Intestinal microbiota and feed design

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Gastrointestinal microbiota is a complex ecosystem with a huge bacterial density and diversity. Despite the vast microbial burden and the close contact between the microbes and the host's cells, normal intestinal microbiota is considered to be beneficial. The aim of the Finnish Research Programme is to characterise intestinal microbiota of production animals and learn to modulate intrinsic microbiota in order to support productivity and animal health. For a simplified and better description of the complex microbiota, Microbial Balance Index (MBI) counted from the proportions of several bacterial groups present in intestinal samples was developed. Cecal samples of the broiler chickens participating in the feed raw material energy evaluation trial were collected. Microbial composition was analysed by using a rapid and bacterial group specific flow cytometric method. MBI was observed to associate with growth (r = 0.38, P = 0.03). The finding suggests that intestinal microbiota is an essential factor affecting animal productivity and well being.

Keywords: Gastrointestinal tract; intestinal microbiota; cytometry

Introduction

Intestinal microbiota is a uniquely complex ecosystem with an exceptionally high microbial density and diversity. Gastrointestinal microbes achieve one of the highest cell densities recorded for any ecosystem, 10^{10} – 10^{12} bacteria in a gram of gut content, and intestinal samples may consist of even hundreds of bacterial species (Conway, 1997). Due to its complexity, the composition of the intestinal microbiota has been poorly known and the microbial composition of the chicken gastrointestinal tract have been reported to be largely unknown (Apajalahti *et al.*, 2004). In domestic animal production, intestinal microbes have traditionally been regarded as harmful for the host animal. Antimicrobial growth promoters (AGPs) have been considered to have beneficial effects on productivity by reducing intestinal microbial load and inhibiting pathogenic microbes. However, the conception of the intestinal microbiota has gradually changed. Gastrointestinal microbes and the host have complex interactions and the bacteria of the normal intestinal microbiota are known to participate for example in the nutrient processing (Bäckhed *et al.*, 2005; Hooper *et al.*, 2002). Non-pathogenic normal microbiota protects the gut from the colonization of harmful microbes, a process known as competitive exclusion. Intestinal bacteria also interact actively with the mucosa associated leukocytes contributing to the healthy maturation of the immune system (Hooper and Gordon, 2001; Umesaki and Setoyama, 2000). Consequently, the host and its normal microbiota are at present perceived to be in a mutualistic relationship where neither partner is harmed, or even in a symbiotic relationship where unique benefits are provided for both parties (Hooper and Gordon, 2001).

The ban in the use of AGPs has generated increasing needs to modulate the microbiota with dietary approaches in a healthy direction (Verstegen *et al.*, 2005). AGPs inhibit specific gastrointestinal infections effectively, but also disturb the natural balance between the host and gut microbes (Dibner and Richards, 2005). In this context, the idea of strengthening the healthy normal

balance between the host and its intestinal microbiota is a fascinating possibility to support resistance against pathogens and to secure good and steady animal productivity. Intestinal bacterial species are known to differ in their substrate preferences and growth requirements and therefore it should be possible to modulate the microbiota by alterations in the diet (Apajalahti *et al.*, 2004). Research on the bacterial composition of the microbiota in different situations has turned out to be in a key position in the development of feeding favouring healthy microbiota. Intestinal microbiota mainly consists of anaerobic bacteria, and it has been difficult to characterise with traditional cultivation methods. However, during the last two decades the advances in microbiological methodology, and particularly the methods based on the detection of nucleic acids of microbes, have enabled a more comprehensive and detailed analysis of intestinal microbiota (Zoetendal *et al.*, 2004).

In the Finnish Research Programme, the objective is to characterise the intestinal microbiota of the production animals and to find out how different feed materials and additives can affect the microbiota, productivity and health. The unique know-how and database acquired have revealed associations between microbial composition and animal productivity, and the future goal is to develop new feeds and feeding programs based on the control of intestinal microbiota. As an example of this microbiota-influenced feeding philosophy, we introduce the concept of Microbial Balance Index (MBI).

Materials and methods

Cecal samples from 31-day-old broiler chickens (n = 72) participating in the feed raw material energy evaluation trial were collected. The samples were collected immediately after the sacrifice and stored frozen up to 48 hours. The broilers had got normal starter feed for three weeks and trial feeds for one week. The six cold-pelleted trial feeds were based on wheat (W), barley (B), dehulled oats (DO), oats (O), wheat and rape seed meal (48 / 50 %) (WRS), or wheat and soy bean meal (48 / 50 %) (WS). The feeds were supplemented with the minerals and vitamins required. Daily weight gains (DWG) of the broilers were determined at the end of the trial.

The cecal bacterial cells were analysed with a rapid and bacterial group-specific method based on flow cytometry (FCM), 16S rRNA hybridization and DNA-staining (Vaahtovuo *et al.*, 2005). The samples were suspended in phosphate-buffered saline (PBS). The bacterial cells were separated with repeated centrifugations, fixed with 4 % paraformaldehyde, washed several times with PBS and re-suspended in 50 % ethanol-PBS. The fixed bacteria were stored at -20ºC until hybridized with oligonucleotide probes. Five 16S rRNA targeted oligonucleotide probes labeled at the 5'-end with indocarbocyanin (Molecular Probes, Eugene, Oregon) were used: Bif662 targeted to genus *Bifidobacterium*; Bacto1080 targeted to *Bacteroides-Porphyromonas-Prevotella* group; Clep866 targeted to *Clostridium leptum* subgroup; Enter1432 targeted to enteric group; and Fprau645 targeted to *Faecalibacterium prausnitzii* group (Doré *et al.*, 1998; Langendijk *et al.*, 1995; Lay *et al.*, 2005; Sghir et al., 2000; Suau *et al.*, 2001). After the hybridization, the bacteria were stained with SYTOX® DNA-stain (Molecular Probes) and analysed with BD FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, California). Bacterial flow cytometry was performed as described previously (Vaahtovuo *et al.*, 2005). The number of hybridized bacteria and the total bacterial counts were determined, and the MBI values were counted with an in-house algorithm (patent pending) from the proportions of bifidobacteria, butyrate-production related *Faecalibacterium prausnitzii* group bacteria, *Bacteroides-Porphyromonas-Prevotella* group bacteria and enteric group bacteria.

Microbial data were analysed with analysis of variance and the correlation between MBI and DWG was calculated by simple regression. In all tests $P \leq 0.05$ was considered to denote a significant difference.

Results and discussion

There were clear growth differences between the trial groups, the average DWG in the feeding groups being: 20 g (W); 36 g (B); 42 g (DO); 45 g (O); 65 g (WRS); and 67 g (WS). An example of an

FCM analysis of cecal sample is presented in Figure 1. FCM analyses revealed significant microbial differences between the feeding groups, which was to be expected, considering the differences in the trial feed compositions. The bacterial counts and the MBI values are presented in Table 1. The total bacterial counts varied from 2.1 x 10^{11} to 4.0 x 10^{11} bacteria per gram of dried cecal content ($P < 0.001$). Of the bacteria studied, *Clostridium leptum* subgroup was the most common and bifidobacteria the second most common, average bacterial counts being 2.6 x 10^{10} and 8.5 x 10^{9} bacteria per gram, correspondingly.

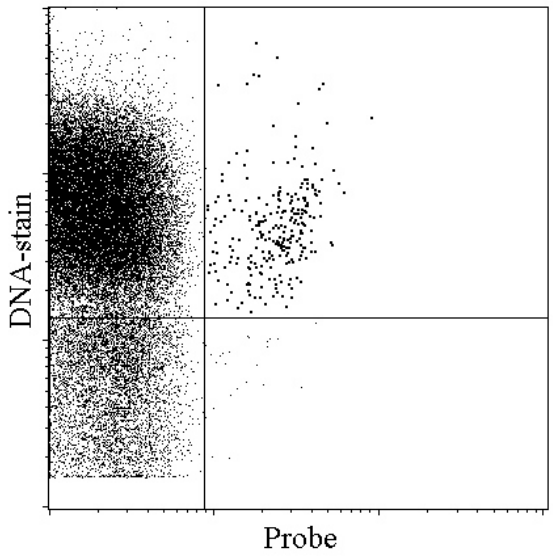

Figure 1. An example of an FCM analysis of cecal sample. In the dot plot, the fluorescence of the probe hybridizing target bacteria is presented in the x direction and the fluorescence of the DNA-stain in the y direction. The hybridized target bacteria are shown in upper right quadrant (dots highlighted) and the unhybridized bacteria in upper left quadrant. The DNA-stain negative and probe negative particles are seen in lower left quadrant and the DNA-stain negative probe positive particles (*i.e.* false-positives) in lower right quadrant. The bacteria are separated from the non-DNA-stained material, and the hybridized target bacteria are discriminated from other bacteria in the sample. All cecal samples were analysed individually. The analyses included at least 20 000 bacteria per sample.

Table 1. The bacterial counts and the MBI values of the feeding groups.

	Feeding group						*P*-value
	W	B	DO	O	WRS	WS	
Total bacteria[a]	3.7 x 10^{11}	3.1 x 10^{11}	2.1 x 10^{11}	4.0 x 10^{11}	2.6 x 10^{11}	3.3 x 10^{11}	***
Bifidobacteria[a]	8.4 x 10^{9}	7.1 x 10^{9}	5.4 x 10^{9}	1.1 x 10^{10}	6.4 x 10^{9}	1.3 x 10^{10}	*
Bacteroides group[a]	2.6 x 10^{9}	1.4 x 10^{9}	9.8 x 10^{8}	3.7 x 10^{9}	1.2 x 10^{9}	2.7 x 10^{9}	***
F. prausnitzii group[a]	5.7 x 10^{9}	3.9 x 10^{9}	2.2 x 10^{9}	1.0 x 10^{10}	4.3 x 10^{9}	6.1 x 10^{9}	**
Enteric group[a]	4.4 x 10^{9}	2.8 x 10^{9}	5.1 x 10^{9}	5.4 x 10^{9}	1.6 x 10^{9}	2.9 x 10^{9}	*
Cl. leptum subgroup[a]	3.4 x 10^{10}	2.4 x 10^{10}	1.1 x 10^{10}	3.2 x 10^{10}	2.5 x 10^{10}	3.3 x 10^{10}	**
MBI	21.5	35.8	17.9	23.0	39.6	32.7	***

Abbreviations: W, wheat; O, oats; DO, dehulled oats; B, barley; WS, wheat and soy bean meal; WRS, wheat and rape seed meal; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.
[a] bacteria per gram of cecal sample (dry matter).

In general, hybridization percentages were somewhat lower than expected, the hybridization percentage of the most common bacterial group, *Clostridium leptum* subgroup, being only 8.5 per cent. However, the proportions of different bacterial groups were in harmony with the literature data, and according to our previous experience, freezing and thawing of the samples may have had effect on the results of oligonucleotide hybridizations (Lu *et al.*, 2003; Zhu and Joerger, 2003). The MBI

reflecting microbial differences comprehensively varied significantly ($P < 0.001$) between the feeding groups and correlated with the DWG ($r = 0.38$, $P = 0.03$; Figure 2).

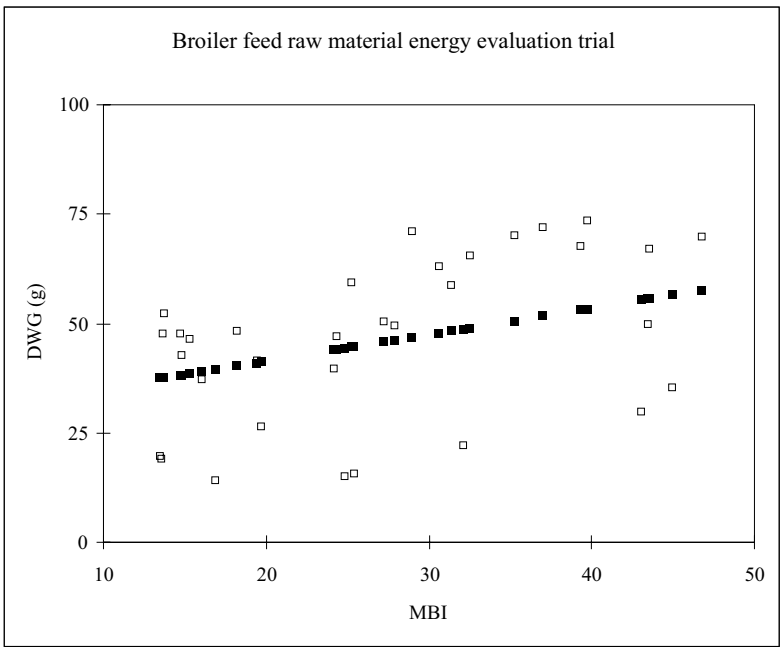

Figure 2. The correlation between DWG and MBI ($r = 0.38$, $P = 0.03$). The DWG and MBI values are presented from each broiler cage (6 cages per feeding). The MBI values are presented in the x direction and the DWG in the y direction. Symbols: □, the measured values; ■, the regression analysis predicted values.

The results demonstrate the possibilities to monitor intestinal microbiota with modern methodology and modulate the microbial composition with dietary measures. MBI was designed to describe the microbiota concisely and in a comprehensive manner. In the current study MBI showed clear microbial differences between the trial groups. It is important to note that due to the systemic nature of the microbiota, even such bacterial species whose proportions are not directly measured, will inevitably influence the MBI. Intestinal microbiota is an adaptive ecosystem, and the changes in some bacterial populations have comprehensive effects on the general microbial balance. Therefore, it is very probable that the microbial differences between the feeding groups extend also to such bacteria that were not targeted with the oligonucleotide probe set used.

The results concerning the association between MBI and growth emphasise the importance of the microbiota in productivity. In addition to expected associations between DWG and nutritional values (*e.g.* protein content) of the test feeds, MBI was found to correlate with DWG. Microbial composition appeared to be a considerable factor influencing digestibility and growth, albeit the differences in the feed compositions were radical. These results are in concordance with the results from piglet and pig production trials, where the differences in the feed compositions have been minor and nutritionally negligible (Vaahtovuo *et al.*, 2006). This indicates that microbial composition can be an independent factor influencing productivity.

The reduction in the use of AGPs has been a challenge for animal nutrition and evoked a widespread interest in finding alternative methods to control intestinal bacteria. The possibility to modulate the microbiota with new feed materials, ingredients or additives towards host-protecting functions thus replacing AGPs, is a topical issue in domestic animal production and creates fascinating possibilities to support animal health. The most probable way to succeed in this is to better learn to understand the inherent nature of the intestinal microecology and to modulate it in a rational and comprehensive manner. Although the knowledge of the effects of such feed additives as pre- and probiotics has increased, fundamental information concerning the microbial impact of different feed raw materials has been incomplete. One of the Finnish Research Programme's objectives is to deepen knowledge in this field. The present results demonstrate how fundamentally the microbiota can be modulated and how profound effects microbial composition has on growth. We predict that in addition to conventional diet formulation parameters, intestinal microbiota will be taken into account in the

future feeding and feed design. With the new technologies and know-how, it will be possible to estimate the microbial compositions that the different kinds of feeds and feed materials will bring about, and to modulate the microbiota effectively in order to enhance animal well-being.

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References

APAJALAHTI, J., KETTUNEN, A. and GRAHAM, H. (2004) Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. *World's Poultry Science Journal* **60**: 223-232.

BÄCKHED, F., LEY, R.E., SONNENBURG, J.L., PETERSON, D.A. and GORDON, J.I. (2005) Host-bacterial mutualism in the human intestine. *Science* **307**: 1915-1920.

CONWAY, P.L. (1997) Development of intestinal microbiota. In: Mackie, R.I., White, B.A. and Isaacson, R.E. (Eds.), Gastrointestinal Microbiology, Volume 2. Chapman & Hall Microbiology Series, New York, p. 3-38.

DIBNER, J.J. and RICHARDS, J.D. (2005) Antibiotic growth promoters in agriculture: history and mode of action. *Poultry Science* **84**: 634-643.

DORÉ, J., SGHIR, A., HANNEQUART-GRAMET, G., CORTHIER, G. and POCHART, P. (1998) Design and evaluation of a 16S rRNA-targeted oligonucleotide probe for specific detection and quantitation of human faecal *Bacteroides* populations. *Systemic and Applied Microbiology* **21**: 65-71.

HOOPER, L.V. and GORDON, J.I. (2001) Commensal host-bacterial relationships in the gut. *Science* **292**: 1115-1118.

HOOPER, L.V., MIDTVEDT, T. and GORDON, J.I. (2002) How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annual Review of Nutrition* **22**: 283-307.

LANGENDIJK, P.S., SCHUT, F., JANSEN, G.J., RAANGS, G.C., KAMPHUIS, G.R., WILKINSON, M.H. and WELLING, G.W. (1995) Quantitative fluorescence *in situ* hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. *Applied and Environmental Microbiology* **61**: 3069-3075.

LAY, C., SUTREN, M., ROCHET, V., SAUNIER, K., DORÉ, J. and RIGOTTIER-GOIS, L. (2005) Design and validation of 16S rRNA probes to enumerate members of the *Clostridium leptum* subgroup in human faecal microbiota. *Environmental Microbiology* **7**: 933-946.

LU, J., IDRIS, U., HARMON, B., HOFACRE, C., MAURER, J.J. and LEE, M.D. (2003) Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. *Applied and Environmental Microbiology* **69**: 6816-6824.

SGHIR, A., GRAMET, G., SUAU, A., ROCHET, V., POCHHART, P. and DORÉ, J. (2000) Quantification of bacterial groups within human fecal flora by oligonucleotide hybridization. *Applied and Environmental Microbiology* **66**: 2263-2266.

SUAU, A., ROCHET, V., SGHIR, A., GRAMET, G., BREWAEYS, S., SUTREN, M., RIGOTTIER-GOIS, L., and DORÉ, J. (2001) *Fusobacterium prausnitzii* and related species represent a dominant group within the human fecal flora. *Systematic and Applied Microbiology* **24**: 139-145.

UMESAKI, Y. and SETOYAMA, H. (2000) Structure of the intestinal flora responsible for development of the gut immune system in a rodent model. *Microbes and Infection* **2**: 1343-1351.

VAAHTOVUO, J., KORKEAMÄKI, M., MUNUKKA, E., VILJANEN, M.K. and TOIVANEN, P. (2005) Quantification of bacteria in human feces using 16S rRNA-hybridization, DNA-staining and flow cytometry. *Journal of Microbiological Methods* **63**: 276-286.

VAAHTOVUO, J., KORKEAMÄKI, M., MUNUKKA, E., HÄMEENOJA, P. and VUORENMAA, J. (2006) Microbial Balance Index – a view on the intestinal microbiota. *Livestock Science*, submitted.

VERSTEGEN, M.W.A., LAN, Y., TAMMINGA, S. and WILLIAMS, B.A. (2005) The role of the commensal gut microbial community in broiler chickens. *World's Poultry Science Journal* **61**: 95-104.

ZHU, X.Y. and JOERGER, R.D. (2003) Composition of microbiota in content and mucus from cecae of broiler chickens as measured by fluorescent *in situ* hybridization with group-specific, 16S rRNA-targeted oligonucleotide probes. *Poultry Science* **82**: 1242-1249.

ZOETENDAL, E.G., COLLIER, C.T., KOIKE, S., MACKIE, R.I. and GASKINS, R.H. (2004) Molecular ecological analysis of the gastrointestinal microbiota: a review. *Journal of Nutrition* **134**: 465-472.