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Host discriminates between probiotics and pathogens: impact of toll like receptor 5-flagellin interaction and evolution

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Abstract: [Objective] Data from ample studies support the idea that the immune homeostasis is crucially dependent on a cross-talk between host immune system and enteric flora in which the host recognizes and responses distinctively to probiotic and pathogenic bacteria. The toll-like receptors (TLRs) and microorganism associated molecular patterns (MAMPs) may play a major role in the host discrimination between probiotics and pathogens, as the recognition of MAMPs by TLRs can activate innate immune response and prime the adaptive immune system. In the TLRs family, TLR5 that responds to flagellin is the only protein-binding TLR, it's much easier to study the flagellin-TLR5 interaction structurally and functionally. The overall aim of this study was to test for a possible contribution of the flagellin-TLR5 crosstalk to the host discrimination between probiotic and pathogenic bacteria. **[Methods]** Using flagellin protein sequences from probiotic and pathogenic bacteria living in gastrointestinal tract, we firstly constructed a phylogenetic tree of flagellin proteins and then aligned the flagellin protein sequences in TLR5 recognition region between probiotic and pathogenic bacteria. **[Results]** We found that probiotic and pathogenic bacteria differed in flagellin protein sequence, particularly in the TLR5 recognition sites. **[Conclusion]** Acclimatization to TLR5 recognition may result in the different TLR5 recognition sites on flagellin between pathogens and probiotics. Moreover, previous studies show that stimulation of basolaterally expressed TLRs results in inflammatory response, but activation of apically expressed TLRs leads to inhibition of the proinflammatory response. Altogether, our findings provide preliminary but encouraging evidence for the existence of crosstalk between flagellin and TLR5 which may be one of the mechanisms for the host discrimination between probiotics and pathogens.

Keywords: Host-flora crosstalk, Flagellin, Toll-like receptor 5, Phylogenetic tree, Sequence alignment

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Toll 样受体 5 和鞭毛蛋白的相互作用影响宿主区分病原菌和益生菌

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摘 要: 【目的】大量的证据表明机体正常的免疫活动在很大程度上依赖于免疫系统和肠道菌群的相互作用，具体表现为免疫系统对病原菌进行免疫清除而对益生菌耐受。其中，免疫系统的 Toll 样受体(Toll-like receptors，TLRs)和来自肠道菌群的微生物相关的分子模型(Microorganism associated molecular patterns，MAMPs)被认为在宿主免疫系统对病原菌和益生菌的区分中发挥了重要作用，因为 TLRs 对 MAMPs 的识别能够激活先天性和获得性免疫反应。在 TLRs 对 MAMPs 的识别中，只有 TLR5 对细菌鞭毛蛋白的识别是基于蛋白-蛋白的相互作用，比较容易对其结合方式进行研究。因此，我们研究的主要目的就是要确定 TLR5 与鞭毛蛋白的相互作用是如何影响宿主区分病原菌和益生菌的。【方法】构建了多种肠道细菌(包括益生菌和病原菌)鞭毛蛋白的系统发育树，并比对了鞭毛蛋白的 TLR5 识别序列。【结果】发现病原菌和益生菌的鞭毛蛋白序列有所不同，尤其是 TLR5 结合并识别的鞭毛蛋白位点。【结论】病原菌和益生菌不同的鞭毛蛋白识别区域可能是鞭毛细菌适应 TLR5 识别下生存的结果，据此宿主能够对病原菌和益生菌进行区分。此外，相关研究表明 TLRs 在肠上皮细胞的分布具有基底侧和顶端的两极性，能够分别引发对病原菌的免疫反应和对益生菌的免疫耐受，从而抵御病原菌的入侵感染、与益生菌和平共处。鞭毛蛋白和 TLR5 蛋白的相互作用反映了肠道菌群和免疫系统在分子层面的相互作用和共同进化，是宿主区分病原菌和益生菌的分子机制之一。

关键词: 宿主-肠道菌群相互作用，鞭毛蛋白，Toll 样受体 5，系统发育树，蛋白序列比对

1 Introduction

Accumulating data emphasize the importance of the cross-talk between host and enteric flora in the fields of immunology, neuroendocrine and neurology, but the mechanisms by which host discriminates between probiotic and pathogenic bacteria remain unclear. How can host recognize the harmful pathogens and launch a defense response, and how can probiotic bacteria transmit their benefits to the host? Microorganism associated molecular patterns (MAMPs) and toll-like receptors (TLRs) expressing on gastrointestinal epithelium and immune cells may be the key factors involved in the host-flora crosstalk, as TLR recognition by MAMP initiates innate immune response and primes the adaptive immune system. TLRs are an important family of innate immune receptors that play a key role in recognizing enteric flora by binding with MAMPs, evolutionarily conserved structures that are required for microbial fitness and are not present in the host[1-2]. There are primarily six families of vertebrate TLRs, including TLR2 family (TLR1, TLR2, TLR6 and TLR10), TLR3 family, TLR4 family, TLR5 family, TLR7 family (TLR7–TLR9), and TLR11 family (TLR11–TLR13)[3]. Each TLR family recognizes and responds to a different set of MAMPs with a distinct ligand-binding mechanism[4]. TLR4 binds lipopolysaccharide (LPS) of gram-negative bacteria, TLR2 family recognizes peptidoglycan from cell wall of gram-positive bacteria, TLR5 detects flagellin monomer of both gram-negative and gram-positive bacteria, and TLR7 family binds nucleic acid and heme motifs[5].

TLR5 is the only protein-binding TLR. Flagellin,

the monomer protein of bacterial flagellum, is demonstrated to be recognized by TLR5[6-7]. Compared with other MAMPs which are complex molecules of carbohydrates or lipids, flagellin is a protein so it is much easier to study its contact with TLR5 structurally and functionally[8]. The fact that the recognition of flagellated bacteria depends on the amino acids contact between flagellin and TLR5 suggests the host discrimination may be attributed to the difference in flagellin protein sequences between probiotic and pathogenic bacteria, most likely in the TLR5 recognition region. In support of this proposition, gastrointestinal ε Proteobacteria pathogens, such as *Helicobacteria pylori* and *Campylobacter jejuni*, that mutate in TLR5 recognition residues on flagellin have been found to evade TLR5 recognition and reduce immune activation[9-11].

On the other hand, TLR5 may also contribute to the host discrimination between probiotics and pathogens. The flagellin-TLR5 interaction possibly forms in the long history of co-evolution between immune system and enteric flora, as the immune tolerance to enteric flora plays a crucial role in speciation[12]. The aim of this study was to assess the impact of flagellin-TLR5 interaction on host discrimination between probiotics and pathogens. This is achieved by constructing phylogenetic tree of flagellin protein sequences, sequence alignment of flagellin proteins between probiotic and pathogenic bacteria, and clarify the possible mechanism by which TLRs discriminately respond to pathogens and probiotics.

2 Materials and methods

2.1 The protein sequences of flagellin

Protein sequences of flagellin were taken from the NCBI database. Flagellin protein sequences from wide range of probiotic and pathogenic flagellated bacteria living in gastrointestinal tract were used in the current study.

2.2 The phylogenetic tree of flagellin protein sequences

The phylogenetic tree of flagellin protein sequences was constructed by using ClustalX and displayed with Molecular Evolutionary Genetics Analysis software (MEGA 5.0B6.1).

2.3 The sequence alignment of flagellin proteins in recognition region

In order to verify whether the TLR5 recognition amino acids on flagellin differ between probiotics and pathogens, the conserved recognition sites should be firstly identified. The crystal structure of zebra fish TLR5 (zTLR5) in complex with D1/D2/D3 fragment of *Salmonella* flagellin (FliC) has recently been solved[13]. The conserved zTLR5-FliC binding sites should be the conserved recognition sites on TLR5 and flagellin, since TLR5 is highly conserved in vertebrate from fish to mammals[6,14-15] and TLR5 recognizes conserved sites on flagellin[11]. Therefore, we investigated the differences in the TLR5 recognition sites on flagellins between probiotics and pathogens following phylogenetic tree construction and protein sequences alignment.

3 Results

3.1 The phylogenetic tree of flagellin protein sequences

As shown in the phylogenetic tree of flagellin proteins (Figure 1), these flagellated bacteria in gastrointestinal tract primarily clustered into three groups: β and γ clades of Proteobacteria, including most pathogenic Enterobacteriaceae like *Salmonella* and *Shigella* species; α and ε clades of Proteobacteria, composed of gastrointestinal pathogenic bacteria such as *Helicobacter* and *Campylobacter* species; clades of Spirochetes and Firmicutes, containing probiotic *Lactobacillus* and *Bacillus* species.

3.2 The sequence alignment of flagellin proteins

As marked in Figure 2, the TLR5 recognition sites on flagellin were highly conserved in β and γ Proteobacteria, but mutated in ε Proteobacteria and Firmicutes probiotics (*Lactobacillus* and *Bacillus*, et al). The mutations primarily located in interface B, a region from 86 aa to 118 aa on flagellin.

4 Discussion

4.1 Impact of TLR5 on flagellin evolution between probiotics and pathogens

The result that the flagellated bacteria clustered into three groups in phylogenetic tree of flagellin protein sequences indicated difference in flagellin protein sequences may exist among β & γ

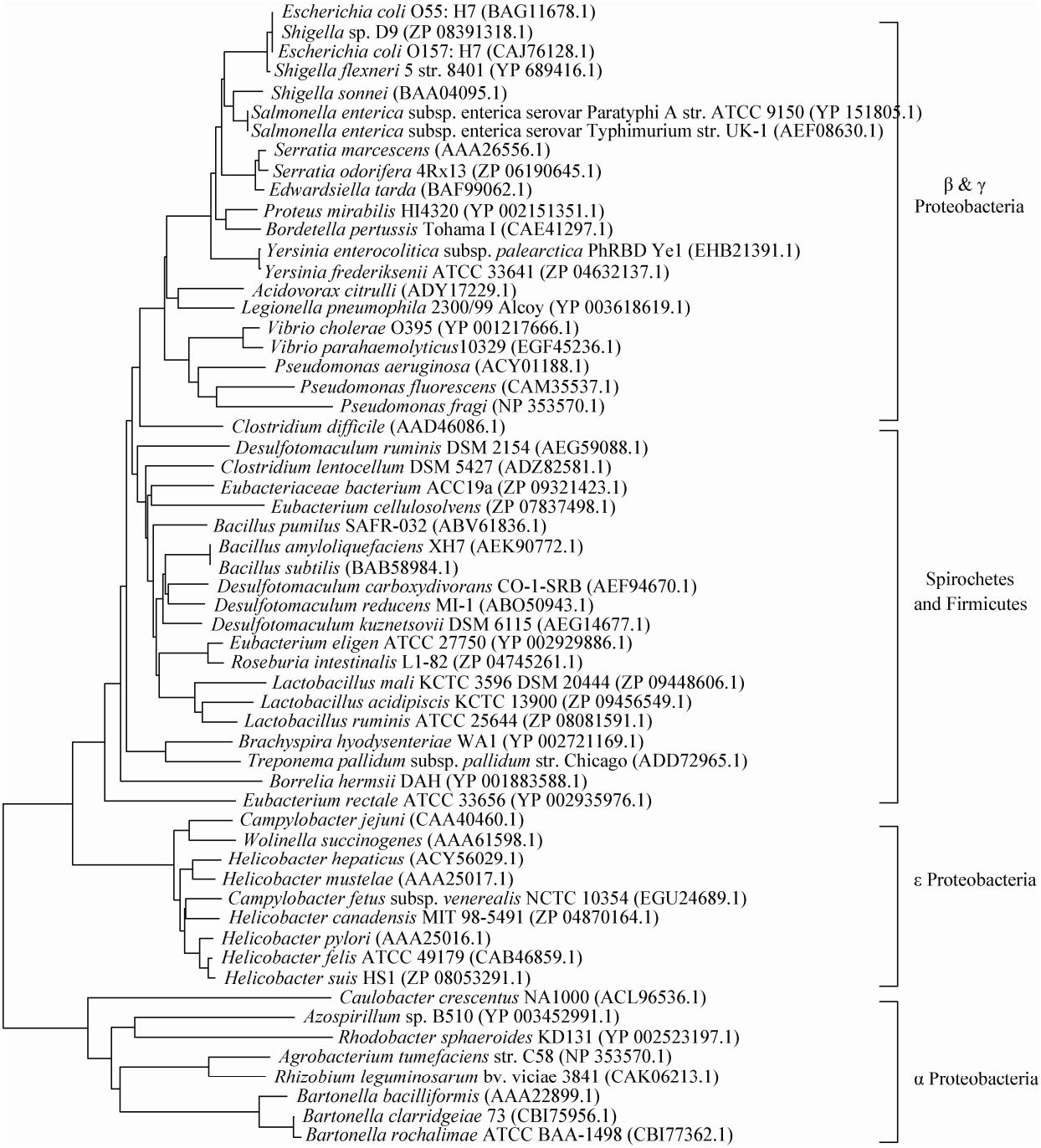

Figure 1 Phylogenetic tree of flagellin protein sequences from flagellated bacteria living in gastrointestinal tract

图 1 胃肠道细菌鞭毛蛋白的系统发育树

Note: The flagellin protein sequences are taken from NCBI database, constructed by using ClustalX and displayed with Molecular Evolutionary Genetics Analysis (MEGA 5.0B6.1). The flagellated bacteria clustered into three groups: β and γ clades of Proteobacteria; α and ε clades of Proteobacteria; and clades of Spirochetes and Firmicutes.

注: 细菌的鞭毛蛋白序列来自于 NCBI 数据库，经过 ClustalX 序列比对后用 MEGA 5.0B6.1 软件作出系统发育树. 根据鞭毛蛋白序列，图中的胃肠道鞭毛细菌主要分为 3 类：β 和 γ 变形菌门，α 和 ε 变形菌门，以及螺旋体和厚壁菌门.

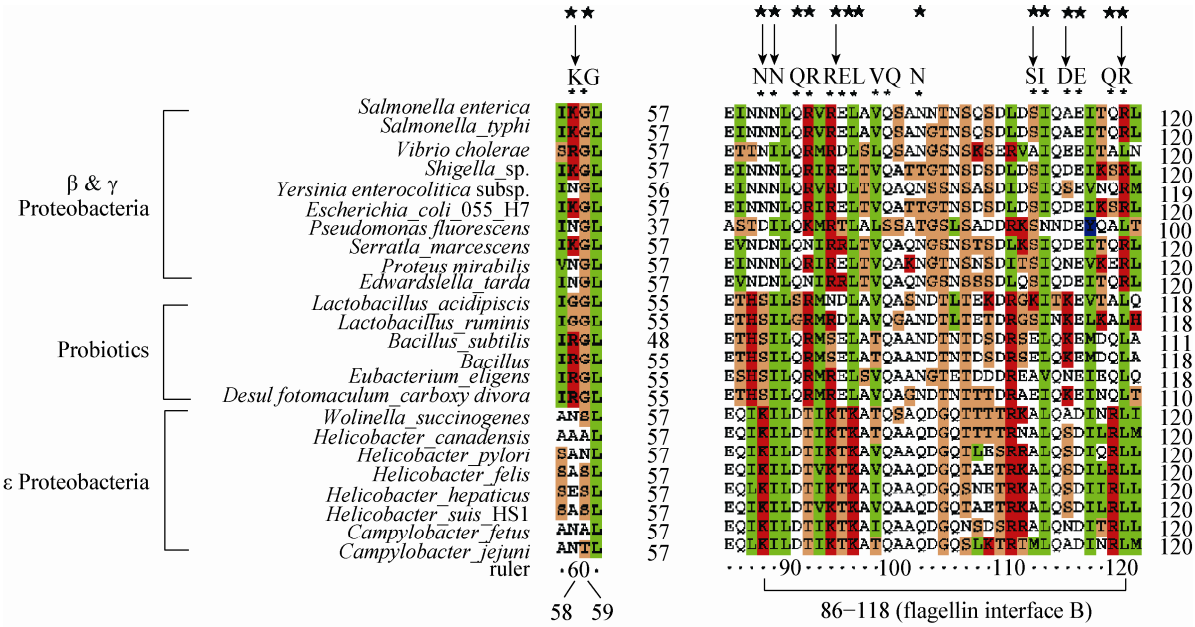

Figure 2 Sequence alignment (ClustalX) of flagellin proteins of probiotic bacteria with those of the pathogens in β & γ Proteobacteria and ε Proteobacteria

图 2 益生菌与 β & γ 和 ε 变形菌门菌(病原菌) 鞭毛蛋白的序列比对(ClustalX)

Note: The capital letters on the top are TLR5 recognition sites on flagellin which are identified in the crystal TLR5-FliC complex. These TLR5 recognition sites are highly conserved across β and γ Proteobacteria, but some sites are mutated in probiotics and ε Proteobacteria. Asterisks (*): TLR5 recognition sites that are conserved in β & γ Proteobacteria flagellin; Down arrow (↓): TLR5 recognition sites that are mutated in probiotic flagellins; Pentacle (★): TLR5 recognition sites that are mutated in ε Proteobacteria flagellins. The binding between 86−118 aa and the compensatory amino acids 58 aa and 59 aa on adjacent flagellin monomer is necessarily required for the flagellar protofilament formation and bacteria motility.

注: 图顶端的大写字母标示的是 TLR5 在鞭毛蛋白上的识别位点(来自于 TLR5-FliC 复合体的晶体结构). TLR5 的这些识别位点在 β 和 γ 变形菌门中高度保守,然而其中的某些位点在益生菌和 ε 变形菌门中发生了突变. 星号(*): β 和 γ 变形菌门鞭毛蛋白上 TLR5 识别的保守位点；向下箭头(↓)：益生菌鞭毛蛋白上 TLR5 识别的突变位点；五角星(★)：ε 变形菌门鞭毛蛋白上 TLR5 识别的突变位点. 细菌鞭毛纤维的形成和运动依赖于鞭毛蛋白 86−118 aa 与相邻鞭毛蛋白上 58 和 59 aa 的相互结合.

Proteobacteria, α & ε Proteobacteria and Firmicutes probiotics (Figure 1). In support of this, growing evidences suggest that flagellins from these three groups of bacteria induce distinct immune responses. In contrast to the well-documented TLR5-stimulatory β & γ Proteobacteria that trigger strong pro-inflammatory response[16-17], the ε Proteobacteria, such as *Helicobacter pylori*, have been observed to evade TLR5 recognition and reduce immune activation as a result of mutations in flagellin conserved amino acids sites[9,11]. Probiotics contributing to the health of the host are extensively tolerated by host's immune system, and their significant effects on immunomodulation and modulation of gut physiology are reported to be partly mediated through flagellin[18-20]. Take together, these data imply that the difference in flagellin protein sequences between probiotics and pathogens may contribute to the host discrimination between probiotics and pathogens by contacting with TLR5 to trigger distinct immune responses.

We further wanted to define the specific flagellin amino acids different between probiotics and pathogens. The symmetric 2:2 flagellin-TLR5 complex consists of two copies of 1:1 complex[13]. The 1:1 complex formation is mediated by the extensive primary binding interface (A, B, A' and B') and its homodimerization to the 2:2 complex is mediated by the secondary dimerization interface (α, α′, and β)[13]. Data from related study have shown that the N-terminal D0-D1 domain of flagellin is critically required for TLR5 agonist activity, since a

FliC (flagellin from *Salmonella typhimurium*) molecule containing the N-terminal D0-D1 domain of FlaA (flagellin from *H. pylori*) was completely inactive[11,21-22]. Then the researchers compared the flagellin N-terminal D0-D1 domain amino acid sequences from TLR5-stimulatory bacteria to bacteria that do not activate TLR5, and they found that the highest proportion of amino acid differences between TLR5-stimulatory and nonstimulatory bacteria was within the D1 domain[11].

The interface B (86 aa to 118 aa) is the only interface that locates in the highly conserved D1 domain of flagellin. Therefore, we mapped the flagellin sequence difference between probiotics and patogens to the TLR5 recognition sites on flagellin in interface B (Figure 2). The interface B is crucial for flagellar filament assembly and motility[8,11], indicating that TLR5 evolutionally detects a common structure in flagellin which is functionally crucial for bacteria motility and hence is evolutionarily conserved. Therefore, the requirement of motility for bacterial colonization and invasion in β and γ Proteobacteria reserves their TLR5 recognition sites on flagellin conserved and preserves their properties of activating pro-inflammatory response. Probiotics are less motile bacteria living peacefully on mucosal surface of intestine[18,23]. Nevertheless, the ε Proteobacteria are not recognized by TLR5 because of mutations in conserved recognition sites on flagellin, yet are highly motile. Mutagenesis studies have demonstrated that preservation of *H. pylori* motility is due to mutations in compensatory sites on the adjacent flagellin monomer, the 58 aa and 59 aa[11]. In our study, alignment results showed that the conserved K58 and G59 in β and γ Proteobacteria mutated to A58 and S59 in most ε Proteobacteria (Figure 2). Interestingly, we also found that probiotics here had G58 and G59 or R58 and G59 on flagellin, suggesting the two kinds of probiotics may have different motile abilities. In support of this, previous study has shown that compared to other *Lactobacillus* species, the *Lactobacillus ruminis* with G58 and G59 on flagellin is a higher motile one known to be autochthonous inhabitant in the mammalian intestine[23]. In the course of tens of thousands of years of co-existence with its human host, *H. pylori* has evolved elaborate adaptations that allow it to persist in the hostile environment of the stomach in the face of a vigorous innate and adaptive immune response[24]. Nonetheless, the host may have other strategies to recognize pathogenic bacteria. In addition to TLRs, a number of other PRRs, including nucleotide-binding oligomerization domain-like receptors (NODs), RIG-like helicases (RLHs) and dectins, are also involved in regulation of intestinal immune responses[25-27]. In support of this, date provide by ample evidences showed that NOD 1 can recognize an *H. pylori*-derived MAMP, i.e. its peptidoglycan[28-29]. $CD4^+$ MHC class II-restricted T-cells are showed to be required for the control of experimental *H. pylori* infections and for the development of vaccine-induced protective immunity[30-32].

In the long history of co-evolution between host's immune system and enteric flora, the selective force of TLR5 recognition may drive the development of specific flagellin sequence in β & γ Proteobacteria, ε Proteobacteria and probiotic bacteria, as the specificity of vertebrate TLR5 arises before Cambrian period allowing it has sufficient time to influence the evolution of flagellins on gut bacteria[33].

4.2 From structure to function: the interaction between TLRs and MAMPs in cellular levels

Activation of the TLRs by MAMPs sequentially activates intracellular molecules such as the cytoplasmic adapter molecule MyD88, leading to the activation of transcription factors, including NF-κB and activator protein-1 (AP-1), which are required for gene transcription and cytokine synthesis[25,34-36]. The different immune responses to pathogens and probiotics may be related to the polarized localization of TLRs on intestinal epithelial cells (IECs), since data have shown that the polarity of IECs has a major role in regulation of immune homeostasis[37-38]. Stimulation of basolaterally expressed TLRs results in inflammatory response via the NF-κB pathway, but activation of apically expressed TLRs leads to inhibition of the NF-κB pathway and does not cause secretion of the proinflammatory cytokines[39-40].

As the basolateral and apical TLRs responses are in line with the TLR-mediated immune responses to pathogenic and probiotic bacteria, it seems reasonable to hypothesize that the TLR expression in polarized IECs has uniquely evolved to efficiently

discriminate between probiotics and pathogens. Indeed, previous studies have revealed that the TLR polarized expression is relevant to the microbiota colonization[41]. Without ligands stimulation, TLR2 and TLR4 constitutively express in the apical pole of IECs, however, they traffic to cytoplasmic compartments near the basolateral membrane after stimulation with lipopolysaccharide (LPS) or peptidoglycan (PNG). What is more, ligands applied basolaterally induce more amount of TLR2 and TLR4 to locate in cytoplasmic compartments than ligand stimulation apically[41]. It consequently suggests that apical pole localization of TLRs is the optimal position to contact with non-invasive probiotics and monitor the enteric flora composition, and the redistribution of TLRs on basolateral membrane efficiently defenses pathogens penetrating the intestinal epithelia barrier.

Our results show that the different recognition sites on flagellin between probiotics and pathogens as well as the polarized localization of TLRs may contribute to host discrimination between probiotics and pathogens. These different TLR5 recognition sites on flagellin may form in a long history of co-evolution between host immune system and enteric flora in which intestinal microbiota evolve to survive under the selective force of immune recognition. The TLR5-flagellin interaction is one of the mechanisms for the host discrimination between probiotics and pathogens, and the compromising outcome of their crosstalk is crucially important for the maintenance of immune homeostasis. Furthermore, the information of specific TLR5 recognition sites on probiotic flagellin may become particularly useful if flagellated bacteria species are to be developed for probiotics, therapeutic, or health-care applications.

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