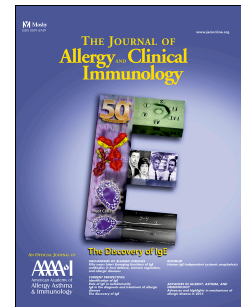


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D-Tryptophan from probiotic bacteria influences the gut microbiome and allergic airway disease

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**1 D-Tryptophan from probiotic bacteria influences the gut microbiome and allergic
2 airway disease**

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47 **Abstract (250/250 words)**

48 **Background** Chronic immune diseases (CIDs), such as asthma, are highly prevalent.
49 Currently available pharmaceuticals improve symptoms, but cannot cure the disease. This
50 prompted demands for alternatives to pharmaceuticals such as probiotics for prevention of
51 allergic disease. However, clinical trials have given inconsistent results. This is at least partly
52 explained by the highly complex crosstalk among probiotic bacteria, the host's microbiota,
53 and immune cells. The identification of a bioactive substance from probiotic bacteria could
54 circumvent this difficulty.

55 **Objective.** To identify and characterize a bioactive, probiotic metabolite for potential
56 prevention of allergic airway disease.

57 **Methods.** Probiotic supernatants were screened for their ability to concordantly lower the
58 constitutive CCL17 secretion of a human Hodgkin lymphoma cell line and prevent
59 upregulation of costimulatory molecules of LPS-stimulated human dendritic cells.

60 **Results.** Supernatants from 13 of 37 tested probiotic strains showed immunoactivity.
61 Bioassay-guided chromatographic fractionation of two supernatants according to polarity,
62 followed by total ion chromatograms and mass spectrometry, yielded $C_{11}H_{12}N_2O_2$ as
63 molecular formula of a bioactive substance. Proton nuclear magnetic resonance and
64 enantiomeric separation identified D-Tryptophan. In contrast, L-Tryptophan and eleven other
65 D-amino acids were inactive.

66 Feeding D-Tryptophan to mice prior to experimental asthma induction, increased numbers of
67 lung and gut regulatory T cells, lowered lung Th2 responses, and ameliorated allergic airway
68 inflammation and hyperresponsiveness. Allergic airway inflammation reduced the gut
69 microbial diversity, which was increased by D-Tryptophan.

70 **Conclusions.** D-Tryptophan is a newly identified product from probiotic bacteria. Our
71 findings support the concept that defined bacterial products might be exploited in novel
72 preventative strategies for CIDs.

73 **Key messages**

- 74 • D-Tryptophan is a newly identified immune modulatory probiotic substance
- 75 • When fed to mice, D-Tryptophan increased the gut microbial diversity and ameliorates
- 76 allergic airway disease
- 77 • While the biology of live probiotic bacteria is very complex, D-Tryptophan has a
- 78 provable mode of action that might be exploited for prevention or treatment of allergic
- 79 diseases.

80

81 **Capsule summary**

82 The complexity of the host-bacterial crosstalk questions therapeutic approaches with live
83 bacteria. By showing that the probiotic substance D-Tryptophan improves airway
84 inflammation, we provide proof-of-principle that defined microbial products might be
85 exploited for therapeutic purposes.

86

87 **Keywords:** D-Tryptophan, probiotic bacteria, bacterial substance, screening, immune
88 modulation, allergic airway disease, gut microbiota

89

90 **List of abbreviations**

91 AAI	Allergic airway disease
92 BALF	Bronchoalveolar lavage fluid
93 CBA	Cytometric Bead Array
94 CCL17	Chemokine ligand 17 (TARC: thymus and activation regulated chemokine)
95 FT-ICR	Fourier transform ion cyclotron resonance
96 LGG	<i>Lactobacillus rhamnosus GG</i>
97 MS	Mass spectrometry
98 PFP	Pentafluorophenyl

99 TIC Total Ion Chromatograms

100 UPLC Ultra Performance Liquid Chromatography

101

102 Introduction

103 Chronic immune diseases such as allergies, inflammatory bowel disease or diabetes are highly
104 prevalent in industrialized countries and a further increase of burden due to non-
105 communicable diseases is expected for the next decades (1). Currently available
106 pharmaceuticals improve symptoms, but cannot cure the disease. Accordingly, there is an
107 increasing demand for proven alternatives to pharmaceutical products from both healthcare
108 professionals and consumers (2).

109 Probiotic bacteria have been shown to modify immune responses *in vitro* (3–5) and in animals
110 (6,7), and are defined as “live microorganisms which when administered in adequate amounts
111 confer a health benefit on the host”. Accordingly, they have been proposed as an alternative to
112 classical therapies for the treatment of immune diseases (8). However, apart from acute
113 infectious diarrhoea (9), clinical trials for different indications such as primary prevention of
114 allergic diseases (e.g. (10–22)) or treatment of chronic inflammatory bowel disease (23) were
115 highly inconsistent. Accordingly, a consensus paper (24) and the European Food Safety
116 Authority (25) stated that a role for probiotic microbes for prevention of allergic
117 manifestations is not established.

118 One important reason for the conflicting results is most likely the complexity of the reciprocal
119 crosstalk between probiotic bacteria and the host’s microbiota and immune cells.

120 Even in healthy individuals, the gut microbiome differs remarkably among individuals
121 (26,27). In addition, the microbiome as well as the immunity can be substantially altered in
122 disease conditions (28). Thus, it is hard to predict the precise functionality of a probiotic strain
123 in individual patients. In addition, there is a lack of mechanistic understanding which is
124 important to establish biological plausibility for any claimed health effect.

125 To overcome these problems, the utilization of specified substances derived from probiotic
126 microbes could provide an attractive alternative. Other than living bacteria with complex fates
127 and response patterns in the host, they should have definable properties with provable mode

128 of action. So far, only very few candidate structures or substances have been demonstrated as
129 bioactive agents and even less with preclinical evidence for therapeutic effects (29).

130 Therefore, the aim of the present study was 1) to establish a screening tool for the detection of
131 Th2-lowering immune activity in probiotic supernatants, 2) to identify a soluble bacterial
132 molecule that mediates this activity, 3) to test the putative substance in a mouse model of
133 allergic airway disease (AAI) and 4) to obtain insight into potential underlying mechanisms.

134 **Materials and Methods**

135 For detailed information on reagents, culture conditions of bacteria and human cells,
136 generation of human monocyte derived dendritic cells (DCs), structural elucidation of D-
137 Tryptophan, cyto/chemokine quantification, murine T cell differentiation, flow cytometry,
138 qRT-PCR, microbiota analysis, isolation of intestinal lamina propria cells and animal
139 experiments (induction of experimental asthma, lung function analyses) see Methods section
140 in this article's Online Repository at www.jacionline.org.

141

142 **Bacterial strains**

143 *Bifidobacteriae*, *Lactobacilli*, *Lactococci*, *E. Coli* Nissle 1917, *Enterococcus faecium*, and
144 *Streptococcus thermophilus* were obtained from different providers (Table E1) All strains
145 were grown until stationary phase and a minimum cell number of 10^8 CFU/ml. Cell free
146 supernatants were obtained by centrifugation (6000 rpm; 5 min; 20° C) followed by filtration
147 through 0.22 μ m pore size surface-modified polyethersulfone membrane (Millipore,
148 Darmstadt, Germany). No bacterial growth was observed when aliquots from supernatants
149 were cultured in bacterial growth medium. Otherwise, supernatants were stored immediately
150 after collection in aliquots at -80 °C until further use.

151

152 **Bioassays for screening for immune modulatory activity in probiotic supernatants**

153 Two biological assays based on down-modulation of costimulatory molecules on human DCs,
154 and of CCL17-secretion by a human Hodgkin lymphoma T cell line (KM-H2) were set up.
155 Human immature DCs were matured with 0.1 μ g/mL lipopolysaccharide (LPS) from *E. coli*
156 (Sigma-Aldrich, St. Louis, USA) in the presence or absence of 200 μ L bacteria free
157 supernatants for 24 h followed by flow cytometric analysis of costimulatory molecules.
158 Similarly, 200 μ L supernatants were added to 3-5 $\times 10^6$ KM-H2 cells for 24 h. Supernatants
159 were collected from KM-H2 by centrifugation and stored at -80 °C until quantification of
160 CCL17. To control for the dilution of KM-H2 culture media with different volumes of

bacterial supernatants, the corresponding amount of blank MRS medium was added. Blank bacterial growth medium and supernatants from *Lactobacillus rhamnosus* DSM 20021, which has no probiotic activity, were used as negative controls in both screening assays.

Animals and oral supplementation with D-Tryptophan

All animal experiments were conducted under the Federal Guidelines for the Use and Care of Laboratory Animals (Az 55.2-1-54-2532-137-13) and was approved by the Government of the District of Upper Bavaria and Schleswig-Holstein (V244-13313/2016 (7-1/10)). Female Balb/c mice from 6-8 weeks old were obtained from Charles River (Sulzfeld, Germany) and housed in individually ventilated cages with two mice each in specific pathogen free conditions. A standard extruded pellet diet and sterile filtered drinking water were provided *ad libitum*. For quantification of D-Tryptophan in mouse sera, D-Tryptophan (Sigma-Aldrich, St. Louis, USA) was dissolved in the drinking water at concentrations of 1.8 mg/dL or 18 mg/dl (approximately 0.09 and 0.9 mg/day per mouse). Control animals received pure water (n=8 per group). No changes in behavior or body weight were noted in the supplemented animals compared to controls. Animals were sacrificed after 14 days and sera were immediately stored at -80 °C until analysis.

For testing prevention of AAI, mice received 50 mM D-Tryptophan starting at least three days before the first sensitization until sacrificing on day 25. For microbiome analyses, the caecum was cut off and immediately stored at -80 °C until further processing.

Statistical analyses

Bioassays and animal experiments

Results of bioassays and animal experiments are given as means with standard deviation.

Student's t test with Dunn's Multiple Comparison Test or Two-way ANOVA with Bonferroni

post test were used where appropriate. Tests applied are given in the respective figure legends.

187 P values ≤ 0.05 were considered significant. (GraphPad Prism Software version 5.0, Inc. La
188 Jolla, CA 92037 USA).

189

190 ***Microbial diversity***

191 Bacterial diversity was assessed by molecular barcoding of 16S rRNA genes in caecum
192 samples of six animals per group. To this end, DNA was directly extracted from the caecum
193 using a kit based protocol (PowerSoil DNA Isolation Kit, MO BIO Laboratories, Carlsbad,
194 CA, USA). 315 bp fragments were amplified within the variable region V5 and V6 of the 16S
195 rRNA gene using S-D-Bact-0785-a-S-18[5'-GGMTTAGATACCCBDGTA-3'] and S-*-Univ-
196 1100-a-A-15 [5'-GGGTYKCGCTCGTTR-3'] as primers (30). Sequencing of amplicons was
197 performed on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using paired end
198 technology (see Supplementary Information, page 9 for details). Sequences are deposited in
199 NCBI accession number PRJNA304109.

200 Reads were analyzed with the software package QIIME (<http://qiime.org>). Operational
201 taxonomic units (OTUs) were picked within the 13_8 version of the Greengenes reference
202 database (31) at a similarity level of 95% sequence identity. Sequences were subsampled to
203 15000 reads per sample, which reflects the number of reads obtained in the sample with the
204 lowest number of reads after quality control. This number was still sufficient to reach a
205 plateau when collectors' curves were calculated on the basis of OTU₉₅. The taxonomy
206 assignment was done using the RDP classifier 2.2 (32). Principal coordinate analysis (PCoA)
207 was generated on the unweighted UniFrac distance matrix using the ape-package within the R
208 software environment (<http://www.r-project.org>) and statistical significance was determined
209 with the Student's *t* test. Alpha-diversity of each sample was measured using the Chao1 metric
210 (Chao A, 1984) and compared between treatments by nonparametric two-sample *t* test (i.e.,
211 using Monte Carlo permutations for significance testing). Beta-diversity was calculated using
212 the phylogenetic method UniFrac (33). The nonparametric analysis of similarity (ANOSIM)

213 was performed to examine the beta-diversity distance matrix for significant differences
214 between groups of samples; differences in OTU abundance between groups were tested for
215 significance by nonparametric ANOVA.

216 Results

217 I. Identification and characterization of a bioactive probiotic substance

218 Screening of crude probiotic supernatants for downregulation of CCL17

219 To develop a high-throughput screening system for the detection of Th2-downregulatory
220 activity in supernatants from probiotic bacteria, we made use of the high constitutive secretion
221 of the Th2-associated CCL17 by the human Hodgkin Lymphoma T cell line KM-H2.

222 To identify the threshold for downregulation of CCL17, KM-H2 cells were incubated with
223 increasing volumes of supernatants from *Lactobacillus rhamnosus* GG (*L. rhamnosus* GG),
224 *Bifidobacterium* BB-420 and *Lactobacillus casei* W56. Supernatants from all three probiotic
225 strains led to a significant dose- and time dependent reduction of CCL17 concentrations to
226 ~30% relative to supernatant from the non-probiotic *Lactobacillus rhamnosus* DSM-20021
227 (**Fig 1A**). The minimum volume (200µl) leading to that reduction was used in all subsequent
228 experiments.

229 As the numerous ingredients of the bacterial culture medium interfered with the detection of
230 specific signals in mass spectrometry, the bacteria were cultivated in less complex medium
231 (CDM1). The potency of supernatants from probiotic strains cultivated in CDM1 versus
232 standard medium to lower CCL17 concentrations was comparable (**Fig E1A, B**). Subsequent
233 testing of supernatants from 37 probiotic strains, revealed that 7 of 21 *Lactobacillus* sp.
234 strains, 5 of 10 *Bifidobacterium* sp. strains, and 1 of 3 *Lactococcus* sp. strains lowered CCL17
235 secretion without affecting cell viability (**Fig E2**). In contrast, none of the *Streptococcus*
236 *thermophilus*, *Enterococcus faecium* or *E. coli* Nissle 1917 strains influenced CCL17 levels
237 (**Fig 2; Table E1**).

238

239 Verification of results from CCL17-based screening assays

240 To confirm the observed immune modulatory activity, we evaluated the efficacy of the
241 probiotic supernatants to lower the expression of co-stimulatory molecules on human

monocyte derived dendritic cells (DC). Upon recognition of antigen, naïve DCs undergo a complex maturation process (34). While fully activated DCs induce adaptive immune responses, incomplete activation leads to tolerance (35). Therefore, we screened for reduced expression of co-stimulatory molecules in the presence of probiotic supernatants. All 13 supernatants that had already been pre-identified as “immune modulatory” in the CCL17-based screen also significantly decreased the percentages of LPS-induced CD83, CD80, CD86 and CD40 expressing mature DCs, whereas the remaining supernatants were inactive on DCs (**Fig 1B**). None of the supernatants affected the viability of DC (**Fig E2**). Thus, both bioassays gave 100% concordant results. For a complete overview of the bioactivity of all strains see table E1.

Fractionation of selected probiotic supernatants yields three bioactive fractions of different polarity

L. rhamnosus GG has been most frequently used in clinical studies (36). Therefore, we selected supernatants from *L. rhamnosus* GG and further of *L. casei* W56 for further enrichment and stepwise chemical characterization of the putative metabolite. During this procedure, each sub-fraction was retested for bioactivity in both the KMH2 and DC bioassays. Bacterial supernatants were subjected to semi-preparative chromatography yielding 11 MeOH/H₂O extracts. The highest immune modulatory activity was found in the 20% fraction along with slightly lower activities in the 40% and 50% MeOH fractions (**Fig 3**). Therefore, we chose this fraction for further purification.

Isolation and identification of the bioactive substance in 20% MeOH/H₂O extracts

Chromatographic sub-fractionation of the 20% MeOH/H₂O fraction yielded ten sub-fractions (**Fig E3A, B**), three of which showed activity in the bioassays. These sub-fractions and their closest neighbours were re-evaluated via reversed phase UPLC-High Resolution TOF MS to

268 generate Total Ion Chromatograms. By identifying similarities in the chromatograms, we
269 identified a substance that, according to peak retention time and molecular mass information,
270 was only present in the bioactive sub-fractions, being highest in sub-fraction 7 from *L. casei*
271 W56 and sub-fraction 6 from *L. rhamnosus* GG (**Fig E4A**). The extracted mass spectrum
272 strongly suggested that this substance was composed of the Tryptophan ions $[2M+H]^+$,
273 $[M+H]^+$ and its fragment $[M+H-NH_3]^+$ (**Fig E4B**).

274 After careful enrichment of the bioactive substance by repeated chromatography runs, the
275 isolated candidate substance of both strains showed bioactivity in both screening assays. High
276 resolution mass spectrometry analyses by FT-ICR-MS confirmed $C_{11}H_{12}N_2O_2$ as the
277 molecular formula of these ions (**Fig E4C, D**). Further analyses by proton NMR provided
278 detailed information on the functional group distribution and molecular structure: The
279 doublets and triplets (δ 7.8-7.0) showed the occurrence of an indole ring. Resonance signals at
280 the region of δ 3.9-3.8 and δ 3.2-3.1 could also be assigned to β -CH and α -CH protons,
281 respectively (**Fig E5**). Thus, there was a close agreement between standard Tryptophan and
282 our bioactive sub-fraction.

283 Since L-Tryptophan is a standard component of the bacterial growth medium, we
284 hypothesized that the bioactivity is related to the D-form of this amino acid. Indeed,
285 enantiomeric separation of the purified sub-fraction confirmed the presence of D- and L-
286 Tryptophan (**Fig E6A**), while the corresponding sub-fraction of blank medium contained
287 solely the L-form (**Fig E6B**).

288

289 **The immune modulatory activity in probiotic supernatants is restricted to the D-form of** 290 **Tryptophan**

291 To verify if the bioactivity was indeed restricted to the D-isomer of Tryptophan, we tested
292 different concentrations of synthetic L- and D-Tryptophan in the CCL17 bioassay. Only D-

293 Tryptophan showed a dose dependent immune activity (**Fig 4**). Moreover, none of twelve
294 other polar and non-polar neutral D-amino acids tested showed any bioactivity (**Table I**).

295

296 **Bacterial supernatants and D-Tryptophan modulate cytokine profiles of enriched** 297 **human DC**

298 To obtain a first insight into mechanisms underlying this bioactivity, we quantified the
299 cytokines secreted by highly enriched DCs (Fig E2D) after treatment with the bacterial
300 supernatants or synthetic D-Tryptophan. All probiotic supernatants and D-Tryptophan
301 strongly induced IL-10 and lowered LPS-induced IFN- γ , IL-12 and IL-5 in these cultures. In
302 contrast, cytokine patterns were unaffected by the control supernatants, and amino acids
303 (**Table II**). Overall this resulted in increased IL-10/IL-12 ratios and – with the exception of
304 BB-46 - in decreased IL-5/IFN- γ ratios.

305

306 **II. Preclinical effects of oral D-Tryptophan supplementation**

307 **D-Tryptophan influences allergic airway inflammation and Th2 immune responses**

308 If it is to be used as an oral intervention in allergic diseases, D-Tryptophan needs to be
309 absorbed from the gut. Oral supplementation of mice with 0.9 mg/day D-Tryptophan
310 increased D-Tryptophan serum levels significantly (**Fig 5A**) indicating enteric uptake and
311 systemic distribution. Pre-treatment of mice with D-Tryptophan for 3 days and throughout
312 experimental “asthma” induction lowered total BALF cells, which was mainly caused by a
313 reduction of eosinophils (**Fig 5B, C**). Furthermore, the supplementation improved airway
314 hyperreactivity to methacholine (**Fig 5D**). As this suggested an involvement of Th2 responses,
315 we analysed lung T cells: D-Tryptophan reduced Il-4 producing T cells and Il4 levels in
316 BALF (trend, Fig 5E, F and E7 for splenic cells) but not Ifn γ -producing Th1 cells.
317 Furthermore, D-Trp treatment significantly increased Helios⁺ Treg while total Foxp3⁺ cells
318 remained unchanged (Fig 5G).

319 To further substantiate these *in vivo* findings we performed T cell differentiation assays *in*
320 *vitro*. In line with the *in vivo* observations, D-Trp reduced Th2 cell differentiation, whereas
321 Th1 differentiation remained unaffected (Fig. 6A, B and Fig E8A). Consequently, *Il4*, *Gata3*
322 expression and *Il13* secretion was reduced, whereas *Ifn γ* expression remained unaffected.
323 Treg, however, showed increased *Foxp3* expression on mRNA and protein level (Fig. 6C and
324 Fig E8B).

325

326 **D-Tryptophan induces gut Tregs and increases intestinal microbial diversity in allergic** 327 **airway inflammation**

328 In addition to the observed pulmonary immune response, the frequency of Foxp3⁺ T cells was
329 locally increased in the colon of supplemented AAI mice compared to non-supplemented AAI
330 mice (**Fig 7A**). Altered gut immunity might be driven directly by D-Tryptophan and/or
331 indirectly via altered gut microbiota.

332 A diversity analysis of bacteria by 16S rRNA based barcoding demonstrated a strongly
333 reduced community richness and diversity at the level of OTU₉₅ in AAI mice (Fig. 7B).
334 Supplementation with D-Tryptophan increased the in bacterial diversity of AAI mice,
335 resulting in comparable alpha-diversity patterns compared to healthy animals. Although the
336 original diversity was not completely restored after D-Trp application, its impact on microbial
337 community composition was significant (Fig E9A).

338 Independent of the health status of the animals D-Tryptophan supplementation all samples
339 were dominated by the phyla Bacteroidetes and Firmicutes (19.4 - 27.7% and 65.9 - 78.4% of
340 the total sequences). As expected, the phylum Firmicutes mainly consisted of members of the
341 order Clostridiales. Other phyla including Actinobacteria and Proteobacteria were also present
342 but at significantly lower abundance. At the family level, Lachnospiraceae, Odoribacteraceae,
343 Rikenellaceae, Ruminococcaceae, S24-7 and an unclassified bacterial family belonging to
344 Clostridiales (Fig E9B) dominated. The latter was mainly present in AAI mice, forming

345 58.6% of the total community. Lachnospiraceae, however, were less abundant in AAI animals
346 (5.5%), compared to controls (13.7%), to D-Tryptophan treated AAI mice (20.6%), or to D-
347 Tryptophan treated mice without AAI (27.5 %). Odoribacteraceae were strongly affected by
348 D-Tryptophan, as their relative abundance tripled in both groups of supplemented animals
349 (3.9 % vs ~1.1%). In contrast, Rikenellaceae showed a decreased abundance in the D-Trp
350 groups (1.1-2.0%) compared to to the control groups (4.6-7.7%). Interestingly,
351 Ruminococcaceae, which were strongly reduced in the control mice affected with AAI
352 (3.7%), recovered by the application of D-Trp (8.9%): this was comparable to the abundance
353 in control group of mice without AAI. Members of the S24-7 family were neither affected by
354 AAI nor by the application of D-Tryptophan. Overall, D-Tryptophan supplementation
355 increased intestinal bacterial diversity in AAI, D-Tryptophan treated mice, such that the
356 bacterial diversity pattern was more comparable to 'healthy' control mice (PBS/PBS). (**Fig**
357 **7B**). Thus, our results suggested that D-Tryptophan treatment reestablishes a “healthy”
358 microbial community genotype in AAI mice.

359 Discussion

360 In the present work we identified for the first time D-Tryptophan as bacterial substance that is
361 produced by the probiotic strains *L. rhamnosus* GG and *L. casei* W56. We demonstrate that D-
362 Tryptophan lowers the production of Th2 cyto- and chemokines in human peripheral and
363 murine immune cells and more importantly prevents full development of AAI when fed to
364 mice. Beneath immune modulation, this may occur also through maintenance of a diverse gut
365 microbiota, which was otherwise lost in animals with experimental asthma.

366 Probiotic bacteria have been shown to modify immune responses *in vitro* (e.g.(3,4)) and in
367 animal studies (e.g. (5,6)), but clear evidence for clinical efficacy in treatment of chronic
368 inflammatory disorders is largely lacking. As the reciprocal interaction of probiotic bacteria
369 with the host's microbiota and immune system is extremely complex, utilization of defined
370 small substances with predictable mode of action might provide an interesting alternative for
371 prevention of allergic disease in individuals at risk.

372 D-amino acids are non-proteinogenic enantiomers of L-amino acids. Until the discovery of
373 free D-aspartate and D-serine in mammalian brain as neurotransmitters in the late 1980s, D-
374 amino acids were considered to play no role in higher organisms. So far, research on D-amino
375 acids in mammals has been mainly restricted to the nervous system, due to the relative
376 abundance of D-aspartate and D-serine in the brain (37) and the difficulty to detect D-amino
377 acids at trace levels (38). Thus, very little is known on D-Tryptophan uptake (39) and
378 metabolism in humans (40) and it has been assumed in the past that higher organisms utilize
379 D-Tryptophan poorly (41). By developing highly sensitive assays, we demonstrated systemic
380 distribution of D-Tryptophan in mice after oral uptake.

381 In contrast to higher organisms, numerous bacteria including probiotic bacteria produce D-
382 amino acids, such as D-glutamate and D-alanine, using them mainly for cross-linking glycan
383 chains in the peptidoglycan wall (42,43).

384 The regulation of bacterial L-Tryptophan biosynthesis and degradation is well known (44). A
385 role for D-Tryptophan in bacterial communication was only recently discovered, by
386 demonstrating its requirement for disassembly of biofilms in *B. subtilis* (45). Other soluble
387 substances produced by probiotic bacteria are less investigated so far (4,46).

388 Humans are potentially exposed to microbial-generated D-amino acids (47) since body
389 surfaces and the environment harbor an abundant and high diversity of microbes (48). Similar
390 to what has already been shown for acyl-homoserine lactones from gram-negative bacteria
391 (e.g.(49–52)), means to recognize and interact with bacterial D-amino acids including D-
392 Tryptophan could have evolved.

393 This hypothesis is supported by several observations: first, human cells used in our bioassays
394 responded to D-Tryptophan, but neither to L-Tryptophan or any other tested D-amino acid;
395 second, at least two surface receptors for D-Tryptophan exist in humans: The G protein-
396 coupled receptor GPR109B (53) is expressed on macrophages, monocytes, adipose tissue and
397 lung (54), and mediates attraction of neutrophils upon binding of D-Tryptophan or its
398 metabolite D-kynurenine. Of note, when we extracted and analysed published transcriptomic
399 data (55), GPR109B was significantly decreased in airway epithelial cells and T cells from
400 patients with asthma as opposed to controls, indicating a potential role for this receptor in
401 allergic disease. (Table E2).

402 The second receptor, SLC6A14 (solute carrier family 6 amino acid transporter member 14,
403 alias ATB^{0,+}) transports D-Tryptophan and four other D-amino acids across epithelial cells
404 (56). As the receptor is expressed in the intestine, SLC6A14 is exposed to high microbial load
405 and diversity. SLC6A14 is further expressed at exceptionally high levels in the fetal lung
406 (own data Fig E10 and (57)). The physiological role of SLC6A14 in fetal life is unknown so
407 far. But it is tempting to speculate a mechanistic link for prenatal intervention trials using
408 probiotic bacteria.

Three enzymes, TDO, indoleamine 2,3-dioxygenase (IDO1) and the more recently discovered IDO2 can metabolize Tryptophan. While TDO is specific for L-Tryptophan, IDO1 channels both D- and L-Tryptophan into the kynurenine pathway. IDO activation leads to Tryptophan depletion and thereby promotes peripheral tolerance (reviewed in (58)), which contrasts our findings. However, IDO1 seems not to be important for the induction of immune tolerance in the airways, but instead promotes Th2 responses via effects on lung DC (59), which we suggest could be counter-acted by D-Trp. In addition, IDO2 which is also expressed on DC (60) and has a slightly different substrate specificity could further modulate D-Tryptophan metabolism (61).

So far, we concentrated on the 20% MeOH fraction for identification of the putative substance as this was the sub-fraction with the highest immune modulatory activity and polarity. Bioactivity was further detected in the 40% and 50% MeOH fractions holding the potential for the discovery of further small immuno-active substances. Our bioassays were designed to detect substances that induce a tolerogenic profile in DCs and decrease the allergy-related chemokine CCL17. Therefore, it is possible that further immune regulatory substances not related to allergic disease were overlooked.

D-Tryptophan could influence immune homeostasis either directly as shown in our screening assays or indirectly by shifting the structure of the microbiome of the host. Apart from the observed immune modulatory properties of D-Tryptophan, we do not have direct mechanistic links explaining the altered gut microbiota or the protection from AAI. However, in line with our own findings, Trompette and colleagues demonstrated that a change in the gut microbiota due to dietary fermentable fibers induces the production of metabolites involved in protection from AAI (62). These metabolites have further been associated with increased frequencies of Foxp3⁺ regulatory T cells (63). The lung microbiota and a population of Foxp3⁺ regulatory T cells have further been shown to protect neonatal mice from exaggerated type 2 immune

434 responses in a murine model of house dust mice induced AAI (64) which supports a role of
435 both immune parameters also in adult mice.

436 In summary, we identified for the first time that D-Tryptophan acts as immune modulatory
437 substance that is produced by probiotic strains. Our results suggest that D-Tryptophan can
438 potentially influence both immune responses and the constituents of intestinal microbiota, and
439 can conceivably reduce the degree of hyperactivity severity of AAI. In addition to immune
440 modulation, this may occur through the maintenance of a diverse gut microbiota, which was
441 otherwise lost in animals with AAI.

442 We conclude that bacteria-derived D-Tryptophan may play a wider role in human health than
443 previously thought. Overall, our findings support the concept that defined bacterial products
444 might provide the basis for future development of preventive strategies of chronic
445 inflammatory disorders.

446

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452

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References

1. World Health Organization. Global status report on noncommunicable diseases 2010 [Internet]. 2010 [cited 2015 Sep 16]. Available from: http://www.who.int/nmh/publications/ncd_report_full_en.pdf
2. Jackson DJ, Hartert T V, Martinez FD, Weiss ST, Fahy J V. Asthma: NHLBI Workshop on the Primary Prevention of Chronic Lung Diseases. *Ann Am Thorac Soc. American Thoracic Society*; 2014 Apr 22;11 Suppl 3:S139–45.
3. Borthakur A, Anbazhagan AN, Kumar A, Raheja G, Singh V, Ramaswamy K, et al. The probiotic *Lactobacillus plantarum* counteracts TNF- α -induced downregulation of SMCT1 expression and function. *Am J Physiol Gastrointest Liver Physiol*. 2010 Oct;299(4):G928–34.
4. Heuvelin E, Lebreton C, Bichara M, Cerf-Bensussan N, Heyman M. A *Bifidobacterium* probiotic strain and its soluble factors alleviate chloride secretion by human intestinal epithelial cells. *J Nutr*. 2010 Jan;140(1):7–11.
5. Mileti E, Matteoli G, Iliev ID, Rescigno M. Comparison of the immunomodulatory properties of three probiotic strains of *Lactobacilli* using complex culture systems: prediction for in vivo efficacy. *PLoS One*. 2009 Jan;4(9):e7056.
6. Kwon H-K, Lee C-G, So J-S, Chae C-S, Hwang J-S, Sahoo A, et al. Generation of regulatory dendritic cells and CD4⁺Foxp3⁺ T cells by probiotics administration suppresses immune disorders. *Proc Natl Acad Sci U S A*. 2010 Feb 2;107(5):2159–64.
7. Fanning S, Hall LJ, Cronin M, Zomer A, MacSharry J, Goulding D, et al. *Bifidobacterium* surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. *Proc Natl Acad Sci U S A*. 2012 Feb 7;109(6):2108–13.
8. Food and Agriculture Organization. Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. *FAO Food Nutr Pap*. 2001;

- 484 9. Allen SJ, Martinez EG, Gregorio G V, Dans LF. Probiotics for treating acute infectious
485 diarrhoea. *Cochrane database Syst Rev*. 2010 Jan;(11):CD003048.
- 486 10. Boyle RJ, Ismail IH, Kivivuori S, Licciardi P V, Robins-Browne RM, Mah L-J, et al.
487 *Lactobacillus GG* treatment during pregnancy for the prevention of eczema: a
488 randomized controlled trial. *Allergy*. 2011 Apr;66(4):509–16.
- 489 11. Kalliomäki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. Probiotics in
490 primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet*.
491 2001 Apr 7;357(9262):1076–9.
- 492 12. Abrahamsson TR, Jakobsson T, Böttcher MF, Fredrikson M, Jenmalm MC, Björkstén
493 B, et al. Probiotics in prevention of IgE-associated eczema: a double-blind,
494 randomized, placebo-controlled trial. *J Allergy Clin Immunol*. 2007 May;119(5):1174–
495 80.
- 496 13. Kukkonen K, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, et
497 al. Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic
498 diseases: a randomized, double-blind, placebo-controlled trial. *J Allergy Clin Immunol*.
499 2007 Jan;119(1):192–8.
- 500 14. Kopp MV, Hennemuth I, Heinzmann A, Urbanek R. Randomized, double-blind,
501 placebo-controlled trial of probiotics for primary prevention: no clinical effects of
502 *Lactobacillus GG* supplementation. *Pediatrics*. 2008 Apr 1;121(4):e850–6.
- 503 15. Wickens K, Black PN, Stanley T V, Mitchell E, Fitzharris P, Tannock GW, et al. A
504 differential effect of 2 probiotics in the prevention of eczema and atopy: a double-blind,
505 randomized, placebo-controlled trial. *J Allergy Clin Immunol*. 2008 Oct;122(4):788–
506 94.
- 507 16. Dotterud CK, Storrø O, Johnsen R, Oien T. Probiotics in pregnant women to prevent
508 allergic disease: a randomized, double-blind trial. *Br J Dermatol*. 2010
509 Sep;163(3):616–23.
- 510 17. Soh SE, Aw M, Gerez I, Chong YS, Rauff M, Ng YPM, et al. Probiotic
511 supplementation in the first 6 months of life in at risk Asian infants--effects on eczema
512 and atopic sensitization at the age of 1 year. *Clin Exp Allergy*. 2009 Apr;39(4):571–8.
- 513 18. Taylor AL, Dunstan JA, Prescott SL. Probiotic supplementation for the first 6 months
514 of life fails to reduce the risk of atopic dermatitis and increases the risk of allergen
515 sensitization in high-risk children: a randomized controlled trial. *J Allergy Clin*
516 *Immunol*. 2007 Jan;119(1):184–91.
- 517 19. Brouwer ML, Wolt-Plompen SAA, Dubois AEJ, van der Heide S, Jansen DF, Hoijsen
518 MA, et al. No effects of probiotics on atopic dermatitis in infancy: a randomized
519 placebo-controlled trial. *Clin Exp Allergy*. 2006 Jul;36(7):899–906.
- 520 20. Bertelsen RJ, Brantsæter AL, Magnus MC, Haugen M, Myhre R, Jacobsson B, et al.
521 Probiotic milk consumption in pregnancy and infancy and subsequent childhood
522 allergic diseases. *J Allergy Clin Immunol*. 2014 Jan;133(1):165–71.e1–8.
- 523 21. Cuello-Garcia CA, Brożek JL, Fiocchi A, Pawankar R, Yepes-Nuñez JJ, Terracciano L,
524 et al. Probiotics for the prevention of allergy: A systematic review and meta-analysis of
525 randomized controlled trials. *J Allergy Clin Immunol*. 2015 Jun 1;
- 526 22. Szajewska H, Shamir R, Turck D, van Goudoever JB, Mihatsch WA, Fewtrell M.
527 Recommendations on probiotics in allergy prevention should not be based on pooling
528 data from different strains. *J Allergy Clin Immunol*. 2015 Aug 29;
- 529 23. Butterworth AD, Thomas AG, Akobeng AK. Probiotics for induction of remission in

- 530 Crohn's disease. *Cochrane database Syst Rev*. 2008 Jan;(3):CD006634.
- 531 24. Fiocchi A, Burks W, Bahna SL, Bielory L, Boyle RJ, Cocco R, et al. Clinical Use of
532 Probiotics in Pediatric Allergy (CUPPA): A World Allergy Organization Position Paper.
533 *World Allergy Organ J*. 2012 Nov;5(11):148–67.
- 534 25. European Food Safety Authority. EFSA News Story: EFSA delivers advice on further
535 808 health claims [Internet]. 2010 [cited 2015 Feb 2]. Available from:
536 <http://www.efsa.europa.eu/en/press/news/nda101019.htm>
- 537 26. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al.
538 Enterotypes of the human gut microbiome. *Nature*. 2011 May 12;473(7346):174–80.
- 539 27. van Baarlen P, Troost F, van der Meer C, Hooiveld G, Boekschoten M, Brummer RJM,
540 et al. Human mucosal in vivo transcriptome responses to three lactobacilli indicate how
541 probiotics may modulate human cellular pathways. *Proc Natl Acad Sci U S A*. 2011
542 Mar 15;108 Suppl :4562–9.
- 543 28. Candela M, Rampelli S, Turrone S, Severgnini M, Consolandi C, De Bellis G, et al.
544 Unbalance of intestinal microbiota in atopic children. *BMC Microbiol*. 2012 Jan;12:95.
- 545 29. Yan F, Cao H, Cover TL, Washington MK, Shi Y, Liu L, et al. Colon-specific delivery
546 of a probiotic-derived soluble protein ameliorates intestinal inflammation in mice
547 through an EGFR-dependent mechanism. *J Clin Invest*. 2011 Jun;121(6):2242–53.
- 548 30. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of
549 general 16S ribosomal RNA gene PCR primers for classical and next-generation
550 sequencing-based diversity studies. *Nucleic Acids Res*. 2013 Jan 7;41(1):e1.
- 551 31. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, et al. An
552 improved Greengenes taxonomy with explicit ranks for ecological and evolutionary
553 analyses of bacteria and archaea. *ISME J*. 2012 Mar;6(3):610–8.
- 554 32. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid
555 assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ*
556 *Microbiol*. 2007 Aug;73(16):5261–7.
- 557 33. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial
558 communities. *Appl Environ Microbiol*. 2005 Dec;71(12):8228–35.
- 559 34. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*.
560 1998;392(6673):245–52.
- 561 35. Probst HC, Muth S, Schild H. Regulation of the tolerogenic function of steady-state
562 DCs. *Eur J Immunol*. 2014;44(4):927–33.
- 563 36. Search of: Lactobacillus. - Results on Map - ClinicalTrials.gov [Internet]. [cited 2015
564 Feb 2]. Available from: <https://clinicaltrials.gov/ct2/results/map?term=Lactobacillus>.
- 565 37. Hashimoto A, Kumashiro S, Nishikawa T, Oka T, Takahashi K, Mito T, et al.
566 Embryonic development and postnatal changes in free D-aspartate and D-serine in the
567 human prefrontal cortex. *J Neurochem*. 1993 Jul;61(1):348–51.
- 568 38. Visser WF, Verhoeven-Duif NM, Ophoff R, Bakker S, Klomp LW, Berger R, et al. A
569 sensitive and simple ultra-high-performance-liquid chromatography-tandem mass
570 spectrometry based method for the quantification of D-amino acids in body fluids. *J*
571 *Chromatogr A*. 2011 Oct 7;1218(40):7130–6.
- 572 39. López-Burillo S, García-Sancho J, Herreros B. Tryptophan transport through transport
573 system T in the human erythrocyte, the Ehrlich cell and the rat intestine. *Biochim*
574 *Biophys Acta*. 1985 Oct 24;820(1):85–94.

- 575 40. LANGNER RR, BERG CP. Metabolism of D-Tryptophan in the normal human subject.
576 J Biol Chem. 1955 Jun;214(2):699–707.
- 577 41. Triebwasser KC, Swan PB, Henderson LM, Budny JA. Metabolism of D- and L-
578 Tryptophan in dogs. J Nutr. 1976 May;106(5):642–52.
- 579 42. Cava F, de Pedro MA, Lam H, Davis BM, Waldor MK. Distinct pathways for
580 modification of the bacterial cell wall by non-canonical D-amino acids. EMBO J. 2011
581 Aug 17;30(16):3442–53.
- 582 43. Lam H, Oh D-C, Cava F, Takacs CN, Clardy J, de Pedro MA, et al. D-amino acids
583 govern stationary phase cell wall remodeling in bacteria. Science. 2009 Sep
584 18;325(5947):1552–5.
- 585 44. Yanofsky C. RNA-based regulation of genes of Tryptophan synthesis and degradation,
586 in bacteria. RNA. 2007 Aug;13(8):1141–54.
- 587 45. Kolodkin-Gal I, Romero D, Cao S, Clardy J, Kolter R, Losick R. D-amino acids trigger
588 biofilm disassembly. Science. 2010 Apr 30;328(5978):627–9.
- 589 46. Thomas CM, Hong T, van Pijkeren JP, Hemarajata P, Trinh D V, Hu W, et al. Histamine
590 derived from probiotic *Lactobacillus reuteri* suppresses TNF via modulation of PKA
591 and ERK signaling. PLoS One. 2012 Jan;7(2):e31951.
- 592 47. Friedman M. Origin, microbiology, nutrition, and pharmacology of D-amino acids.
593 Chem Biodivers. 2010 Jun;7(6):1491–530.
- 594 48. Structure, function and diversity of the healthy human microbiome. Nature. 2012 Jun
595 14;486(7402):207–14.
- 596 49. Hooi DSW, Bycroft BW, Chhabra SR, Williams P, Pritchard DI. Differential immune
597 modulatory activity of *Pseudomonas aeruginosa* quorum-sensing signal molecules.
598 Infect Immun. 2004 Nov;72(11):6463–70.
- 599 50. Smith RS, Kelly R, Iglewski BH, Phipps RP. The *Pseudomonas* autoinducer N-(3-
600 oxododecanoyl) homoserine lactone induces cyclooxygenase-2 and prostaglandin E2
601 production in human lung fibroblasts: implications for inflammation. J Immunol. 2002
602 Sep 1;169(5):2636–42.
- 603 51. Ritchie AJ, Yam AOW, Tanabe KM, Rice SA, Cooley MA. Modification of in vivo and
604 in vitro T- and B-cell-mediated immune responses by the *Pseudomonas aeruginosa*
605 quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone. Infect Immun.
606 2003 Aug;71(8):4421–31.
- 607 52. Chhabra SR, Harty C, Hooi DSW, Daykin M, Williams P, Telford G, et al. Synthetic
608 analogues of the bacterial signal (quorum sensing) molecule N-(3-oxododecanoyl)-L-
609 homoserine lactone as immune modulators. J Med Chem. 2003 Jan 2;46(1):97–104.
- 610 53. Irukayama-Tomobe Y, Tanaka H, Yokomizo T, Hashidate-Yoshida T, Yanagisawa M,
611 Sakurai T. Aromatic D-amino acids act as chemoattractant factors for human
612 leukocytes through a G protein-coupled receptor, GPR109B. Proc Natl Acad Sci U S A.
613 2009 Mar 10;106(10):3930–4.
- 614 54. Wise A, Foord SM, Fraser NJ, Barnes AA, Elshourbagy N, Eilert M, et al. Molecular
615 identification of high and low affinity receptors for nicotinic acid. J Biol Chem. 2003
616 Mar 14;278(11):9869–74.
- 617 55. Kicic A, Hallstrand TS, Sutanto EN, Stevens PT, Kobor MS, Taplin C, et al. Decreased
618 fibronectin production significantly contributes to dysregulated repair of asthmatic
619 epithelium. Am J Respir Crit Care Med. 2010 May 1;181(9):889–98.

- 620 56. Hatanaka T, Huang W, Nakanishi T, Bridges CC, Smith SB, Prasad PD, et al. Transport
621 of D-serine via the amino acid transporter ATB(0,+) expressed in the colon. *Biochem*
622 *Biophys Res Commun.* 2002 Feb 22;291(2):291–5.
- 623 57. Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, et al. A gene atlas of the
624 mouse and human protein-encoding transcriptomes. *Proc Natl Acad Sci U S A.* 2004
625 Apr 20;101(16):6062–7.
- 626 58. Munn DH, Mellor AL. Indoleamine 2,3 dioxygenase and metabolic control of immune
627 responses. *Trends Immunol.* 2013 Mar;34(3):137–43.
- 628 59. Xu H, Oriss TB, Fei M, Henry AC, Melgert BN, Chen L, et al. Indoleamine 2,3-
629 dioxygenase in lung dendritic cells promotes Th2 responses and allergic inflammation.
630 *Proc Natl Acad Sci U S A.* 2008 May 6;105(18):6690–5.
- 631 60. Lob S, Konigsrainer A, Schafer R, Rammensee H-G, Opelz G, Terness P. Levo- but not
632 dextro-1-methyl Tryptophan abrogates the IDO activity of human dendritic cells.
633 *Blood.* 2008 Feb 15;111(4):2152–4.
- 634 61. Pantouris G, Serys M, Yuasa HJ, Ball HJ, Mowat CG. Human indoleamine 2,3-
635 dioxygenase-2 has substrate specificity and inhibition characteristics distinct from
636 those of indoleamine 2,3-dioxygenase-1. *Amino Acids.* 2014 Sep;46(9):2155–63.
- 637 62. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, et
638 al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and
639 hematopoiesis. *Nat Med.* Nature Publishing Group, a division of Macmillan Publishers
640 Limited. All Rights Reserved.; 2014 Feb;20(2):159–66.
- 641 63. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, et al. The
642 microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis.
643 *Science.* 2013 Aug 2;341(6145):569–73.
- 644 64. Gollwitzer ES, Saglani S, Trompette A, Yadava K, Sherburn R, McCoy KD, et al. Lung
645 microbiota promotes tolerance to allergens in neonates via PD-L1. *Nat Med.* 2014
646 Jun;20(6):642–7.

649 **Table I. Percentage of surface marker expressing mature DCs treated with synthetic D-**
 650 **amino acids***

	D-Ala	D-His	D-Isoleu	D-Leu	D-Met	D-Phe
CD83	97.7±2.3	103.1±0.3	100.5±1.1	97.1±2.4	102.4±2.2	99.6±2.0
CD86	99.2±2.1	102.5±0.4	99.8±1.2	101.9±1.3	102.2±2.5	99.2±3.3
CD80	98.3±2.6	102.0±0.9	98.2±1.5	100.3±1.6	100.4±0.2	92.4±3.5
CD40	102.3±3.4	101.4±3.2	100.4±2.4	100.4±1.7	102.7±0.6	100.4±2.7
HLA-DR	98.1±1.1	99.9±0.9	100.1±0.3	98.0±10.0	98.9±2.0	98.0±3.2
	D-Pro	D-Ser	D-Thr	D-Trp	D-Tyr	D-Val
CD83	100.9±0.4	100.8±0.3	102.6±0.5	7.6±3.3	101.6±0.6	102.1±1.2
CD86	101.2±1.9	101.1±2.8	102.1±0.7	24.1±2.7	102.2±0.9	101.8±0.9
CD80	100.3±0.1	100.1±2.8	100.8±0.4	12.1±1.7	101.6±0.4	99.6 ±2.0
CD40	99.2±1.3	100.8±1.1	100.6±1.5	15.2±6.5	100.4±1.3	101.4±2.4
HLA-DR	98.4±2.6	98.6±0.6	97.1±3.9	88.9±3.0	98.4±2.4	100.0±1.0

651 *DCs were stimulated with LPS (0.1µg/ml) in the presence of the indicated D-amino acids

652 (10µM). Percentages of CD83, CD86, CD80 or CD40 expressing DCs were assessed.

653 Three independent experiments (mean percentages ± SD, relative to LPS-induced expression).

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Table II: Cytokine regulation by probiotic supernatants or D/L-Tryptophan in human LPS treated DCs*

	IL-10		IL-5		IFN- γ		IL-12		Ratios			
	[pg/ml]		[pg/ml]		[pg/ml]		[pg/ml]		IL-10/IL-12		IL-5/IFN- γ	
	-	LPS	-	LPS	-	LPS	-	LPS	-	LPS	-	LPS
Medium	3.20	2.90	14.70	68.30	112.50	2238.80	102.10	2092.80	0.031	0.001	0.131	0.031
DSM-20021	6.80	4.80	33.60	55.90	330.00	2520.50	447.80	2217.30	0.015	0.002	0.102	0.022
LGG	432.90	787.90	9.10	5.40	372.70	105.70	79.20	106.90	5.466	7.370	0.024	0.051
LA-2	107.30	591.70	8.00	10.30	111.60	437.70	89.30	238.00	1.202	2.486	0.072	0.024
LA-5	81.30	305.70	7.60	8.00	113.30	531.80	87.50	331.10	0.929	0.923	0.067	0.015
LC-01	452.40	924.50	7.90	2.40	109.30	211.30	76.90	67.80	5.883	13.636	0.072	0.011
BB-12	234.90	735.70	11.00	10.90	75.40	437.00	91.50	228.20	2.567	3.224	0.146	0.025
BB-46	813.50	1230.70	14.00	13.60	13.50	637.90	95.10	202.30	8.554	6.084	1.037	0.021
BB-420	450.40	915.40	8.80	8.40	81.50	783.70	102.50	356.90	4.394	2.565	0.108	0.011
L-Trp	5.70	4.90	12.00	61.40	45.00	2031.50	88.30	1993.00	0.065	0.002	0.267	0.030
D-Trp	56.90	202.50	10.30	20.60	21.90	1129.50	82.50	871.90	0.690	0.232	0.470	0.018
L-Pro	**	6.00	14.80	57.70	88.90	2133.90	99.80	1938.00	**	0.003	0.166	0.027
D-Pro	5.90	4.00	15.80	69.10	92.60	2295.40	90.60	1911.90	0.065	0.002	0.171	0.030

*DCs were stimulated in the presence or absence of LPS (0.1 μ g/ml) with supernatants from 200 μ l bacterial cell free supernatants or Tryptophan enantiomers (10 μ M) for 14h. Non-probiotic DSM-20021 and blank medium (CDM1) were used as negative control. D/L-prolin and L-Tryptophan were used as controls for D-Tryptophan. ** below detection limit

FIGURE LEGENDS**Figure 1. Screening of supernatants from different of probiotic strains for immune activity on human cells**

A, Dose-dependent capacity of bacterial supernatants from *Lactobacillus rhamnosus* GG (●), *Bifidobacterium* BB-420 (▼) and *Lactobacillus casei* W56 (■) to lower CCL17 secretion of human Hodgkin lymphoma KM-H2 cells. Negative control: non-probiotic *Lactobacillus* DSM-20021(▲). Three independent experiments in duplicates (mean percentages $\pm$ SD, relative to CCL17 secretion of untreated KM-H2 cells). Student's t test; $**p \leq 0.005$, $***p \leq 0.0005$. **B**, Capacity of supernatants from *Lactobacillus rhamnosus* GG, *Bifidobacterium* BB-420, *Lactobacillus casei* W56 or non-probiotic *Lactobacillus* DSM-20021 to prevent full upregulation of costimulatory molecules and HLA-DR on LPS stimulated human monocyte derived dendritic cells. +/- with/without bacterial supernatant. Five independent experiments (mean percentages $\pm$ SD relative to LPS alone). Dunn's Multiple Comparison Test; $**p < 0.01$, $***p < 0.001$.

Figure 2. Overview on the ability of bacterial supernatants from all 37 strains to lower CCL17 secretion of KM-H2 cells.

Shaded bars: non-probiotic *Lactobacillus* DSM-20021 (negative control); *L. rhamnosus* GG was included as positive control in all experiments with strains other than Lactobacilli. White bars: untreated KMH2 and medium controls

Three independent experiments in duplicates (mean percentages $\pm$ SD, relative to CCL17 secretion of untreated KM-H2). Student's t test; $**p < 0.005$ and $***p < 0.0005$.

Figure 3. Capacity of subfractions of probiotic supernatants to lower CCL17 secretion in KM-H2 cells.

Subfractions with different polarity (MeOH/H₂O gradient chromatography) from supernatants of *Lactobacillus rhamnosus* GG (top), *Lactobacillus casei* W56 (middle), Negative controls: Non-probiotic DSM-20021 and blank CDM1 medium (bottom). Three independent experiments in duplicate (mean percentages $\pm$ SD, relative to constitutive CCL17 secretion of untreated KM-H2 cells). Student's t test; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Figure 4. Effect of Tryptophan L- and D-isomers on CCL17 secretion by KM-H2 cells.

KM-H2 cells were stimulated with different concentrations of synthetic L- and D-isomers of Tryptophan followed by CCL17 quantification in KM-H2 culture media after 24h. (●) D-Tryptophan, (◆) L-Tryptophan. Three independent experiments in duplicates (mean percentages $\pm$ SD, relative to constitutive CCL17 secretion of untreated KM-H2 cells). Student's t test; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Figure 5. Oral D-Tryptophan reduces allergic airway inflammation

A, Serum D-Tryptophan in mice receiving D-Tryptophan (50 mM) in drinking water or water only (UPLC-MS peak areas). Note the different scales for D-Tryptophan (black bars) and L-Tryptophan (grey bars). Welch Test, mean $\pm$ SD, ** $p = 0.006$, *** $p = 0.004$. **B**, Total number of cells in BALF. **C**, Differential cell count. **D**, Measurement of airway resistance to increasing doses of methacholine. Two-way ANOVA with Bonferroni post-test. **E**, Geometric mean (fold change) of Ifn γ and Il4 in lung-derived CD3⁺CD4⁺ lymphocytes. **F**, Il4 levels in BALF of mice, assessed by CBA. **G**, Helios⁺ Tregs of lung-derived CD3⁺CD4⁺ Foxp3⁺ lymphocytes. (**B,C,F**) $n = 8$ mice per group, Mann-Whitney U. (**D,G**) $n = 6-12$ mice per group, Student's T-Test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6. D-Trp influences *in vitro* primary T cell differentiation

729 Primary, murine splenocytes were differentiated towards, Th1 **(A)**, Th2 **(B)**, and Treg **(C)** with
730 respective cytokine mixes under the presence of 0, 10 or 50 μ M D-Trp (dissolved in water).
731 Differentiation was assessed by flow cytometry, qRT-PCR and CBA for Il13 and Il5 protein
732 levels from culture supernatants. Graphs depict fold changes to differentiated cells not treated
733 with D-Trp. n=3-4 independent experiments, Mann-Whitney U, *p<0.05

734

735 **Figure 7. Oral D-Tryptophan supplementation increased gut Treg and the intestinal**
736 **bacterial community in mice with AAI**

737 **A**, Percentage of Foxp3⁺ cells within CD3⁺CD4⁺ T cells in lamina propria of the colon. n=6-
738 12 mice per group, Student's *t* test.***P<0.0001. **B**, Alpha diversity of bacterial communities.
739 Shannon diversity index was used to estimate bacterial diversity for each treatment. Wilcoxon
740 rank-sum test.

Figure 1

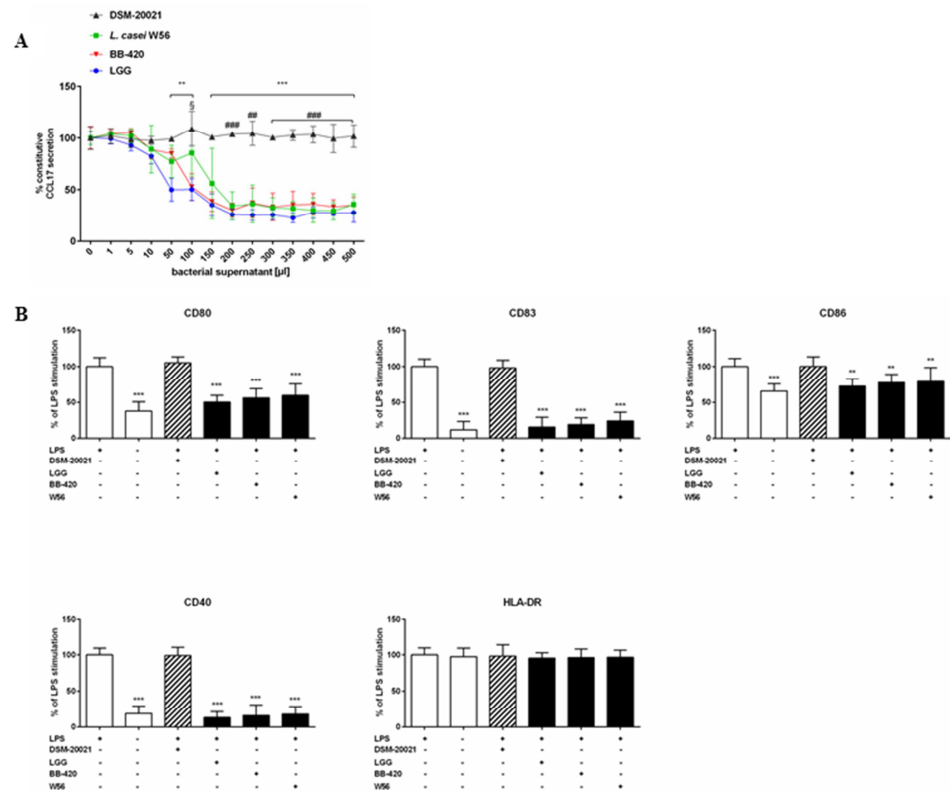


Figure 2

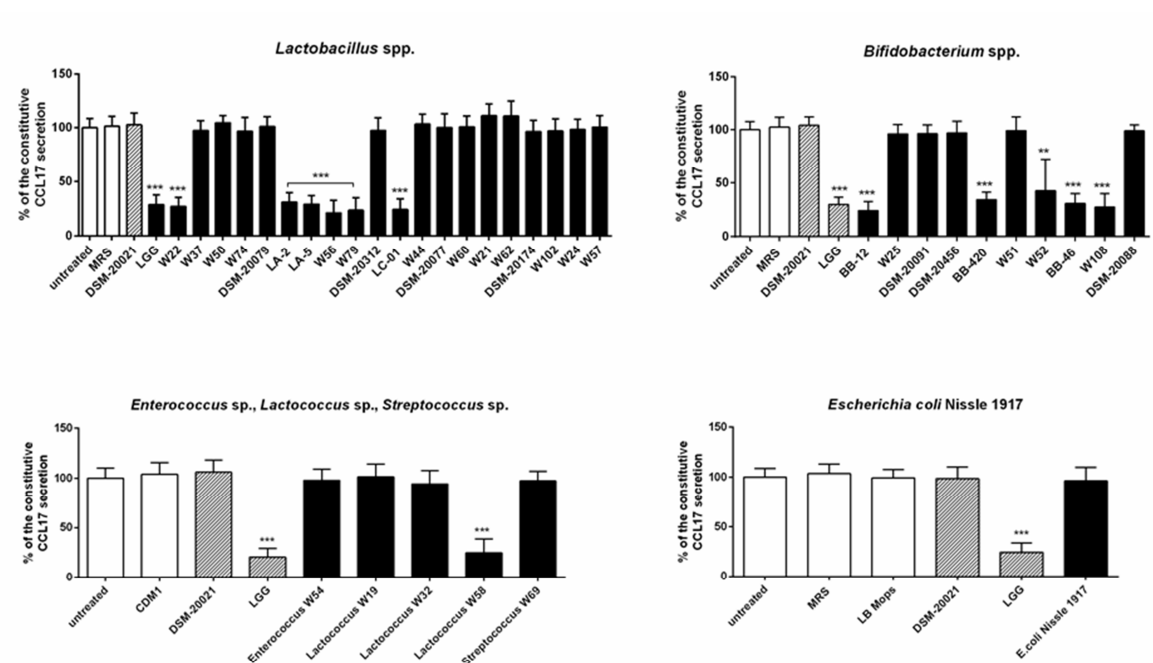


Figure 3

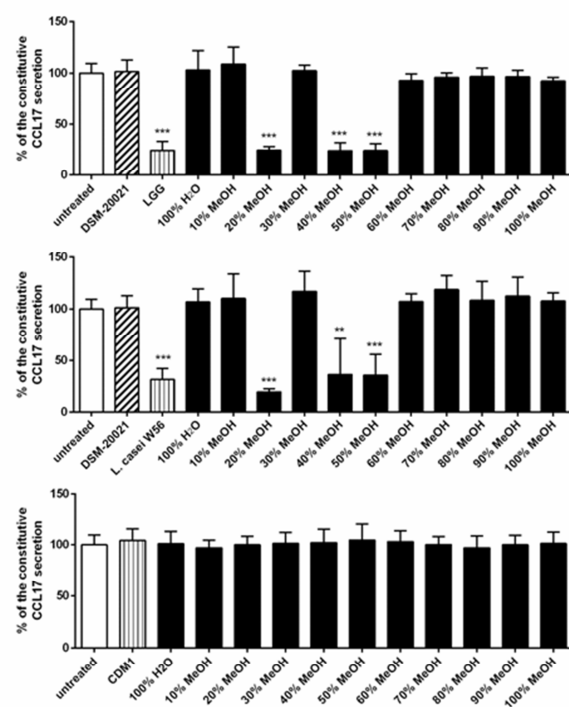


Figure 4

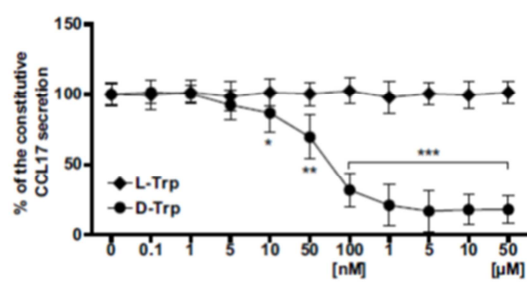


Figure 5

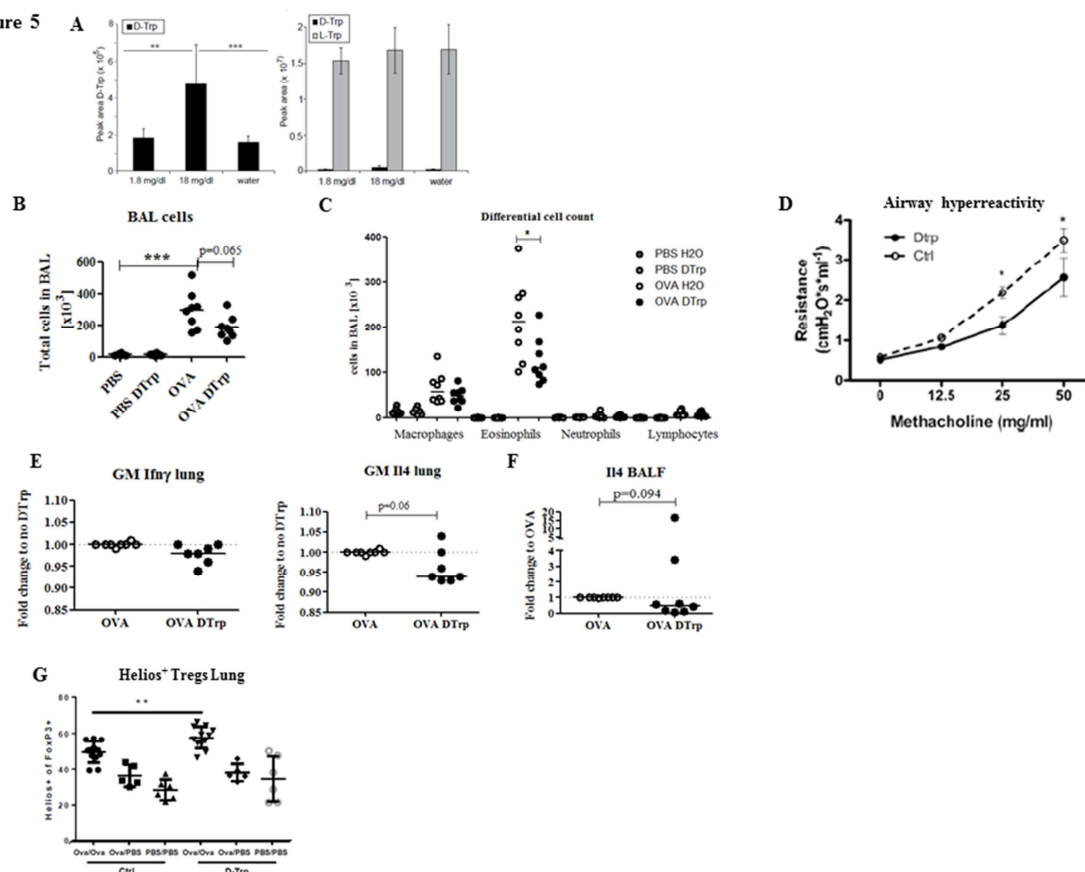


Figure 6

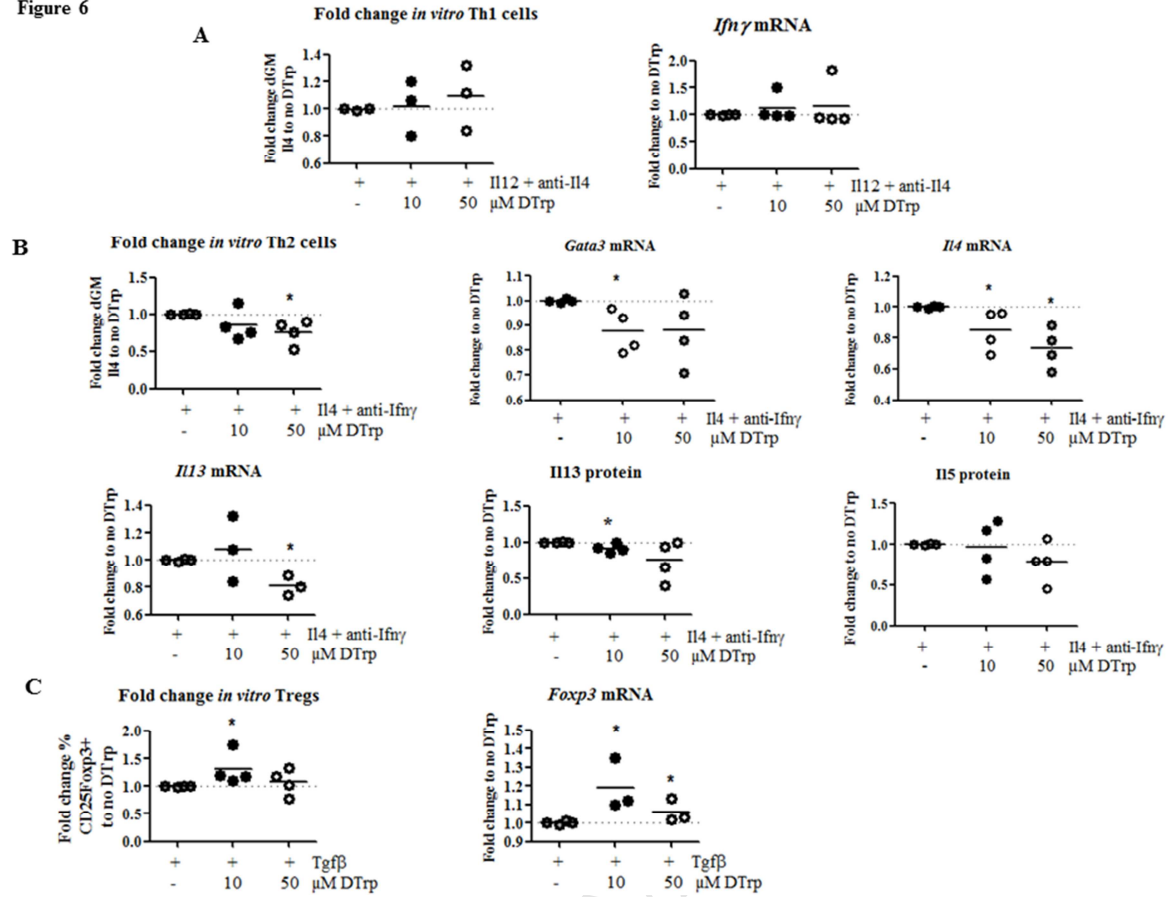
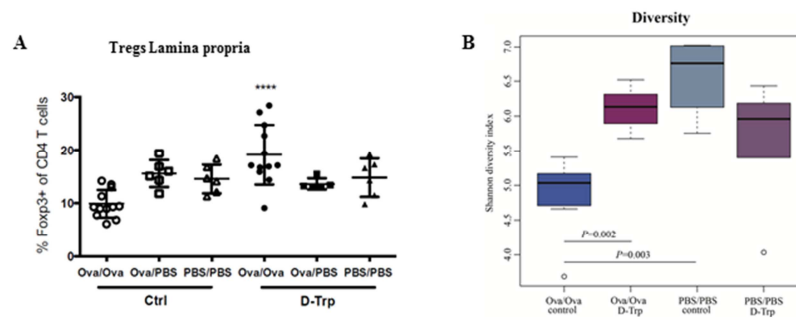


Figure 7



D-Tryptophan from probiotic bacteria influences the gut microbiome and allergic airway disease

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Materials and Methods

Reagents

L-Tryptophan and D-amino acids (A, F, H, I, L, M, P, S, T, V, W, Y) were purchased from Carl Roth GmbH, Karlsruhe, Germany.

Growth conditions of bacteria and collection of supernatants

For the primary screen of bioactivity, probiotic strains were grown in complex de Man-Rogosa-Sharpe (MRS) medium (Applichem, Darmstadt, Germany) at 37 °C under microaerobic conditions in an Incubator (Thermo Fisher Scientific, Waltham, USA). For metabolite analyses, the strains were grown in modified defined medium CDM1 (1) which contains L-Tryptophan among 19 other L-amino acids, at 37 °C. In contrast to the original medium, we omitted Tween 80, as it is known to interfere with mass spectrometric analyses (2). *E. Coli Nissle 1917* was grown aerobically in Luria-Bertani (LB)-Medium on a rotary shaker (New Brunswick Scientific, Enfield, USA) (200 rpm) at 37 °C.

Generation of human monocyte-derived dendritic cells

Peripheral venous blood was obtained from healthy volunteers aged between 20 and 50 years after informed consent. Individuals with allergic disease, acute infection or taking any medication 20 days prior to blood sampling or any history of smoking smokers (personal interview) were excluded prior to blood sampling. All experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood by density gradient centrifugation (2000 rpm, 22 °C, 20 min) (Biocoll, Biochrom AG, Berlin, Germany). Adherent monocytes were obtained from PBMC via plastic adherence in 12-well plates (Nunc, Wiesbaden, Germany) in 1.5 mL very low endotoxin (VLE) RPMI 1640 medium (Biochrom AG, Berlin, Germany) supplemented with 5% human serum type AB (CELLect[®], MP Biomedicals[™], Eschwege, Germany) and 1% glutamine (Gibco[®] Invitrogen, Karlsruhe, Germany) for 1 h. Granulocyte macrophage colony-stimulating factor (GM-CSF) and Interleukin (IL)-4 (CellGenix, Freiburg, Germany) were added on days 1, 3, and 6 at 100 ng/mL and 20 ng/mL, respectively, yielding 2-3 x 10⁶ immature DCs per well. Purity and viability of DCs was assessed by flow cytometry (Fig E2A-C).

Human T cell line KM-H2

The human Hodgkin lymphoma T cell line (KM-H2) was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. 3-5 x 10⁶ KM-H2 cells were grown in 4 mL RPMI 1640 per well in 6-well-plates (Nunc, Wiesbaden, Germany). All eukaryotic cells were cultured in 5% CO₂ at 37 °C (HeraCell 240 and Heraeus BDD 6220, Thermo Fisher Scientific, Waltham, USA). Viability of cells after treatment with bacterial supernatants was assessed by trypan blue, 7-AAD and CellTiter-Blue[®] staining. (Fig E2A-C)

Isolation of intestinal lamina propria cells

Total colons were excised from mice, removed from mesenteries, opened longitudinally and cut into 2 cm long pieces that were incubated for 30 min in 30 mM EDTA in PBS on ice and then shaken vigorously with PBS to remove epithelial cells. After further cutting into small pieces with sharp scissors these were digested at 37 °C in RMPI supplemented with 1 mg/mL collagenase D (Roche Diagnostics, Mannheim, Germany) and 10 mg/ml DNase I (Sigma-Aldrich, St. Louis, USA). Cells were separated by a 40/80% (w/v) Percoll (GE Healthcare, Chalfont St. Giles, UK) density centrifugation step and washed prior to staining for flow cytometry analysis.

Differentiation of murine primary T cells

Murine spleens from female Balb/c mice were filtered through a 40 µm filter to generate single cell suspensions. These were then subjected to red blood cell lysis by using RBC lysis buffer (Biolegend, San Diego, USA) according to the manufacturer's recommendations. Afterwards, naïve CD4⁺ T cells were isolated via magnetic-activated cell sorting (MACS) using the naïve CD4⁺ T cell isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). The resulting naïve CD4⁺ T cells were cultured in 96 wells plates (1x10⁶ cells/ml Medium) in TexMACS medium supplemented with 10% FCS, 0.01 mM 2-Mercaptoethanol, rIL-2 (10 ng/ml) as well as MACSiBead particles with anti-CD3 and anti-CD28 (1x10⁶ beads per 1x10⁶ cells) (all Miltenyi Biotec). Cells were differentiated over a period of 6 days towards Th1 (10 ng/ml IL12 and 10 µg/ml anti-IL4, derived from Cytobox Th1), Th2 (10 ng/ml IL4 and 10 µg/ml anti-Ifnγ, derived from Cytobox Th2) and Treg (5 ng/ml) (all Miltenyi Biotec). Differentiation of cells was assessed by flow cytometry (see Fig E8 for gating strategy), enhanced cytometric bead array (CBA) from cell culture supernatants and qRT-PCR. Prior to CBA and flow cytometry, cells were

stimulated with monensin (0.7 μ L/mL, BD Golgi Stop, BD Bioscience, Heidelberg, Germany) for 4 h at 37 °C in the presence of Ionomycin (500 ng/ml, Sigma Aldrich, Missouri, USA) and PMA (5ng/ml, Sigma Aldrich, Missouri, USA).

Flow cytometry

Costimulatory molecules on human DCs

The following primary monoclonal antibodies were used: CD83 fluorescein isothiocyanate (FITC); CD1a phycoerythrin (PE); CD86-FITC; CD80-PE; CD14 allophycocyanine (APC); CD40-APC; CD3-peridinin chlorophyll protein (PerCP); HLA-DR-PerCP; mouse immunoglobulins G₁ (IgG₁) mouse immunoglobulins G₁ (IgG₁) κ were used as isotype controls using the corresponding fluorochromes (all purchased from BD Biosciences, Heidelberg, Germany). To account for donor dependent variability of DC surface markers, all data were normalized to the expression induced by LPS which was set to 100% after subtraction of background values (FACS Canto, FACS DIVA software, Version 5.0.3, BD Biosciences, Heidelberg, Germany).

Murine DC subsets and CD4⁺ T cells

Single cell suspensions of splenocytes were obtained by filtering spleens through a 70 μ m strainer. Samples were stained with CD3-PB (1:200, Biolegend, San Diego, USA), CD4-APC-H7 (BD, 1:400), CD11c- APC-Cy7 (1:100), Mhc-II-Percp-Cy5 (1:100), CD11b-FITC (1:100), CD80-AF647, CD40-APC (1:100). For intracellular cytokines, total spleen cells were stimulated with anti-CD3 (4 μ g/mL, BD Bioscience, Heidelberg, Germany) and anti-CD28 (30 ng/mL, BD Bioscience, Heidelberg, Germany) for 16 h. 1×10^6 of total lung cells were stimulated for 16h with MACSiBead particles coated with anti-CD3 and anti-CD28 using the T cell activation/expansion kit (Miltenyi Biotec, Bergisch Gladbach, Germany) in TexMACS medium (Miltenyi Biotec) according to the manufactures

recommendations. Prior to staining, cells were treated with monensin (0.7 μ L/mL, BD Golgi Stop, BD Bioscience, Heidelberg, Germany) for 6 h at 37 °C and intracellularly stained for IL-4-PE (1:100, eBioscience, Vienna, Austria according to the manufacturer's protocols. Intracellular staining for Foxp3 was performed by using the Foxp3/Transcription Factor Staining buffer set (eBiosciences, Vienna, Austria) according to the manufacturer's recommendations.

The analysis was performed on an LSRII, with DIVA TM Software v8.0 (BD Bioscience, Heidelberg, Germany), kindly provided by the core unit fluorescence cytometry of the Research Center Borstel

Assessment of cytokines by cytometric bead array

Levels of IL4, IL5 and IL13 were analysed in BALF and cell culture supernatants using an enhanced cytometric bead array (eCBA, Fex Set Kits, BD Biosciences, Franklin Lakes, New Jersey, USA) according to the manufacturer's guidelines.

Murine regulatory T cells

Cells were preincubated with Fc-Block (BD Bioscience, Heidelberg, Germany) for 5 min and stained for 20 min with the following antibodies: FITC-conjugated anti-CD3, Alexa-Fluor700-conjugated anti-CD4 and APC-eFluor780-conjugated CD45.2. For intracellular staining, cells were fixed and permeabilized with a commercially available fixation/permeabilization buffer (eBioscience, Vienna, Austria). LIVE/DEAD fixable Aqua dead stain kit (Invitrogen, Carlsbad, USA) was used prior to fixation. Intracellular staining was performed with PerCP-Cy5.5-conjugated anti-Foxp3 and Helios. Gates were set on live CD45⁺CD3⁺CD4⁺ T cells. All antibodies were from eBioscience (Vienna,

Austria). Cells were analyzed with a flow cytometer (Fortessa and LSRII, BD Bioscience, Heidelberg, Germany) and analyzed with Flowjo software (Flowjo LLC, Ashland, USA).

Quantification of CCL17 and cytokines in eukaryotic cell culture supernatants

CCL17 was quantified in cell culture supernatants of KM-H2 cells using ELISA reagent Quantikine CCL17/TARC, (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions (ELISA reader MRXII, Thermo Fisher Scientific, Waltham, USA).

For cytokine analyses, cell-free culture supernatants were collected from DCs after 24 h incubation with either probiotic supernatants or D-Tryptophan, and were stored in aliquots at -80 °C before analysis. For probiotic supernatants, blank CDM1 was used as medium control. For D-Tryptophan we used L-Tryptophan, D- and L-Proline as control. IL-5, IFN- γ , IL-12, and IL-10, were quantified by a multiplex assay (Milliplex Human Cytokine Immunoassay, Millipore GmbH Schwalbach, Germany) as described by the manufacturer.

Bioassay-guided fractionation of probiotic supernatants and structural elucidation of D-Tryptophan

Each fractionation step was controlled and driven by the results from the bioassays.

Fractionation according to polarity

Bioactive cell-free supernatants were first fractionated using stepwise gradient elution in solid phase extraction cartridges. We applied 6 mL of bacteria free supernatants from *Lactobacillus casei* W56 and *Lactobacillus rhamnosus* GG and CDM1 medium (control) into SPE-C₁₈ cartridges (1 g, Mega Bond Elut, Varian, Agilent Technologies, Santa Clara,

USA). We eluted in 10 steps using 2 mL of methanol/water solutions from 0% to 100% MeOH. Each resulting eluate was divided in two equal volumes and dried in a SpeedVac (SpeedVac Concentrator, Savant SPD 121P, Thermo Fisher Scientific, Waltham, USA) for further bioassay experiments and chemical analyses. MeOH/water extracts that showed bioactivity were resolved in 500 μ L of 10% MeOH/water solution and further subjected to a second fractionation using a pentafluorophenyl chromatographic column (Kinetex PFP 1.7 μ m, 2.1 x 150 mm, Phenomenex, Torrance, USA) in order to have a complementary selectivity to C₁₈ phase. A nonlinear gradient in 10 min from 5 to 25% B, 14 min to 100% B at 40 °C with 0.180 mL/min flow rate (Mobile phase A: 10% MeOH/H₂O; B: 100% MeOH) was applied.

To this end, we coupled an Ultra Performance Liquid Chromatography system (UPLC-PDA, Waters, UK) to an automatic fraction collector (TriVersa NanoMate, Advion BioSciences, Ithaca, USA) to originate new sub-fractions, which were retested in our bioassays. UPLC and collection methods were defined for the bioactive 20% MeOH/water extract according to its chromatogram at λ =200nm in an attempt to collect single peaks or at least, reduce the complexity present in each collected sub-fraction. To obtain a large volume of each sub-fraction, the separation and collection process was repeated 15 times. The results from the bioassays drove the chemical characterization of the newly obtained bioactive sub-fractions.

Structural elucidation of the bioactive compound present in 20% MeOH extracts

The bioactive sub-fractions and their nearest neighbors collected from the second step fractionation in PFP columns were reevaluated via UPLC (Acquity, Waters, Elstree, UK)

coupled to high resolution TOF Mass Spectrometer (maXis URH-TOF, Bruker Daltonics, Bremen, Germany) to identify a candidate compound by comparing peak retention time and m/z values between chromatograms. Here, we used reversed phase chromatography (BEH-C₁₈ 1.7 μ m, 2.1 x 150 mm, Waters) and gradient elution from 0 to 100% B in 8 min at 40 °C (A: 10% MeOH / 0.1% formic acid / water; B: 0.1% formic acid / MeOH; flow rate: 0.4 mL/min). Total Ion Chromatograms were obtained in electrospray ionization (ESI) positive mode. We isolated the candidate compound by repeated chromatographic runs followed by peak collection until 1 ml of volume was obtained to purify the bioactive sub-fraction for further molecular formula assignment by high resolution FT-ICR-MS in positive and negative ESI mode (APEX-Qe 12 Tesla, Bruker Daltonics, Bremen, Germany) and for structural elucidation by proton NMR (UltraShield Plus 800 MHz, Bruker Biospin, Billerica, USA).

Chiral separation of amino acid enantiomers in fractionated supernatants

After chemical characterization of the bioactive sub-fraction, it was important to know whether both isomers of Tryptophan were present in the solution. Therefore, we applied a derivation technique using o-phthaldialdehyde (OPA) and N-isobutyryl-L-cysteine (IBLC) (Sigma-Aldrich, St. Louis, USA) according to a previously described method(3). Standard solutions containing D- and L-Tryptophan and our bioactive sub-fraction were then analyzed by reversed phase chromatography using a small diameter UPLC column (BEH-C₁₈ 1.7 μ m, 1.0 x 150 mm, Waters, Elstree, UK) with isocratic elution at 45% B for 3 min at 60 °C (A: 20 mM sodium acetate; B: 7% acetonitrile in MeOH; flow rate: 0.1 mL/min) coupled to a fluorescence detector (λ =300 nm for the excitation; λ =445 nm for the emission).

Enantiomeric separation of D-Tryptophan in murine sera

The investigator (C.M.) was blinded to the murine intervention groups. Due to the abundance of interfering proteins a protein precipitation was done. For this purpose the sera were thawed on ice and 20 μ L of each sample were vigorously shaken with 80 μ L 4 ° C methanol (Chromasolve, Fluka, St. Louis, USA) and centrifuged (15,000 x g at 4 °C for 15 min). The supernatants were taken, evaporated and resolved in water before injection. The derivation was performed as described for bacterial supernatants with some modifications as recently published (2). For quantification human serum was spiked with different concentrations (0.005-0.15 μ g/mL) of D-Tryptophan and randomly analyzed. The enantiomeric ratio (peak area D-Tryptophan/peak area L-Tryptophan) was calculated, and was observed to follow a linear regression ($y=0.0406x+0.0102$, $R^2>0.98$).

Induction of allergic airway inflammation

Female 6-8 week old Balb/c mice (Charles River Laboratories, Wilmington, USA) were sensitized i.p. using 10 μ g of ovalbumin (grade VI; Sigma Aldrich, St. Louis, USA) or PBS (controls) in alum (Pierce Chemical Co, Rockfort, USA) at day 0, 7 and 14 and challenged intranasally under isoflurane narcosis with 10 μ g of ovalbumin in 20 μ l PBS or PBS only (controls).

Lung Function

Animals were anesthetized i.p. with ketamine (140 mg/kg) and xylazine (7 mg/kg), tracheostomized, intubated (18G tube), placed on a warming plate and ventilated with a tidal volume of 10 mL/kg at a frequency of 150 breaths/minute and a positive end-expiratory pressure of 2 cm H₂O on a Buxco R/C system (Buxco Research Systems, Wilmington, USA). To assess airway hyperreactivity, the mice were challenged with

metacholine in physiological saline generated with an in-line nebulizer and administered directly with increasing concentrations (0, 12.5, 25, 50 mg/mL) by the ventilator for 20 seconds. Resistance (R) and Compliance (C) were measured continuously for 2 min and the average was calculated and plotted against concentration.

Gene expression analysis in murine fetal lungs

Fetal lungs were collected from animals delivered via cesarean section at embryonic day 18.5 (Balb/c). Total RNA was isolated employing the miRNeasy Mini (Qiagen, Venlo, Netherlands) including digestion of remaining genomic DNA. The Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA) was used to assess RNA quality and only high quality RNA ($RIN \geq 8.7$) was used for microarray analysis. For mRNA profiling, 30 ng total RNA was amplified using the Ovation PicoSL WTA System V2 in combination with the Encore Biotin Module (Nugen, San Carlos, USA). Amplified cDNA was hybridized on an Affymetrix Mouse Gene ST 2.1 array plate. Staining and scanning was done according to the Affymetrix expression protocol including minor modifications as suggested in the Encore® Biotin protocol (NuGen, San Carlos, USA).

Bacterial 16S rRNA gene amplification and diversity analysis

Diversity analysis of 16S rRNA genes was performed by amplicon sequencing. In the first PCR reaction, bacterial genomic DNA was subjected to 16S rRNA gene amplifications using the primer S-D-Bact-0785-a-S-18[5'-GGMTTAGATACCCBDGTA-3'] and S-*Univ-1100-a-A-15 [5'-GGGTYKCGCTCGTTT-3'] as already mentioned. The reaction mixture of 25 μ L in total was composed of 5 ng $\times$ μ L⁻¹ template DNA, 10 μ M of each primer, 10 mM dNTPs (Fermentas, Vilnius, Lithuania), 5% of dimethyl

sulfoxide (Sigma-Aldrich, St. Louis, USA), 5 U x μ L⁻¹ of FastStart High Fidelity Polymerase (Roche Diagnostics, Mannheim, Germany), 10x FastStart Buffer, and nuclease-free water (Life Technologies, Carlsbad, CA, USA). The PCR started with an initialization at 95°C for 5 min, followed by 28 cycles of denaturation at 94°C for 45 sec, annealing at 44°C for 45 sec, and elongation at 72°C for 45 sec. A final elongation step at 72°C for 5 min completed the PCR reaction. To minimize contamination with primer dimers, generated fragments were cut out of the gel after standard agarose gel electrophoresis and purified with the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany).

During the second PCR, Illumina sequencing adapters as well as dual indices were attached to the purified amplicons using the Nextera XT Index Kit (Illumina, San Diego, CA, USA). The reaction volume was 50 μ L in total and contained 5 μ L of genomic DNA, 5 μ L of each Nextera XT Index Primer, 25 μ L 2x KAPAHiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA), and 10 μ L of nuclease-free water (Life Technologies, Carlsbad, CA, USA). The PCR reaction was performed according to the following thermal profile: 95°C for 3 min, followed by 8 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and finalized by 72°C for 5 min. The PCR products were cleaned up with the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA), DNA was quantified and the DNA quality was controlled using the 2100 Bioanalyzer Instrument (Agilent Technologies, Santa Clara, CA, USA), and sequenced with the MiSeq instrument (Illumina, San Diego, CA, USA).

Gene expression analysis

Total RNA was isolated from homogenized lung tissue or cell culture using the

miRNeasy Micro Kit according to manufacturer's instructions (Qiagen, Venlo, Netherlands). Concentrations were determined using a NanoDrop® ND-1000 (NanoDrop Technologies, Erlangen, Germany) spectrophotometer. mRNA was transcribed to cDNA with the QuantiTect Rev. Transcription kit (Qiagen, Venlo, Netherlands) and PCR for specific genes was performed on a LightCycler 480 platform with Light Cyclyer 480 SYBR Green I Mastermix (Roche, Mannheim, Germany). Detailed qPCR primer sequences are listed in table E3.

References

1. Savijoki K, Suokko A, Palva A, Varmanen P. New convenient defined media for [(35)S]methionine labelling and proteomic analyses of probiotic lactobacilli. *Lett Appl Microbiol.* 2006 Mar;42(3):202–9.
2. Müller C, Fonseca JR, Rock TM, Krauss-Etschmann S, Schmitt-Kopplin P. Enantioseparation and selective detection of D-amino acids by ultra-high-performance liquid chromatography/mass spectrometry in analysis of complex biological samples. *J Chromatogr A.* 2014 Jan 10;1324:109–14.

Figure legends**Figure E1. Kinetic and volumes of supernatants from probiotic bacteria able to lower CCL17 secretion from KM-H2 cells.**

A, Capacity of supernatants from bacteria cultured in rich MRS medium to lower CCL17 secretion of KM-H2 cells

B, Capacity of supernatants from bacteria cultured in restricted CDM1 medium to lower CCL17 secretion of KM-H2 cells

(■ 2 h; ● 6 h; ▲ 12 h; ◆ 24 h; ▼ 48 h). Data are shown each from three independent experiments in duplicate (mean percentages $\pm$ SD, relative to constitutive CCL17 secretion of untreated KM-H2).

Figure E2. Viability of KM-H2 cells and primary DCs

Viability analysis of KM-H2 cells (upper panel) and primary DCs (lower panel) after treatment with probiotic supernatants with trypanblue (**A**), 7-AAD staining (**B**) and photometric analysis after treatment with CellTiter-Blue® reagent (**C**). **D**, Purity assessment of primary DCs via flow cytometry before (left panel) and after LPS stimulation (right panel) (representative image). (right panel)

Figure E3. UPLC-PDA chromatogram of 20% MeOH/H₂O extract from L. casei W56 supernatant

A, Sub-fractions and their time of collection were decided based on the peaks observed in the chromatogram. **B**, Immune modulatory activity was observed for sub-fractions 7, 8 and 9. Chromatographic conditions: Kinetex PFP column 1.7 μ m particle size, 2.1 x 150mm. Nonlinear gradient in 10 min from 5 to 25% B, 14 min to 100% B at 40 °C with 0.180 mL/min flow rate (Mobile phase A: 10% MeOH/H₂O; B: 100% MeOH).

Figure E4. UPLC-URH-TOF MS analyses of *L. casei* W56 and LGG**bioactive sub-fractions and their nearest neighbors.**

A, Total ion chromatograms in ESI+ using reversed phase chromatography (C18 column: 1.7 μm , 2.1 x 150 mm) showing a peak at t_R 4.2 min for all bioactive sub-fractions. **B**, Extracted mass spectra corresponding to the chromatographic time range of 4.1-4.3 min. The same signal profile was observed for all bioactive sub-fractions, where m/z 409.1875, 205.0971 and 188.0702, strongly agree with the Tryptophan ions $[2M+H]^+$, $[M+H]^+$ and its fragment $[M+H-NH_3]^+$ (<http://www.massbank.jp/>). **C**, and **D**, FT-ICR-MS spectra of the purified bioactive sub-fraction 7 of LGG in ESI negative mode and ESI positive mode respectively. The assigned molecular formula is $C_{11}H_{12}N_2O_2$ with an error <0.01 ppm.

Figure E5: Proton NMR spectra of probiotic bacterial supernatant and L-Tryptophan.

The upper panel of both figures shows an enlargement of the shaded areas in the lower panel.

A, 800 MHz ^1H NMR spectrum of probiotic bacterial supernatant; note the low sample concentration (asterisk: ^{13}C satellites at 0.5% intensity of H_2O with 99.95% ^2H). Aromatic spin systems can be recognized, including J-couplings, whereas aliphatic nuclei show partial splitting due to low signal to noise (S/N) ratio.

B, 800 Hz ^1H NMR spectrum of L-Tryptophan in CD_3OD with aromatic (yellow) and aliphatic (green) spin systems indicated (note: D- and L-Tryptophan produce identical NMR spectra in achiral solvents).

Figure E6. Comparative UPLC-FLD chromatogram from the purified bioactive sub-fraction.

Comparative UPLC-FLD chromatogram from the purified bioactive sub-fraction.

Panel a: Subfraction 7 from LGG; Panel b: corresponding sub-fraction of blank

CDM1 medium. Enantiomer separation of Tryptophan derivatized with OPA-IBLC

shows the presence of D-Tryptophan only in the bioactive sub-fraction.

Figure E7: Oral D-Tryptophan supplementation ameliorates allergic airway

inflammation

A, Treatment scheme for induction of allergic airway inflammation, D-Trp was supplied in drinking water from day -3 in respective groups. **B**, Measurement of airway resistance to increasing doses of methacholine. **C**, Percent IL-4^+ and IL-13^+ cells within spleen $\text{CD3}^+\text{CD4}^+$ T cells. **D**, CD40^+ and CD80^+ on spleen $\text{CD11b}^{\text{high}}$ DCs, Box and whisker plots: Maximum and minimum values (whiskers), the upper and lower quartiles (boxes) and median (horizontal line). **(A)** 7-8 mice/group, mean $\pm$ SD, Two-way ANOVA with Bonferroni post-test. * $p < 0.05$, *** $p < 0.001$.

Figure E8. In vitro differentiation of primary T cells

Murine naïve CD4^+ cells were differentiated in vitro for 6 days with respective cytokines.

Gating strategy for analysis of **(A)** Th1 & Th2 cells as assessed by $\text{CD4}^+\text{Ifn}\gamma^+$ or

$\text{CD4}^+\text{IL4}^+$, respectively, and induced Tregs as $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$ cells **(B)**.

Representative images of $n=4$ independent experiments.

Figure E9. Influences of D-Tryptophan supplementation on the intestinal bacterial composition in healthy and diseased mice.

A, An unweighted UniFrac distance matrix based on OTU counts was used to perform Principal Coordinate Analysis (PCoA). The generated scatterplot indicates dissimilarities between individual samples. Statistical significance was determined with Student's t test,

P=0.001. All results are based on 95%-similarity OTUs. OTU, operational taxonomic unit. PC, principal coordinate. Ova, ovalbumin. PBS, phosphate buffered saline. **B**, Proportion of dominant bacteria (>0.05% abundance) in the intestinal tract of healthy and diseased mice. Pie charts were generated to visualize the relative distribution of the most abundant bacteria at the family level.

Figure E10. Expression of Slc6a family members in lungs of neonatal mice.

Sketch-quantile normalized linear intensity (mean $\pm$ SD). Background levels (dashed line). Male and female neonates (n=4 each).

TABLES

Table E1: Bacterial strains used in this study

Bacterial strain	Provider	Bioactivity on ¹	
		KM-H2	DC
<i>Lactobacillus rhamnosus</i> DSM-20021	Leibniz Institute DSMZ	-	-
<i>Lactobacillus rhamnosus</i> GG	Valio Ltd, Helsinki, Finland	+	+
<i>Lactobacillus acidophilus</i> W22	Winlove Bioindustries BV	+	+
<i>Lactobacillus acidophilus</i> W37	Winlove Bioindustries BV	-	-
<i>Lactobacillus acidophilus</i> W50	Winlove Bioindustries BV	-	-
<i>Lactobacillus acidophilus</i> W74	Winlove Bioindustries BV	-	-
<i>Lactobacillus acidophilus</i> DSM-20079	Leibniz Institute DSMZ	-	-
<i>Lactobacillus acidophilus</i> LA-2	Chr. Hansen	+	+
<i>Lactobacillus acidophilus</i> LA-5	Chr. Hansen	+	+
<i>Lactobacillus casei</i> W56	Winlove Bioindustries BV	+	+
<i>Lactobacillus casei</i> W79	Winlove Bioindustries BV	+	+
<i>Lactobacillus paracasei</i> DSM-20312	Leibniz Institute DSMZ	-	-
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> LC-01	Chr. Hansen	+	+
<i>Lactobacillus gasseri</i> W44	Winlove Bioindustries BV	-	-
<i>Lactobacillus gasseri</i> DSM-20077	Leibniz Institute DSMZ	-	-
<i>Lactobacillus helveticus</i> W60	Winlove Bioindustries BV	-	-
<i>Lactobacillus plantarum</i> W21	Winlove Bioindustries BV	-	-
<i>Lactobacillus plantarum</i> W62	Winlove Bioindustries BV	-	-
<i>Lactobacillus plantarum</i> DSM-20174	Leibniz Institute DSMZ	-	-
<i>Lactobacillus rhamnosus</i> W102	Winlove Bioindustries BV	-	-
<i>Lactobacillus salivarius</i> W24	Winlove Bioindustries BV	-	-
<i>Lactobacillus salivarius</i> W57	Winlove Bioindustries BV	-	-
<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> BB-12	Chr. Hansen, Horsholm, Denmark	+	+
<i>Bifidobacterium breve</i> W25	Winlove Bioindustries BV, Amsterdam, Netherlands	-	-

Bacterial strain	Provider	Bioactivity on ¹	
		KM-H2	DC
<i>Bifidobacterium breve</i> DSM-20091	Leibniz Institute DSMZ- German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany	-	-
<i>Bifidobacterium bifidum</i> DSM-20456	Leibniz Institute DSMZ	-	-
<i>Bifidobacterium lactis</i> BB-420	Danisco, Niebüll, Germany	+	+
<i>Bifidobacterium lactis</i> W51	Winclove Bioindustries BV	-	-
<i>Bifidobacterium lactis</i> W52	Winclove Bioindustries BV	+	+
<i>Bifidobacterium longum</i> BB-46	Chr. Hansen	+	+
<i>Bifidobacterium longum</i> W108	Winclove Bioindustries BV	+	+
<i>Bifidobacterium longum subsp. infantis</i> DSM-20088	Leibniz Institute DSMZ	-	-
<i>Enterococcus faecium</i> W54	Winclove Bioindustries BV	-	-
<i>Lactococcus lactis</i> W19	Winclove Bioindustries BV	-	-
<i>Lactococcus lactis</i> W32	Winclove Bioindustries BV	-	-
<i>Lactococcus lactis</i> W58	Winclove Bioindustries BV	+	+
<i>Streptococcus thermophilus</i> W69	Winclove Bioindustries BV	-	-
<i>Escherichia coli</i> Nissle 1917	Ardeypharm GmbH, Herdecke, Germany	-	-

¹Ability of bacterial cell free supernatants to lower CCL17 secretion by KMH2 cells (left) and to reduce LPS-induced up-regulation of costimulatory molecules on human monocyte derived dendritic cells (DC, left)

393 **Table E2:****Table E2. Regulation of GPR109B (NIACR2) in asthmatic or atopy subjects compared to controls ***

Experimental conditions	GEO Acc.	p value	fold change
Airway epithelial cells from children with atopic asthma vs. healthy children	GSE18965	0.0447	-2.95
Airway epithelial cells 4hr after saline exposure - atopy and asthma subjects vs. control	GSE8190	0.0042	-1.53
CD4+ T cells isolated from blood of patients with non-severe asthma vs. healthy subjects	GSE31773	1.8E-5	-2.29
CD8+ T cells isolated from blood of patients with non-severe asthma vs. healthy subjects	GSE31773	0.0002	-1.91

* Data for GPR109B association with asthma was extracted from the Disease Atlas Database (www.nextbio.com).

Table E3: primer sequences used for qRT-PCR

Name	fwd 5'→3'	rev 5'→3'
<i>Gata 3</i>	CTTATCAAGCCCAAGCGAAG	CATTAGCGTTCCTCCTCCAG
<i>IL4</i>	GGATGCGACAAAAATCACTTG	TTGGAAGCCCTACAGACGAG
<i>IL13</i>	Mm_IL13_1_SG QuantiTect Primer Assay (Qiagen)	
<i>Ifny</i>	AGGTCAACAACCCACAGGTC	GAATCAGCAGCGACTCCTTT
<i>Foxp3</i>	TCAAGTACCACAATATGCGACC	TAGGCGAACATGCGAGTAAAC

Figure E1

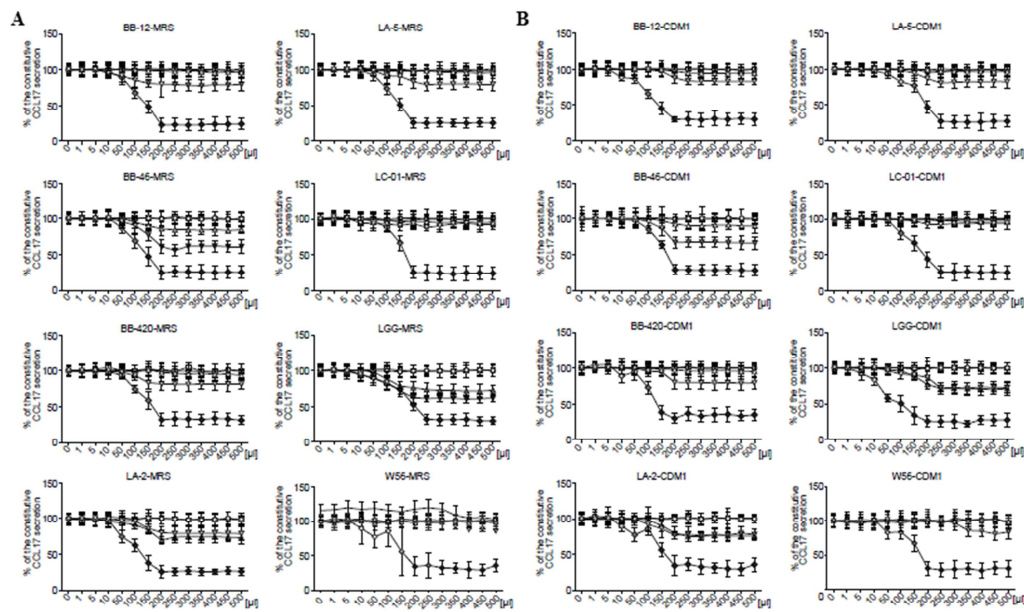


Figure E2

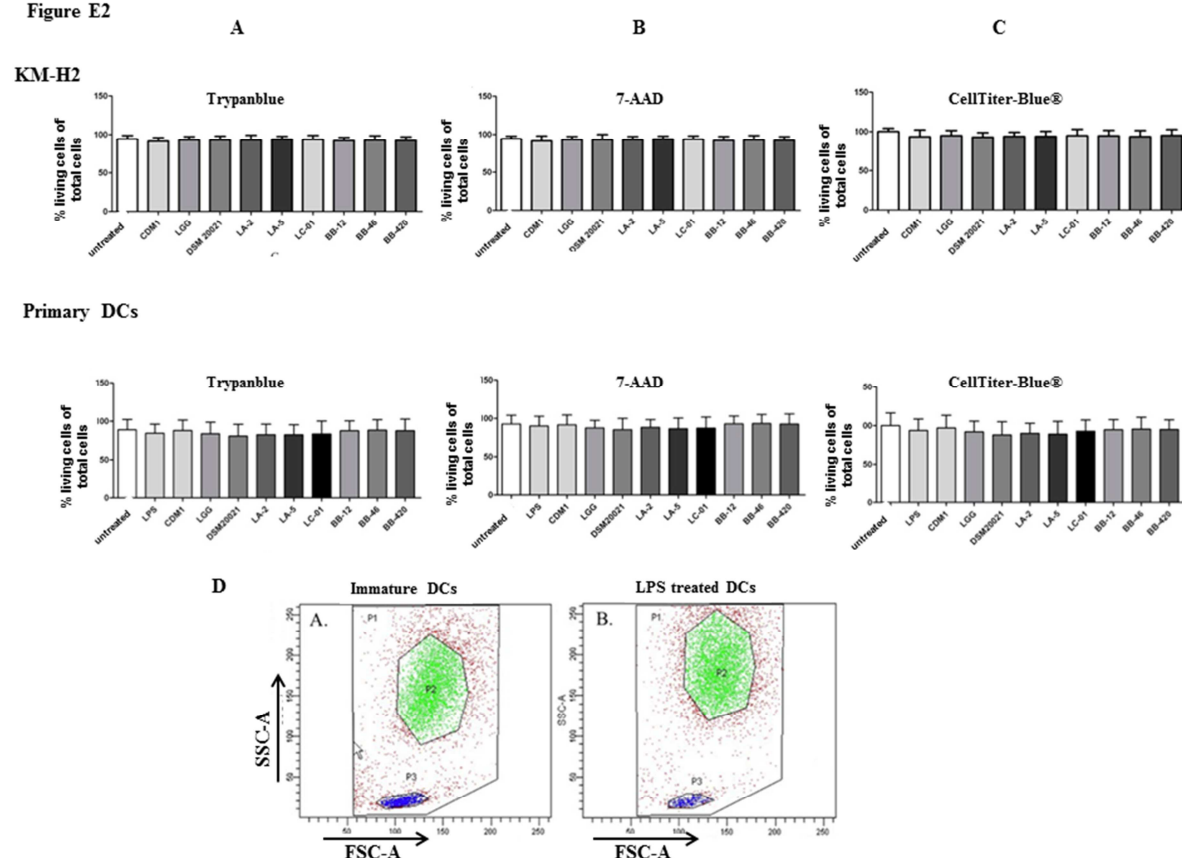


Figure E3

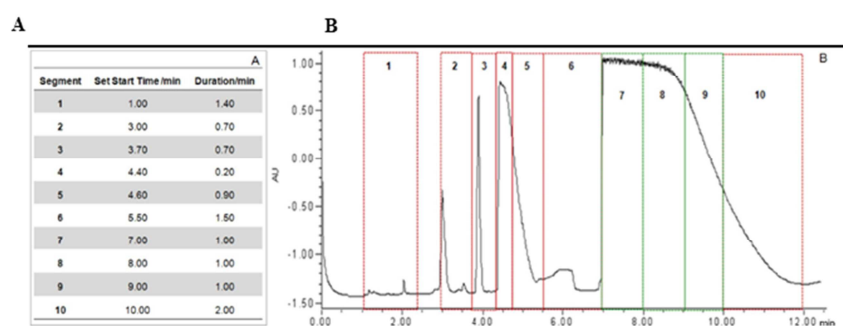


Figure E4

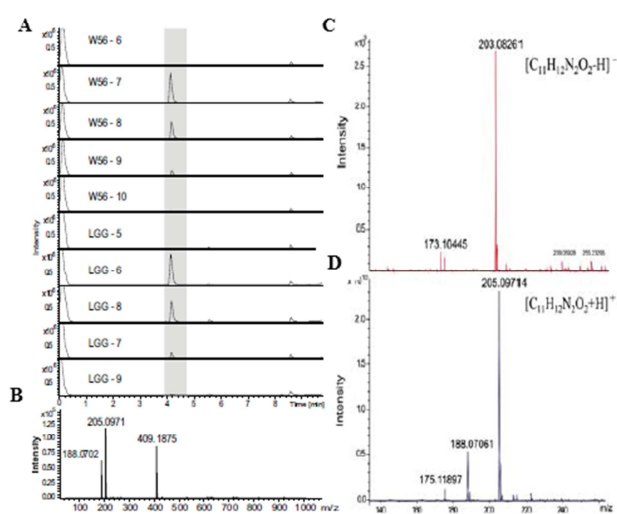


Figure E5

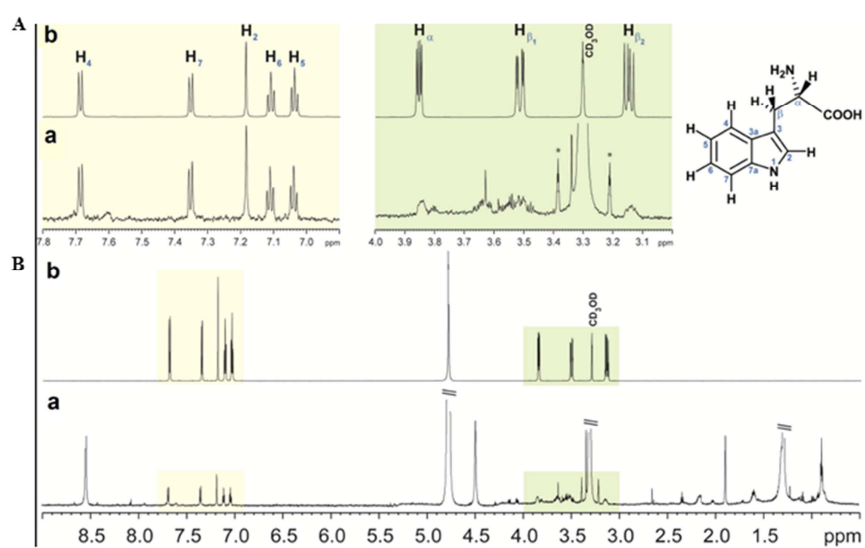


Figure E6

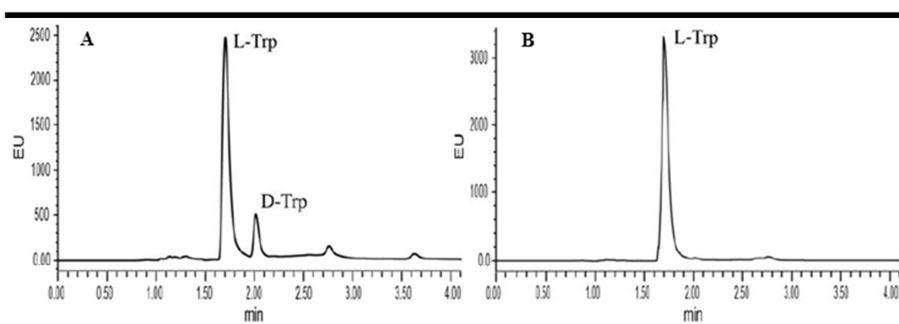


Figure E7

Figure E8

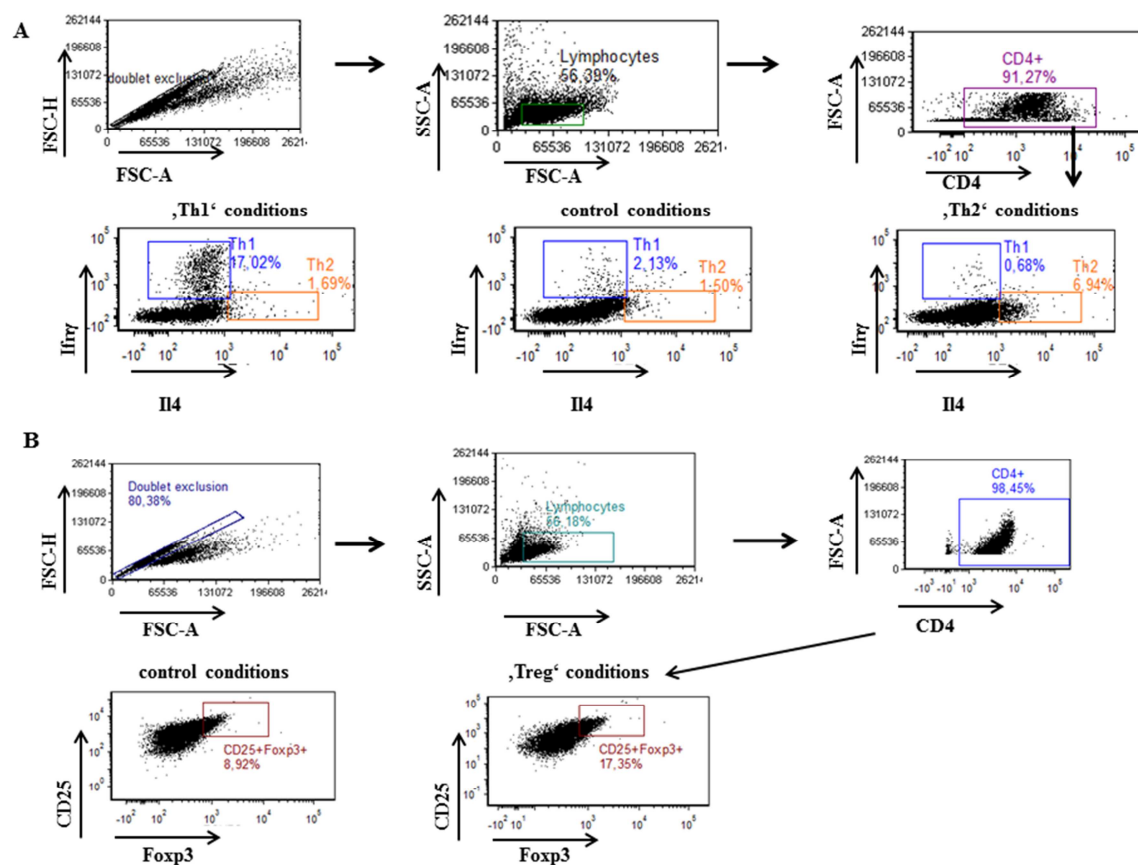


Figure E9

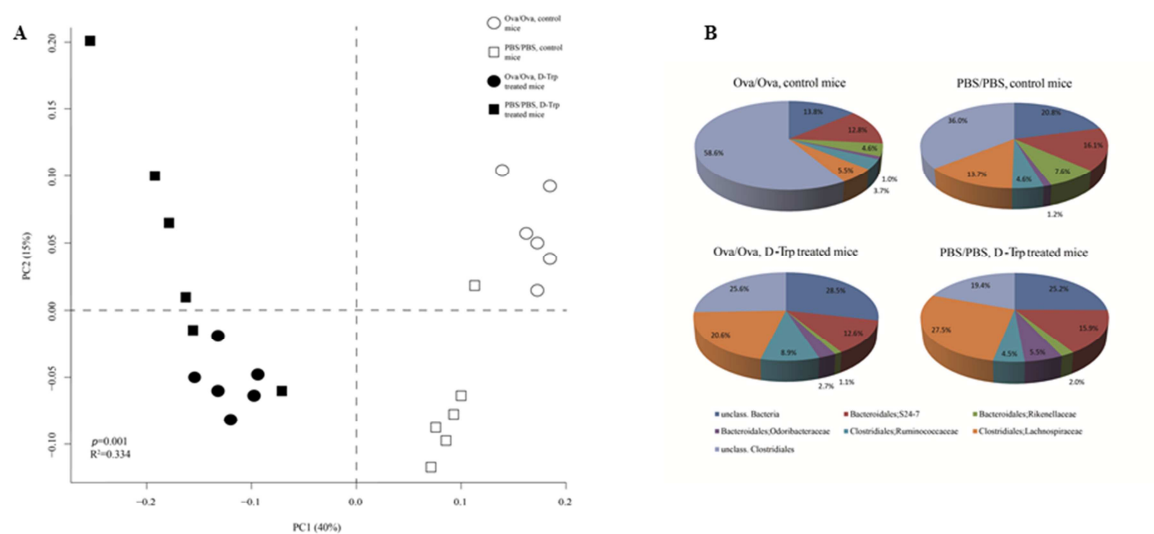


Figure E10

