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SEGMENTED FILAMENTOUS BACTERIA: KEY DRIVERS OF THE MUCOSAL IMMUNE MATURATION

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SUMMARY

Association of germ-free mice with a complete intestinal microbiota promotes the development of the mucosal immune system. *Clostridium*-related, segmented filamentous bacteria (SFB) are non-cultivable, commensal bacteria that strongly adhere to the epithelial cells of the ileum and of Peyer's patches at which time they competitively inhibit adhering pathogens. Colonizing SFB induce IgA-producing cells in the lamina propria of the small intestine and αβ-T-cell-receptor bearing intra-epithelial lymphocytes. As a consequence of this immuno-activation, adherence of SFB declines in time. The level of immune activation exceeds that of other non-pathogenic bacterial strains or non-host microbiota. In conclusion, SFB are key players in the maturation of the murine immune system of the gut. In this review, taxonomic data, interactions with the host and its immune system, and factors influencing colonization are highlighted.

INTRODUCTION

The bacterial community in the mammalian gastrointestinal tract comprises an estimated several hundreds of different species and as many as 10^{11}-10^{12} bacteria per gram of content in the colon. Many species are difficult or as yet impossible to culture, and it is only recently that techniques are available to describe the complex intestinal microbial ecosystem by molecular techniques without the need to culture bacteria (*Vaughan* et al., 2000; *Dethlefsen* et al., 2008).

Bacterial colonization in the small intestine is far lower than in the colon, and in major part concentrated to the terminal part the ileum. So-called Segmented Filamentous Bacteria (SFB) belong to the most remarkable bacterial species in the ileum. These bacteria are defined on the basis of their morphology and habitat: Long chains ('filaments') of spore-forming bacterial cells ('segments') that are attached with one end of the filament to the intestinal wall (Figure 1). In mammals, they preferentially adhere to the epithelial cells of the ileum and of Peyer's patches, small lymphoid organs involved in antigen sampling from the intestinal lumen. Despite intense association with the gut wall, SFB generally do not possess pathogenic characteristics.

Because of the intimate relationship of SFB with the host and non-pathogenic nature, it has been speculated for years that they might increase host resistance against intestinal infections (*Glick* et al., 1978; *Porvaznik* et al.,

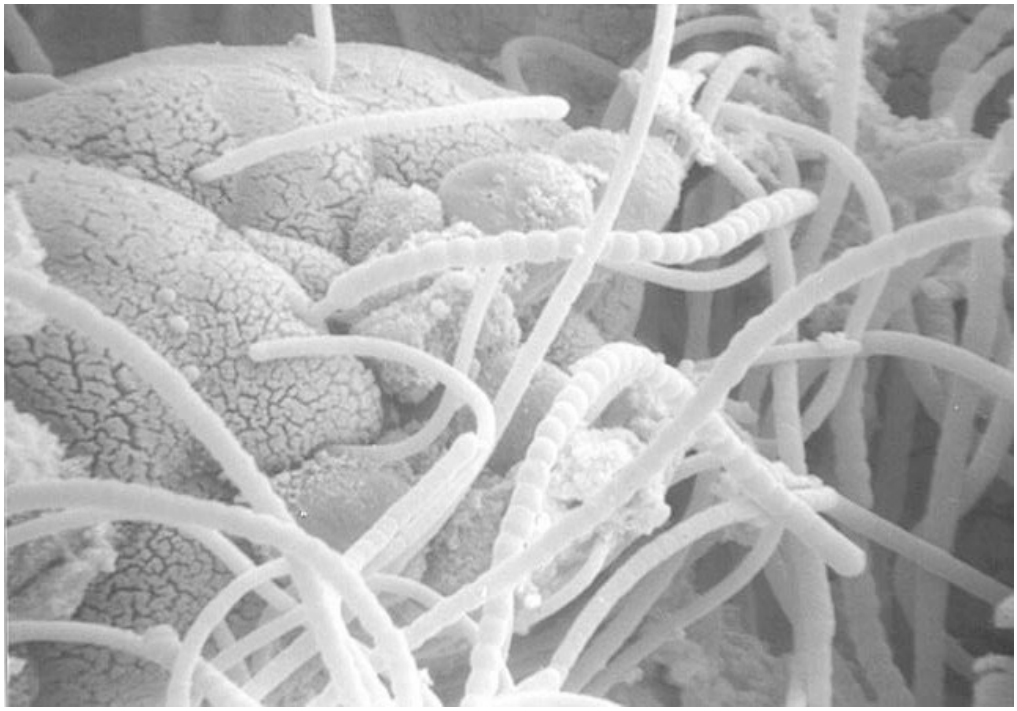

Figure 1: Scanning electron microscopic image of segmented filamentous bacteria in mice. Filaments are attached to epithelial cells without any signs of inflammation. Two morphotypes can be distinguished: smooth filaments and filaments with a beaded appearance.

1979). Several studies have demonstrated an important role for these bacteria in the maturation of the mucosal immune system. SFB are recognized to strongly stimulate IgA production (*Klaasen* et al., 1993a; *Snel* et al., 1997), and intra-epithelial lymphocytes (*Umesaki* et al., 1995) in the small intestine when germ-free mice become colonized with these bacteria.

PHYLOGENY AND TAXONOMIC STATUS

Intestinal spore-forming bacteria with a segmented filamentous appearance that attach to the intestinal wall were originally described in mice (*Hampton* and *Rosario*, 1965) and chickens (*Fuller* and *Turvey*, 1971), but have now been found in a wide range of animals (Table 1). Although never cultured *in vitro*, monocultures of SFB have been established by using intestinal homogenates of donor mice that were treated with filtered ethanol, diluted and administered intra-ileally to recipient germ-free mice (*Klaasen* et al., 1991a; *Umesaki* et al., 1995). These cultures have been used to determine the 16S ribosomal RNA sequence of SFB and for subsequent phylogenetic analysis (*Snel* et al., 1994).

Analysis of the available ribosomal RNA sequences from SFB in mice, rats and chicken revealed that these bacteria form a natural group, which is peripherally related to the genus *Clostridium* sensu stricto (group I *Clostridium*) (*Snel* et al., 1995). Later de

Table 1: Overview of animal species in which intestinal spore-forming bacteria with a segmented filamentous appearance that attach to the intestinal wall have been observed

Species	Site	Adhesion and morphology revealed by microscopy	16S rRNA sequence data described
Mice	ileum	fluorescent *in situ* hybridisation using SFB- specific probe (*Snel* et al., 1994); scanning electron microscopy (*Hampton* and *Rosario*, 1965; *Davis* and *Savage*, 1974)	*Snel* et al., 1994, 1995; *Imaoka* et al., 1997
Rats	ileum	(*Tannock* et al., 1984)	*Snel* et al., 1995; *Imaoka* et al., 1997
Chicken	ileum, proximal caecum	(*Fuller* and *Turvey*, 1971; *Pearson* et al., 1982; *Angel* et al., 1990; *Allen*, 1992)	*Snel* et al., 1995; *Gong* et al., 2007
Dog	ileum	scanning electron microscopy (*Davis* et al., 1977; *Hoskins* et al., 1982; *Klaasen* et al., 1993b)	
Cat	ileum	scanning electron microscopy (*Gregory* et al., 1985)	
Rabbit	ileum	scanning electron microscopy (*Heczko* et al., 2000)	
Sheep	ileum	scanning electron microscopy (*Gregory* et al., 1985)	
Rainbow trout	distal intestine	fluorescent *in situ* hybridisation using SFB- specific probe (*Urdaci* et al., 2001)	*Urdaci* et al., 2001
Carp	small intestine	light microscopy (*Klaasen* et al., 1993b)	
Horse	ileum	scanning electron microscopy (*Lowden* and *Heath*, 1995)	
Pig	ileum	*Sanford*, 1991	
Crab-eating monkey	ileum and caecum	light microscopy (*Klaasen* et al., 1993b)	*Imaoka* et al., 1997
Rhesus monkey	ileum	light microscopy (*Klaasen* et al., 1993b)	
Vervet monkey	ileum	scanning electron microscopy (*Bruorton* et al., 1991)	
Humans	faeces, ileum	light microscopy (*Klaasen* et al., 1993b)	hybridization with SFB-specific probe (*Child* et al., 2006)

rived sequences from rainbow trout (*Urdaci* et al., 2001) and crab-eating monkeys (*Imaoka* et al., 1997) fall in the same group. Since these bacteria cannot be cultured, they are not submitted to any culture collection, and can only be described on the basis of their phylogeny and morphology. Therefore, as a provisional genus name, "*Candidatus* Arthromitus" was proposed (*Snel* et al., 1995).

SFB are host specific: Ileal bacterial preparations containing SFB did only lead to outgrowth in ex-germ-free mice and rats when they where derived from the same species (*Tannock* et al.,

1984). Also faecal preparations from SFB-mono-associated mice did rarely lead to attachment to the ileum of rats, although colonization of the caecal content was observed (unpublished data). Because of this host specificity, species names "*Candidatus* Arthromitus muris", "*Candidatus* Arthromitus ratti" and "*Candidatus* Arthromitus galli" were suggested for SFB in mice, rats and chickens respectively (*Snel* et al., 1995)

A few studies have described SFB-like microorganisms in invertebrate species, such as the hindgut of termites (*Margulis* et al., 1990) and cockroaches (*Bracke* et al., 1979). Nevertheless, later studies revealed that the segmented, filamentous microorganisms in these species were in fact *Bacillus cereus* (*Margulis* et al., 1998). The authors concluded that *B. cereus* and its close relatives, easily isolated from soil and grown on nutrient agar, enjoy filamentous growth in moist, nutrient-rich intestines of healthy arthropods and similar habitats. This illustrates that firm conclusions on the presence of SFB can only be drawn when genetic information such as ribosomal RNA sequence data are available together with microscopic evaluation.

The wide range of vertebrate animal species in which SFB is found (Table 1), including birds, fish and mammals, suggest that bacteria from this group are present in humans too. Although an SFB-specific 16S ribosomal RNA targeted probe SFB did hybridize with bacteria in a continuous culture model inoculated with human faeces (*Child* et al., 2006), and long filamentous organisms are observed by light microscopy in human ileal biopsies (*Klaasen* et al., 1993b), conclusive evidence that SFB form a natural part of the intestinal microbiota in humans is still lacking.

MICROSCOPIC OBSERVATIONS

As being related to *Clostridium*, SFB are endospore-forming microorganisms (*Chase* and *Erlandsen*, 1976). As expected for sporeformers, SFB are resistant to treatment with chloroform or ethanol, but surprisingly sensitive to temperatures above 55°C. Microscopic analysis of SFB revealed that its spores contain two daughter cells instead of one as observed in most spore-forming species. Multiple daughter cells in single spores have also been reported in *Metabacterium* and *Epulopiscium* species (*Angert* et al., 1996). Although *Metabacterium polyspora* is a common member in the guinea pig caecal microbiota, it is like *Epulopiscium* spec. not directly related to SFB.

Filaments of SFB in mice are either smooth or have a beaded appearance. One end of the filament is attached to the epithelial wall with a special structure, described as holdfast (Figure 2). A life cycle is proposed in which spores release holdfasts that subsequently adhere to the ileum and grow out to filaments of 50-1000 μm (*Klaasen* et al., 1992a).

Several electron microscopic studies in mice describe Gram-positive rod-shaped bacteria adhering to the SFB filaments in mice (*Koopman* et al., 1987; *Klaasen* et al., 1992a). The exact nature of these bacteria is unknown, although it is speculated that they may be *Lactobacillus* species (*Koopman* et al., 1987). Similar sub-colonization of SFB by rod-shaped bacteria is found in chickens (unpublished data).

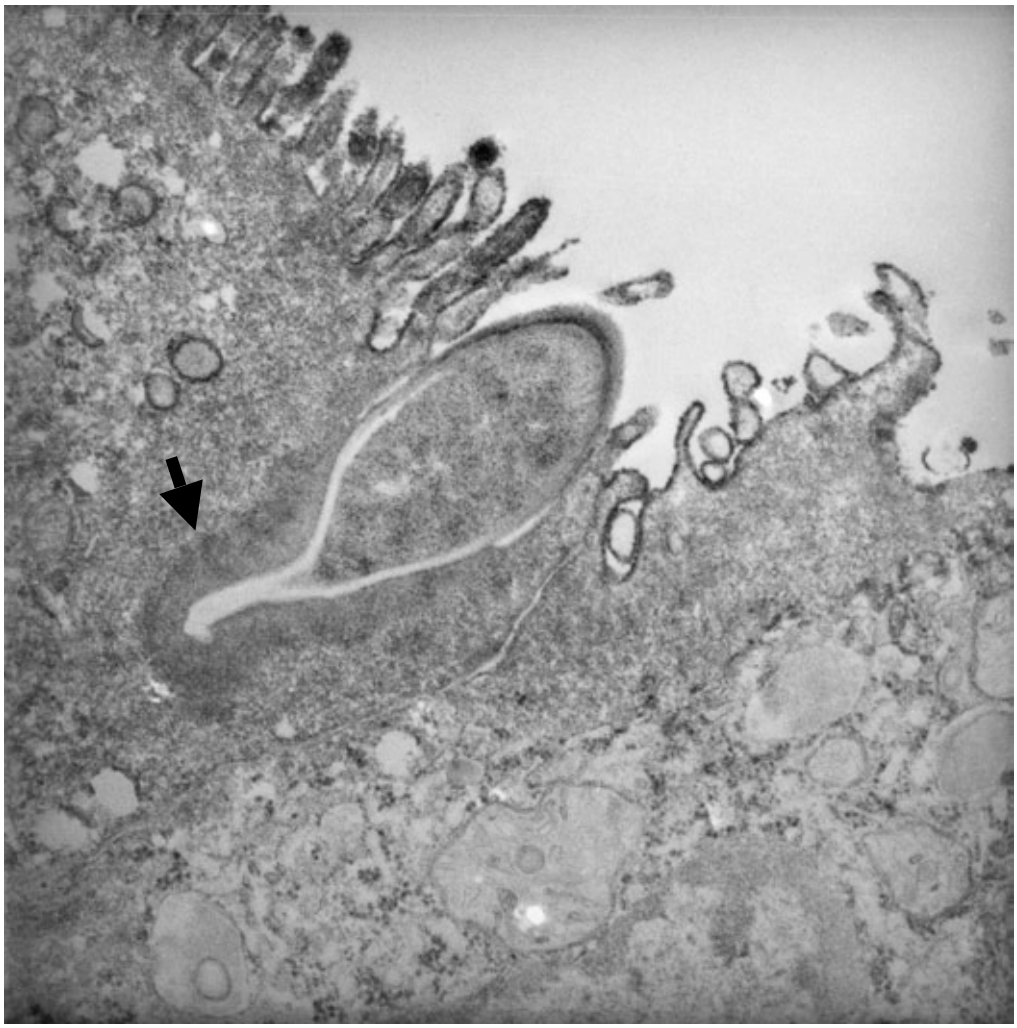

Figure 2. Transmission electron microscopic image of an epithelial cell containing an SFB holdfast. The brush border membrane of the epithelial cell is unaffected. The host cell has responded by accumulation of actin at the site of attachment (arrow).

ATTACHMENT AND EPITHELIAL RESPONSES

SFB adhere to the epithelial cells of the ileum with a special structure described as holdfast (Figure 2). The shape of this holdfast can vary from bean-, teardrop, to bulb-shaped (*Blumershine* and *Savage*, 1978). Using transmission electron microscopy, it is demonstrated that attachment of SFB causes an invagination of the plasma membrane and displacement of the microvilli at the site of attachment. The host cell responds to adhesion with the accumulation of polymerized actin, similar as seen after attachment of enteropathogenic *Escherichia coli*. In contrast to infection with pathogenic bacteria, the microvilli of the brush border remain intact (*Jepson* et al., 1993).

Attachment is not restricted to regular epithelial cells of the ileum:

SFB also adhere to the follicle associated epithelium of Peyer's patches in the ileum, specialized lymphoid organs as part of the mucosal immune system (*Jepson* et al., 1993; *Snel* et al., 1998). Within the follicle-associated epithelium, membranous cells (M-cells) are involved in the continuous sampling of antigens from the lumen. Although rarely seen, SFB are capable to adhere to M-cells of mice (*Jepson* et al., 1993; *Meyerholz* et al., 2002), and even extend from an M-cell into intimate association with an intra-epithelial mononuclear cell (*Meyerholz* et al., 2002).

Epithelial gene expression was examined by microarrays and compared to gene expression induced by the probiotic strains *Lactobacillus casei* Shirota and *Bifidobacterium breve* Yakult after 3 days of mono-association of germfree mice (*Shima* et al., 2008). Most pronounced effects were found in the ileum where SFB far more than the two probiotics differentially expressed 942 genes (478 more than 2-fold upregulated and 464 more than 2-fold downregulated compared to germ-free animals) versus 362 for *Lactobacillus* (183 up and 179 down) and 264 for *Bifidobacterium* (75 up and 189 down). Surprisingly, the overlap in differentially expressed genes by these 3 strains was limited. It was found that the many of the upregulated genes belonged to the functional categories cell communication, defence and immunity, metabolism, and transport.

In ex-germ-free mice associated with SFB, fucosylation of asialo GM1 glycolipid occur in the small intestinal epithelial cells (*Umesaki* et al., 1995). Using the genomics approach, it was demonstrated that in particular, alpha(1-2) fucosyltransferase was induced in the gut epithelium after mono-association with SFB, but not after mono-association with a *Lactobacillus* or *Bifidobacterium* strain (*Shima* et al., 2008). Another interesting gene induced by SFB is pancreatitis-associated protein (PAP or RegIIIγ). This gene encodes a C-type lectin, and is even found to be induced after mono-association of germ-free severe combined immunodeficient (SCID) mice with SFB as an innate response to microbial colonization (*Keilbaugh* et al., 2005). A recent study described antimicrobial activity of the gene product, and suggested a role in gut homeostasis in order to maintain symbiotic host-microbe interactions (*Cash* et al., 2006).

Host epithelial cells in germ-free mice are known for their low expression of major histocompatibility complex II (MHC-II) molecules on the apical surface which is rapidly induced after conventionalization with a complete microbiota. After attachment, SFB can be phagocytised into the epithelial cells of the ileum and intracellularly processed by heterophagy (*Yamauchi* and *Snel*, 2000). Mono-association with SFB results in expression of MHC-II, but this is not seen after mono-association with related spore-forming bacteria from the genus Clostridium (*Umesaki* et al., 1995, 1999).

ROLE OF SFB IN HOST RESISTANCE

Several studies have suggested that SFB may increase the resistance of the host to infectious diseases. Newborns acquire adaptive and innate immunity through maternal sources, either via the transplacental route before birth or via the milk after birth. This process, referred to as passive immunity, provides a number of defence factors such as immunoglobulins, lactoferrin, lyso-

zyme, cytokines, and chemokines. In mice it is shown that the immune status of the dam also influence the development of the systemic and mucosal adaptive immune system of newborn animal (*Kramer* and *Cebra*, 1995). At this stage, the role of SFB is limited since they are not part of the intestinal microbiota of suckling mice.

During the weaning phase, high levels of SFB can be observed. Colonization of mice starts at about 3 weeks of age of the animals, at which the animals shift their diets from milk to solid food (*Blumershine* and *Savage*, 1978; *Koopman* et al., 1987). The high colonization level of SFB in weaning animals may competitively lead to reduced colonization levels of food-borne pathogens. Indeed, such an effect was observed in rats orally infected with *Salmonella enteritidis* (*Garland* et al., 1982). Here, a reduction of surface colonization by these pathogens was found in the presence of SFB. However, in another study, using either *S. enteritidis* or *Enterobacter cloacae* as the challenging microorganisms, the presence of SFB did not lead to significantly reduced translocation of pathogens (*Klaasen* et al., 1992b)

It is known that SFB are only abundantly colonizing the epithelium shortly after weaning in mice (*Davis* and *Savage*, 1974; *Klaasen* et al., 1992a; *Snel* et al., 1998) and for about 10 days after hatching in chicks (*Yamauchi* et al., 1990). During this period, the mucosal immune system is strongly stimulated, resulting in the induction of high levels of IgA plasma cells in the gut lamina propria and secretory IgA in gut secretions (*Klaasen* et al., 1993a), and αβ-T-cell-receptor bearing intra-epithelial lymphocytes (*Umesaki* et al., 1995). Since SFB colonization is temporary abundant in immunocompetent mice, whereas they persist in athymic nude mice (*Snel* et al., 1998) as well as in IgA-deficient mice (*Suzuki* et al., 2004), it is strongly suggested that it is the induced immune response leads to self-limiting colonization levels. A self-limiting response has been reported for translocation of the Gram-negative bacterium *Morganella morganii*, the number of translocating bacteria begins to drop with the onset of a specific IgA response while colonization of the intestinal lumen is unaffected (*Shroff* et al., 1995). The induction off IgA and other components of the mucosal immune system contribute to enhanced resistance to salmonella in mice. Young adult mice infected with *S. typhimurium* had a prolonged survival when mono-colonized with SFB compared to germ-free mice. Prolonged survival was not seen in mice mono-associated with *Clostridium innocuum* (unpublished data).

The influence of SFB on resistance to enteropathogens because of its high bacterial density during weaning and stimulation of the immune response post-weaning is schematically represented in Figure 3.

FACTORS INFLUENCING SFB COLONIZATION

Because of the role of SFB in maturation of the host immune system during the weaning period, it is of importance to understand factors either stimulating or suppressing the presence of these bacteria in the gut.

A few studies focussed on antimicrobial drugs in relation to SFB colonization. These studies are hampered by the inability to culture these bacteria *in vitro*. Penicillin was the first drug for which sensitivity of SFB was demon

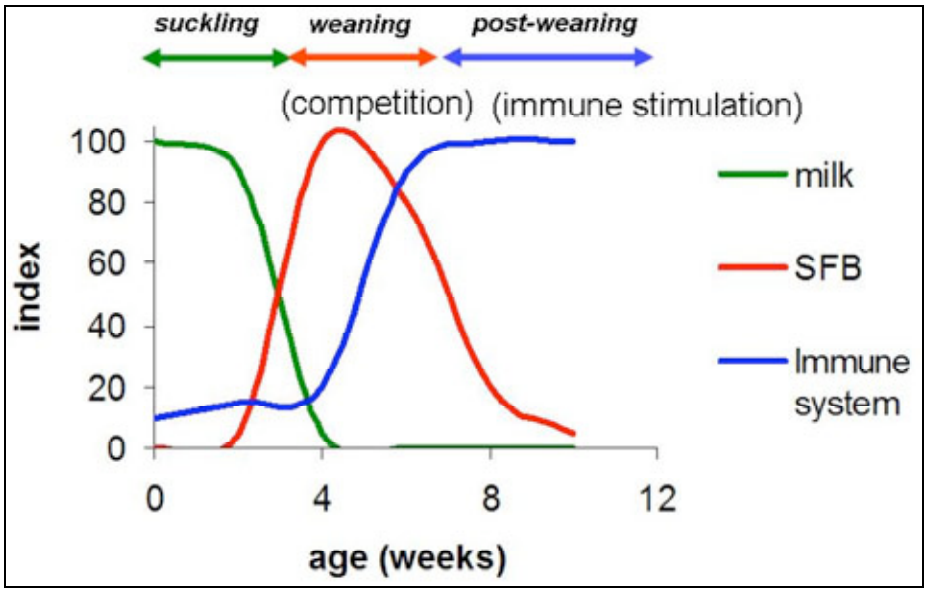

Figure 3. Schematic overview of the defence against enteropathogenic microorganisms. SFB contributes to competitive exclusion by high colonization levels during weaning and stimulates immune maturation after weaning.

strated (*Davis* and *Savage*, 1976). Only one study attempted to investigate antibiotic sensitivity of SFB in a systematic way and demonstrated that these bacteria were sensitive to amoxicillin, doxycyclin, ciprofloxacin, clindamycin, streptomycin, and cefotaxim when added to the drinking water (*Klaasen* et al., 1991b). Colonization was studied by microscopic evaluation of ileal scrapings of conventional animals, whereas at present mono-associated mice would have been available for these studies (*Klaasen* et al., 1991a). It has not been demonstrated whether antibiotic treatment of mono-associated animals would prevent maturation of the immune system. In conventional animals, treatment of mice with selective antibiotics is known to inhibit Th17 cell differentiation in the lamina propria and is accompanied by increase in Foxp3+ regulatory T cells (*Ivanov* et al., 2008).

Information on dietary influences of SFB colonization is scarce. In mice, the composition of the diet is a factor creating significant differences in SFB colonization. Nevertheless, an attempt to identify key dietary components by using purified diets failed since SFB colonization was completely inhibited (*Klaasen* et al., 1992c). So far, effects of macronutrients and their influence on SFB colonization are not systematically investigated.

Addition of red kidney beans (*Phaseolus vulgaris*) to the diet of 6-7 wk old mice stimulated colonization of SFB (*Klaasen* et al., 1991c, 1992c). Although phytohaemagglutinin (PHA) from red kidney beans is known to stimulate overgrowth of *Escherichia coli* (*Pusztai* et al., 1993), it is not likely that this lectin is responsible for the effect since also boiled red kidney beans, in which PHA was inactivated, led to the observed stimulation (*Klaasen* et al., 1991c). Besides, no increase of SFB was reported in a study in which purified PHA was added to the diet (*Banwell* et al., 1985).

CONCLUSIONS

Segmented filamentous bacteria are a group of bacteria that are found in the ileum of several mammals, birds and fish. Here they have a profound effect on maturation of the immune system. This does not only affect colonization of SFB itself, but also that of pathogenic microorganisms such as *Salmonella* and *E. coli*.

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