Probiotics during and after antibiotics

- Designer probiotics are capable of more than reducing AAD -

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Abstract

Each individual has a unique, relatively stable, intestinal microbiota which plays an important role in human health. One of the side effects of antibiotic intake is a disturbance of this microbiota which can result in antibiotic-associated diarrhoea (AAD). It was widely assumed that these disturbances were short-term but recently medium and long-term disturbances have been described. The exact clinical consequences of these microbiota disturbances are not yet clear but they are associated with a multitude of disorders such as IBS and allergy. Knowledge of the effect of antibiotics on the intestinal microbiota and ways of restoring their unique composition are therefore of clinical importance. A RTC performed by the Maastricht University Medical Centre showed that the probiotic Ecologic® AAD was not only able to reduce AAD but also to restore the intestinal microbiota towards the pre-antibiotic state.

Introduction

The gastrointestinal tract (GI-tract) comprises a complex bacterial ecosystem (i.e. the intestinal microbiota) colonizing the entire length of the gut and containing approximately hundred times as many genes as the human genome[1]. The intestinal microbiota contains approximately 10^{14} bacteria and is composed of more than 1000 different species[2], of which anaerobes are a hundred times more abundant. In addition, the composition of the microbiota differs both along the gastrointestinal tract and from lumen to mucosa[3].

The microbiota plays an important role in human physiology. Firstly, the intestinal microbiota aids in the break-down of non-digested and indigestible polysaccharides, and supplies essential substrates like vitamins and short-chain fatty acids. It also provides colonization resistance by competing for substrates and adhesion sites and by producing antibacterial substances, thereby preventing the overgrowth of potential pathogens. Secondly, the intestinal microbiota affects mucosal barrier function by influencing the metabolism, proliferation and survival of the intestinal epithelial cells, the production and composition of mucus and by the strengthening of tight junctions (level 2)[4]. Moreover there is an active cross-talk between the intestinal microbiota and the immune system via epithelial cells, specialised M cells and lamina propria DCs[5-6].

Though marked variations are present between individuals, within adults the intestinal microbiota is found to be relatively stable over time[7-8]. However, several factors among which the use of antibiotics, can disturb this microbiota.

Antibiotics and antibiotic-associated side effects

Ever since the discovery of the first antibiotic, penicillin, by Alexander Flemming in 1928, antibiotics have become one of the cornerstones in the prevention and treatment of infectious diseases. However, their use is not without side effects as it may lead to the development of antibiotic resistance or cause a disturbance of the intestinal microbiota. Moreover, antibiotics can have direct allergic and toxic effects on the intestinal mucosa, direct effects on immune cell function, and pharmacological effects on intestinal motility[9-11].

One of the most common and clinically manifest side effects of antibiotic use is antibiotic-associated diarrhoea (AAD), which can occur shortly after antibiotic intake to up to 8 weeks after cessation[12-13]. The incidence of AAD ranges from 5-39%, depending on the definition of diarrhoea, the route of administration, the type of antibiotic used and host factors[13]. In general, broad-spectrum antibiotics, amoxycillin, amoxycillin/clavulanic acid, clindamycin and cephalosporines are associated with a high risk of AAD[14]. AAD can be divided into two types: non-specific AAD, which is usually mild and CDAD, which can lead to severe pseudomembranous colitis[15]. *Clostridium difficile* is thought to be the causative agent in up to 20% of AAD[16]. However, the exact pathogenesis of the majority of cases of AAD is not

clear and is attributed to a disturbance of the intestinal microbiota. Overviews of the ecological disturbances due to different types of antibiotics have been published by Edlund *et al.* and Sullivan *et al.*[17-18]. In these reviews it was generally found that the microbiota was only temporarily disturbed, returning to its original composition 1-2 months after cessation. However, mainly cultivation-based techniques were used, while it is estimated that less than 25% of the intestinal bacterial populations can be cultured[2 19-20]. The application of culture-independent methods based on 16S rRNA provides a better and more comprehensive insight into the diversity of the intestinal microbiota and the ecological disturbances due to antibiotic treatment[21-25]. Nowadays, literature data suggests that short-term use of antibiotics can also have long-term consequences on the intestinal ecology. Jernberg *et al.* showed that a 7-day clindamycin intake caused short-term disturbances in the total bacterial profiles. In addition, large and persistent changes were found in the *Bacteroides* community, which did not return to its original status within two years post-treatment[24]. A similar finding was reported by Dethlefsen *et al.* who found that 5 days of ciprofloxacin reduced the diversity and stability of one third of the bacterial taxa in the gut. Although the majority of the microbial communities returned to pre-treatment values after four weeks, several taxa failed to recover within six months[23]. Recently, Lindgren et al. showed that clindamycin treatment had a prolonged impact on *Enterococus* spp. variation[26]. Moreover, a study in mice using qPCR showed that total bacterial numbers returned to normal within 1 week after cessation of antibiotic intake, but alterations in *Bacteroides* and segmented filamentous bacteria persisted more than 3 weeks[27].

Antibiotic-induced disturbances of the intestinal microbiota can result in important functional differences in the microbial metabolome (i.e. de collective biochemical output of the microbiota), loss of colonisation resistance, a decreased intestinal barrier function and can influence the immune responses of the host[9].

The clinical consequences of these antibiotic induced microbiota perturbation for the host are not yet clear. There is however, accumulating evidence indicating that a disturbance of the intestinal microbiota plays an important role in a multitude of disorders ranging from allergies to diarrhoea, irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) and colorectal cancer[4 28-30]. A disturbance of this intestinal microbiota due to antibiotics has been associated with local inflammation and a disturbed immunological functioning of the host[28 31-32]. In addition, a number of epidemiological studies have demonstrated an association between the use of antibiotics during early childhood and an increased risk for acquiring allergy or asthma[33-36]. Also, alteration of the intestinal microbiota due to antibiotic treatment was found to induce or exacerbate IBS[37]. Thus, knowledge of the effect of antibiotic on the intestinal microbiota and ways of restoring their unique composition are of clinical importance.

Probiotics for antibiotic-associated side effects

Probiotics are defined as "*live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host*". The treatment and/or prevention of AAD by probiotics have been subject of many trials and are reviewed in several meta-analyses and some systematic reviews (Table 1). The meta-analyses by Cremonini, D'Souza, Szajewska (2005 and 2006) and Johnston found combined relative risks of 0.37 - 0.44 in favour of probiotic over placebo treatment in the prevention of AAD[38-42]. McFarland[43] and Sazawal[44] both conducted large meta-analyses (25 and 19 studies, respectively) including many different strains and types of probiotics. Both meta-analyses also showed that probiotics were efficacious in preventing AAD with a RR of 0.43 and 0.48, respectively. Moreover, McFarland stratified by probiotic type and showed that only *S. boulardii*, LGG and probiotic mixtures showed significant efficacy[43]. Sazawal analysed the use of probiotics in the prevention of acute diarrhoea (n=34). Subgroup analyses were performed on type of diarrhoea (AAD:19 studies), age groups, products and quality of trial and showed that only type of diarrhoea and product influenced the effect[44].

Next to several meta-analysis also three systematic reviews were published[45-47]. In the systematic review by Hawrelak, evaluating only papers on LGG, 4 of 6 studies found a significant reduction of AAD, one study found a significant decrease in the number of days with diarrhoea and one study was negative[45]. Scheike reviewed 23 trials and found that probiotics were associated with a relative reduction of AAD of 52% compared to placebo with a number to treat (NTT) of 8[47]. Despite statistical heterogeneity between studies, detailed analysis revealed that the effects of probiotics were consistent. Moreover, a stratified analysis revealed that *S. boulardii*, LGG and multispecies products were more effective than monospecies products. Furthermore, no relation between trial quality and magnitude of effect on probiotic treatment was found and no adverse events were reported[47]. The Cochrane database of systematic reviews published a review on probiotics for the prevention of paediatric AAD. The per protocol analysis showed a pooled relative risk of 0.49 in favour of probiotic use. However, the intention to treat analysis showed non-significant results[46].

Only a limited number of studies investigated the effect of probiotic in the prevention of CDAD. As approximately 20% of AAD is CDAD[16], this is probably due to the large sample size required to detect a significant difference.

Thus there is compelling evidence that probiotics can be efficacious in the treatment and prevention of AAD. However, few studies have investigated the mechanism of action on the (potential) efficacy of probiotics which included the effect on antibiotic induced perturbations of the intestinal microbiota. Most that have, have focussed on survival of ingested probiotic

strains, emergence of antibiotic resistance or prevention of disturbances in specific bacterial groups[48-52]. In addition, mainly culture-dependent approaches were used and the effect on the total microbiota was rarely investigated.

Recently, a study was conducted by the Maastricht University Medical Centre (MUMC) investigating the effect of a multispecies probiotics (Ecologic® AAD) on bacteriological, immunological and clinical parameters in healthy volunteers during and after amoxycillin use. The study showed that there is a clear association between the disruption of the intestinal microbiota and the development of diarrhoea. Moreover, the study showed that intake of Ecologic® AAD significantly reduced the occurrence of diarrhoea-like bowel movements. This was probably due to a better restoration of the microbiota as a intestinal microbiota more similar to the pre-antibiotic state was observed in the volunteers treated with Ecologic® AAD compared to placebo[53-54]. The latter was also observed in a study looking at the effect of a probiotic mixture during amoxicillin/clavulanic acid intake in healthy volunteers using a combination of culture and terminal restriction fragment length polymorphism. The results of both studies suggest therefore that a restoration of the intestinal microbiota is one of the mechanisms of probiotics efficacy.

Conclusion

It is becoming accepted that antibiotic treatment can cause medium to long term ecological disturbances of the intestinal microbiota even if AAD is not present. Even thought the clinical consequences of these antibiotic induced microbiota perturbations are not yet clear, accumulating evidence indicates an important role in a multitude of disorders. Probiotics have not only shown to be efficacious in preventing AAD but also in restoring these antibiotic induced microbiota perturbations.

Large prospective human intervention trial are needed to determine to what extent antibiotic induced disturbances of the intestinal microbiota are permanent, what their clinical implications are and if probiotic supplementation is able to either prevent or reverse a prolonged imbalance. In addition, it would be interesting to identify host-related, microbiota-related or environmental risk factors to characterize subjects at risk and who would benefit from probiotic intake.

References

1. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. *Science* 2006;312(5778):1355-9.
2. Rajilic-Stojanovic M, Smidt H, de Vos WM. Diversity of the human gastrointestinal tract microbiota revisited. *Environ Microbiol* 2007;9(9):2125-36.
3. Isolauri E, Salminen S, Ouwehand AC. Microbial-gut interactions in health and disease. Probiotics. *Best Pract Res Clin Gastroenterol* 2004;18(2):299-313.
4. Dethlefsen L, Eckburg PB, Bik EM, Relman DA. Assembly of the human intestinal microbiota. *Trends Ecol Evol* 2006;21(9):517-23.
5. Galdeano CM, de Moreno de LeBlanc A, Vinderola G, Bonet ME, Perdigon G. Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. *Clin Vaccine Immunol* 2007;14(5):485-92.
6. Shida K, Nanno M. Probiotics and immunology: separating the wheat from the chaff. *Trends Immunol* 2008;29(11):565-73.
7. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. *Science* 2005;308(5728):1635-8.
8. Zoetendal EG, Rajilic-Stojanovic M, de Vos WM. High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. *Gut* 2008;57(11):1605-15.
9. Beaugerie L, Petit JC. Microbial-gut interactions in health and disease. Antibiotic-associated diarrhoea. *Best Pract Res Clin Gastroenterol* 2004;18(2):337-52.
10. Hogenauer C, Hammer HF, Krejs GJ, Reisinger EC. Mechanisms and management of antibiotic-associated diarrhea. *Clin Infect Dis* 1998;27(4):702-10.
11. Van Vlem B, Vanholder R, De Paepe P, Vogelaers D, Ringoir S. Immunomodulating effects of antibiotics: literature review. *Infection* 1996;24(4):275-91.
12. Bartlett JG. Antibiotic-associated diarrhea. *Clin Infect Dis* 1992;15(4):573-81.
13. McFarland LV. Epidemiology, risk factors and treatments for antibiotic-associated diarrhea. *Dig Dis* 1998;16(5):292-307.
14. McFarland LV. Epidemiology of infectious and iatrogenic nosocomial diarrhea in a cohort of general medicine patients. *Am J Infect Control* 1995;23(5):295-305.
15. Wilkins TD, Lyerly DM. Clostridium difficile testing: after 20 years, still challenging. *J Clin Microbiol* 2003;41(2):531-4.
16. Pillai A, Nelson R. Probiotics for treatment of Clostridium difficile-associated colitis in adults. *Cochrane Database Syst Rev* 2008(1):CD004611.
17. Edlund C, Nord CE. Effect on the human normal microflora of oral antibiotics for treatment of urinary tract infections. *J Antimicrob Chemother* 2000;46 Suppl 1:41-8; discussion 63-5.
18. Sullivan A, Edlund C, Nord CE. Effect of antimicrobial agents on the ecological balance of human microflora. *Lancet Infect Dis* 2001;1(2):101-14.
19. Tannock GW. Analysis of the intestinal microflora using molecular methods. *Eur J Clin Nutr* 2002;56 Suppl 4:S44-9.
20. Zoetendal EG, Vaughan EE, de Vos WM. A microbial world within us. *Mol Microbiol* 2006;59(6):1639-50.
21. De La Cochetiere MF, Durand T, Lalande V, Petit JC, Potel G, Beaugerie L. Effect of antibiotic therapy on human fecal microbiota and the relation to the development of Clostridium difficile. *Microb Ecol* 2008;56(3):395-402.
22. De La Cochetiere MF, Durand T, Lepage P, Bourreille A, Galmiche JP, Dore J. Resilience of the dominant human fecal microbiota upon short-course antibiotic challenge. *J Clin Microbiol* 2005;43(11):5588-92.
23. Dethlefsen L, Huse S, Sogin ML, Relman DA. The Pervasive Effects of an Antibiotic on the Human Gut Microbiota, as Revealed by Deep 16S rRNA Sequencing. *PLoS Biol* 2008;6(11):e280.
24. Jernberg C, Lofmark S, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *Isme J* 2007;1(1):56-66.

25. Young VB, Schmidt TM. Antibiotic-associated diarrhea accompanied by large-scale alterations in the composition of the fecal microbiota. *J Clin Microbiol* 2004;42(3):1203-6.
26. Lindgren M, Lofmark S, Edlund C, Huovinen P, Jalava J. Prolonged impact of a one-week course of clindamycin on Enterococcus spp. in human normal microbiota. *Scand J Infect Dis* 2009;41(3):215-9.
27. Croswell A, Amir E, Teggatz P, Barman M, Salzman NH. Prolonged impact of antibiotics on intestinal microbial ecology and susceptibility to enteric Salmonella infection. *Infect Immun* 2009;77(7):2741-53.
28. Othman M, Aguero R, Lin HC. Alterations in intestinal microbial flora and human disease. *Curr Opin Gastroenterol* 2008;24(1):11-6.
29. Penders J, Stobberingh EE, van den Brandt PA, Thijs C. The role of the intestinal microbiota in the development of atopic disorders. *Allergy* 2007;62(11):1223-36.
30. Shreiner A, Huffnagle GB, Noverr MC. The "Microflora Hypothesis" of allergic disease. *Adv Exp Med Biol* 2008;635:113-34.
31. Rautava S, Isolauri E. The development of gut immune responses and gut microbiota: effects of probiotics in prevention and treatment of allergic disease. *Curr Issues Intest Microbiol* 2002;3(1):15-22.
32. Preidis GA, Versalovic J. Targeting the human microbiome with antibiotics, probiotics, and prebiotics: gastroenterology enters the metagenomics era. *Gastroenterology* 2009;136(6):2015-31.
33. Droste JH, Wieringa MH, Weyler JJ, Nelen VJ, Vermeire PA, Van Bever HP. Does the use of antibiotics in early childhood increase the risk of asthma and allergic disease? *Clin Exp Allergy* 2000;30(11):1547-53.
34. Farooqi IS, Hopkin JM. Early childhood infection and atopic disorder. *Thorax* 1998;53(11):927-32.
35. Johnson CC, Ownby DR, Alford SH, Havstad SL, Williams LK, Zoratti EM, et al. Antibiotic exposure in early infancy and risk for childhood atopy. *J Allergy Clin Immunol* 2005;115(6):1218-24.
36. Wickens K, Pearce N, Crane J, Beasley R. Antibiotic use in early childhood and the development of asthma. *Clin Exp Allergy* 1999;29(6):766-71.
37. Spiller R, Campbell E. Post-infectious irritable bowel syndrome. *Curr Opin Gastroenterol* 2006;22(1):13-7.
38. Cremonini F, Di Caro S, Covino M, Armuzzi A, Gabrielli M, Santarelli L, et al. Effect of different probiotic preparations on Anti-Helicobacter pylori therapy-related side effects: A parallel group, triple blind, placebo-controlled study. *Am J Gastroenterol* 2002;97(11):2744-9.
39. D'Souza AL, Rajkumar C, Cooke J, Bulpitt CJ. Probiotics in prevention of antibiotic associated diarrhoea: meta- analysis. *Bmj* 2002;324(7350):1361.
40. Johnston BC, Supina AL, Vohra S. Probiotics for pediatric antibiotic-associated diarrhea: a meta-analysis of randomized placebo-controlled trials. *Cmaj* 2006;175(4):377-83.
41. Szajewska H, Mrukowicz J. Meta-analysis: non-pathogenic yeast Saccharomyces boulardii in the prevention of antibiotic-associated diarrhoea. *Aliment Pharmacol Ther* 2005;22(5):365-72.
42. Szajewska H, Ruszczynski M, Radzikowski A. Probiotics in the prevention of antibiotic-associated diarrhea in children: A meta-analysis of randomized controlled trials. *J Pediatr* 2006;149(3):367-72 e1.
43. McFarland LV. Meta-analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of Clostridium difficile disease. *Am J Gastroenterol* 2006;101(4):812-22.
44. Sazawal S, Hiremath G, Dhingra U, Malik P, Deb S, Black RE. Efficacy of probiotics in prevention of acute diarrhoea: a meta-analysis of masked, randomised, placebo-controlled trials. *Lancet Infect Dis* 2006;6(6):374-82.

45. Hawrelak JA, Whitten DL, Myers SP. Is Lactobacillus rhamnosus GG effective in preventing the onset of antibiotic-associated diarrhoea: a systematic review. *Digestion* 2005;72(1):51-6.
46. Johnston BC, Supina AL, Ospina M, Vohra S. Probiotics for the prevention of pediatric antibiotic-associated diarrhea. *Cochrane Database Syst Rev* 2007(2):CD004827.
47. Scheike I, Connock M, Taylor R, Fry-Smith A, Ward D. Probiotics for the prevention of antibiotic associated diarrhea: a systematic review. *Department of Public Health and Epidemiology* 2006.
48. Jernberg C, Sullivan A, Edlund C, Jansson JK. Monitoring of antibiotic-induced alterations in the human intestinal microflora and detection of probiotic strains by use of terminal restriction fragment length polymorphism. *Appl Environ Microbiol* 2005;71(1):501-6.
49. Madden JA, Plummer SF, Tang J, Garaiova I, Plummer NT, Herbison M, et al. Effect of probiotics on preventing disruption of the intestinal microflora following antibiotic therapy: a double-blind, placebo-controlled pilot study. *Int Immunopharmacol* 2005;5(6):1091-7.
50. Plummer SF, Garaiova I, Sarvotham T, Cottrell SL, Le Scouiller S, Weaver MA, et al. Effects of probiotics on the composition of the intestinal microbiota following antibiotic therapy. *Int J Antimicrob Agents* 2005;26(1):69-74.
51. Sullivan A, Barkholt L, Nord CE. Lactobacillus acidophilus, Bifidobacterium lactis and Lactobacillus F19 prevent antibiotic-associated ecological disturbances of Bacteroides fragilis in the intestine. *J Antimicrob Chemother* 2003;52(2):308-11.
52. Sullivan A, Johansson A, Svenungsson B, Nord CE. Effect of Lactobacillus F19 on the emergence of antibiotic-resistant microorganisms in the intestinal microflora. *J Antimicrob Chemother* 2004;54(4):791-7.
53. Koning CJ, Jonkers D, Stobberingh E, Out TA, StockbrUgger R. Amoxycillin and multispecies probiotic intake affect cytokine production in healthy volunteers. *Gastroenterol* 2007;132(4 suppl 2):s1266.
54. Koning CJM. Multispecies probiotics and antibiotic-associated side effects - pathophysiological and clinical evidence. University Maastricht, the Netherlands 2010.
55. D'Souza AL, Rajkumar C, Cooke J, Bulpitt CJ. Probiotics in prevention of antibiotic associated diarrhoea: meta-analysis. *Bmj* 2002;324(7350):1361.
56. Cremonini F, Di Caro S, Nista EC, Bartolozzi F, Capelli G, Gasbarrini G, et al. Meta-analysis: the effect of probiotic administration on antibiotic-associated diarrhoea. *Aliment Pharmacol Ther* 2002;16(8):1461-7.
57. Szajewska H, Ruszczynski M, Radzikowski A. Probiotics in the prevention of antibiotic-associated diarrhea in children: a meta-analysis of randomized controlled trials. *J Pediatr* 2006;149(3):367-72.

Table 1. Meta-analyses and systematic reviews on the effect of probiotics in AAD

Reference	Number of trials	Adults/ of subjects	children	probiotics included	RR (95% CI)	Remarks
Meta-analyses						
D'Souza 2002[55]	9	1214	mixed	mix of single and multispecies	0.37 (0.26-0.53)	
				▪ 4 *S. boulardii* trials	0.39 (0.25-0.62)	
				▪ 5 'non-yeast' trials	0.34 (0.19-0.61)	
Cremonini 2002[56]	7	881	mixed	*Lactobacillus* spp. and *S. boulardii*	0.40 (0.27-0.57)	
Szajewska 2005[41]	5	1076	mixed	*S. boulardii*	0.43 (0.230.78)	Only trial with *S. boulardii* were included
Szajewska 2006[57]	6	766	children	mix of single and multispecies	0.44 (0.25-0.77)	
Mcfarland 2006[43]	25	2810	mixed	mix of single and multispecies	0.43 (0.31-0.58)	
				▪ 6 *S. boulardii*	0.37 (0.26-0.52)	
				▪ 6 *L. rhamnosus* GG	0.31 (0.13-0.72)	
				▪ 6 Single strain probiotics	0.46 (0.21-1.03)	
				▪ 7 Multiple strain probiotics	0.51 (0.38-0.68)	
	6 CDD	354	adults	mix of single and multispecies	0.59 (0.41-0.85)	
Johnston 2006[40]	6	707	children	mix of single and multispecies	0.43 (0.25-0.75)	No significant results with ITT
Sazawal 2006[44]	19	2050	mixed	mix of single and multispecies	0.48 (0.35-0.65)	Trial was on acute diarrhoea (n=34), but subgroup analyses was performed for AAD (n=19)
Systematic reviews						
Hawrelak 2005[45]	6	692	mixed	*L. rhamnosus* GG	0.13 (0.02-0.94)/ 0.32 (0.09-1.11)	Only trials with LGG were included and RR range based on 4 of 6 positive studies.
Scheike 2006[47]	23	3365	mixed	mix of single and multispecies	0.48 (0.37-0.63)	
Johnston 2007[46]	10	1986	children	mix of single and multispecies	0.49 (0.32-0.74)	No significant results with ITT