve* in the premature infant**. *J Pediatr Gastroenterol Nutr* 2017, **65**:449-455.

42. Garrido D, Ruiz-Moyano S, Kirmiz N, Davis JC, Totten SM, Lemay DG, Ugalde JA, German JB, Lebrilla CB, Mills DA: **A novel gene cluster allows preferential utilization of fucosylated milk oligosaccharides in *Bifidobacterium longum* subsp. *longum* SC596**. *Sci Rep* 2016, **6**:35045.

43. Frese SA, Hutton AA, Contreras LN, Shaw CA, Palumbo MC, Casaburi G, Xu G, Davis JCC, Lebrilla CB, Henrick BM *et al.*: **Persistence of supplemented *Bifidobacterium longum* subsp. *infantis* EVC001 in breastfed infants**. *mSphere* 2017, **2**.

44. Afrazi A, Branca MF, Sodhi CP, Good M, Yamaguchi Y, Egan CE, Lu P, Jia H, Shaffiey S, Lin J *et al.*: **Toll-like receptor 4-mediated endoplasmic reticulum stress in intestinal crypts induces necrotizing enterocolitis**. *J Biol Chem* 2014, **289**:9584-9599.

45. Patel RM, Denning PW: **Therapeutic use of prebiotics, probiotics, and postbiotics to prevent necrotizing enterocolitis: what is the current evidence?** *Clin Perinatol* 2013, **40**:11-25.

46. Patel RM, Denning PW: **Intestinal microbiota and its relationship with necrotizing enterocolitis**. *Pediatr Res* 2015, **78**:232-238.

47. Ganguli K, Collado MC, Rautava J, Lu L, Satokari R, von Ossowski I, Reunanen J, de Vos WM, Palva A, Isolauri E *et al.*: ***Lactobacillus rhamnosus* GG and its SpaC pilus adhesin modulate inflammatory responsiveness and TLR-related gene expression in the fetal human gut**. *Pediatr Res* 2015, **77**:528-535.

48. Meng D, Zhu W, Ganguli K, Shi HN, Walker WA: **Anti-**
• **inflammatory effects of *Bifidobacterium longum* subsp *infantis* secretions on fetal human enterocytes are mediated by TLR-4 receptors**. *Am J Physiol Gastrointest Liver Physiol* 2016, **311**:G744-G753.
This study demonstrated that probably a glycan or acyl-glycan in the conditioned media of*Bifidobacterium longum* subspecies *infantis* is able to modulate TLR-4 dependent signalling in human cells and could thereby play a critical role in the protective effect of this probiotic in NEC, since TLR-4 is considered to play a significant role in induction of NEC.