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Orally administered multispecies probiotic formulations to prevent uro-genital infections: a randomized placebo-controlled pilot study

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Abstract
Purpose The aim of this study was to evaluate in the vagina of 60 pre-menopausal women the detection of orally administered multispecies probiotic formulations showing anti-microbial properties in test in vitro.
Methods A randomized, double-blind, three-arm parallel pilot study was carried out on 60 pre-menopausal women. Subjects were randomly divided in three groups (F_1, F_2, F_3). Each group received a daily oral administration of probiotic mixtures (for 14 days and at the day 21, 7 days after the wash-out) containing: *Lactobacillus acidophilus* and *Lactobacillus reuteri* (F_1), or *Lactobacillus plantarum*, *Lactobacillus rhamnosus* and *Bifidobacterium animalis subsp. lactis* (F_2), or placebo (F_3), respectively. Vaginal swabs were collected at four experimental times, at t0 and at t7, t14 and t21 days, and analyzed by qPCR. At the same time, the anti-microbial activity of the probiotic formulations was verified by assays in vitro against microorganisms as *Escherichia coli* and *Candida albicans*.
Results *L. acidophilus* and *L. reuteri* as well as *L. plantarum*, *L. rhamnosus* and *B. lactis* were significantly increased on 7 days in the groups administered with F_1 and F_2, respectively, compared to group F_3. A similar significant trend was observed on 21 days, 7 days after the wash-out. F_1 and F_2 showed coherent anti-microbial properties.
Conclusion Both probiotic formulations F_1 and F_2, chosen because of their anti-microbial activity against pathogens responsible for vaginal dysbiosis and infections, led to vaginal detection and enhancement of the amount of species of formulates when orally administered. This work provides the basis for further clinical investigations of the F_1 and F_2 capacity to prevent or treat uro-genital infections.

Keywords Probiotics · *Lactobacillus* · *Bifidobacterium* · Vaginal colonization · qPCR · Uro-vaginal infections

Introduction

Probiotics are, according to the FAO and WHO's definition, "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [1]. Several health benefits of probiotics are known: their administration is beneficial in preventing and curing different types of diarrhea [2]; they are used in the treatment of inflammatory intestinal diseases [3] and it has been demonstrated that they help preventing from allergies [4]. Usually, the gut is the target organ for probiotic formulations, but recently other organs and tissues have been related to probiotics, such as skin, hair [5], the oral cavity [6] and the vagina [7], by showing their positive effects for human health. In the specific case of vagina, the microbial species play an important role in the maintenance of health and prevention from infections throughout several mechanisms: occupation of specific adhesion sites of the uro-vaginal epithelium, maintenance of a low pH and production of anti-microbial metabolites such as acids, bacteriocins, hydrogen peroxides and anti-adhesive polysaccharides [8]. In physiological conditions, the vagina

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hosts mainly *Lactobacillus* spp. which concurs to a healthy microbiota, hence preventing from colonization of pathogenic bacteria and fungi [9, 10]. On the contrary, the depletion of vaginal lactobacilli facilitates the overgrowth of diverse species such as *Gardnerella vaginalis*, *Atopobium vaginae*, *Candida albicans*, *Escherichia coli*, responsible for vaginal dysbiosis and uro-genital infections [9–12]. Different probiotics were reported [13–15] to restore the normal vaginal homeostasis by colonization of lactobacilli, when administered topically.

Recent works [16, 17] also suggest that oral administration of lactobacilli and bifidobacteria would colonize both the intestinal and vaginal mucosal surfaces. This opens a new prospect for probiotic therapy: the challenge is to develop probiotics formulation to treat the uro-genital infections and to reduce the recurrences of disease in time, thanks to the capacity to control the pathogenicity of other microbes and to restore the normal ecological balance in vagina. However, these studies did not describe the efficacy of oral administration in term of presence and persistence of probiotic at the vaginal mucosa.

In this context, the aim of the present work is to evaluate in the vagina of 60 pre-menopausal healthy women the detection of orally administered multispecies probiotic formulations showing anti-microbial properties in test in vitro. A formulation F_1 containing *L. acidophilus* PBS066 and *L. reuteri* PBS072 and the other F_2 composed by *L. plantarum* PBS067, *L. rhamnosus* PBS070 and *B. animalis* subsp. *lactis* PBS075 were compared with the placebo (F_3). Data on these two mixtures supported the anti-microbial activity exerted by the above-mentioned single strains by assays in vitro using cell-free supernatants against microorganisms as *E. coli* and *C. albicans*.

The pilot study developed in this paper represents an example to investigate how oral consumption of probiotics formulations can lead to increased levels of the consumed species in the vagina of the women recruited in the study that showed anti-microbial activity against microorganisms potentially involved in uro-genital infections.

Materials and methods

Strains, probiotic formulations and culture conditions

This study comprised five strains of *Lactobacillus* spp. and *Bifidobacterium* spp. supplied from a private collection (Principium Europe Srl) (Table 1).

Unless otherwise specified, *Lactobacillus* spp. strains were cultured in deMan, Rogosa and Sharpe (MRS) medium. For *Bifidobacterium* spp. strains the MRS medium was supplemented with 0.3 g/L L-cysteine hydrochloride monohydrate (cMRS) (Sigma-Aldrich). The cultures were incubated at 37 °C under microaerophilic or anaerobic conditions using anaerobic atmosphere generation bags (Anaerogen, Oxoid).

Two different formulations containing lactobacilli and bifidobacteria of the study (mix F_1 and mix F_2) or placebo (mix F_3) were prepared (Table 2). The composition of the probiotic mix F_1 was as follows: 5×10^9 CFU *L. acidophilus* PBS066 (40 mg as lyophilized), 5×10^9 CFU *L. reuteri* PBS072 (30 mg as lyophilized), 320 mg inulin, 5 mg silica, 5 mg talc. The F_2 composition was as follows: 5×10^9 CFU *L. plantarum* PBS067 (12 mg as lyophilized), 5×10^9 CFU *L. rhamnosus* PBS070 (20 mg as lyophilized), 5×10^9 CFU *B. animalis subsp. lactis* PBS075 (60 mg as lyophilized), 298 mg inulin, 5 mg silica, 5 mg talc. Placebo (F_3) composition was as follows: 390 mg inulin, 5 mg silica, 5 mg talc.

Both single strains and formulations were tested for their anti-microbial activity [18]. As antagonistic microorganisms for anti-microbial activity assays, *E. coli* ATCC 25922 and *C. albicans* ATCC 10231 were employed. With the exception of *C. albicans*, maintained in Sabouraud (Oxoid) agar (1.5% w/v Agar Technical, Oxoid) medium, the antagonists were cultured in Tryptic Soy Agar (TSA; Oxoid) at 37 °C in aerobiosis.

Assessment of the anti-microbial activity

Inhibitory activity of formulates not-neutralized cell-free supernatant on the growth of antagonistic microorganisms

Over-night MRS (or cMRS) cultures were centrifuged at 12,000×*g* at 4 °C for 10 min. The pHs of the supernatants were recorded and measured. An aliquot of the not-neutralized (NN) supernatant fractions was filtered with 0.22 µm pore filter membranes to remove any residual bacterial cell.

The antimicrobial activities were detected by measuring the growth inhibition of *E. coli* ATCC 25922 and *C. albicans* ATCC 10231 in liquid cultures in the presence of the NN cell culture supernatants. Single colonies from freshly streaked plates of the antagonists were resuspended in saline solution at a concentration of 10^6 CFU/mL. The assay was performed in 96-well plates. Each well contained: double concentrated TSB, 10% (v/v) cell suspension of antagonistic strains, 25% (v/v) of NN cell culture supernatants and distilled water up to 150 µL as final volume.

Positive controls were prepared by substituting to NN cell culture supernatants an equal volume of not-inoculated MRS medium. *Bifidobacterium* sp. strain NN supernatant was used as Ref. [18]. Plates were incubated at 37 °C in aerobiosis for 24 h. At regular times, the cultures were

Table 1 List of the strains used in this study, deposit number and the most relevant antimicrobial activities describe in Presti et al. [18], 2015

Strain	Deposit number	Antimicrobial activity vs	Code
Lactobacillus rhamnosus PBS070	DSM 25568	*C. albicans; E. faecalis; P. aeruginosa; S. aureus; E. coli*	LRh
Lactobacillus plantarum PBS067	DSM 24937	*C. albicans; E. faecalis; P. aeruginosa; S. aureus; E. coli*	LP
Bifidobacterium animalis subsp. *lactis* PBS075	DSM 25566	*E. faecalis; P. aeruginosa; E. coli*	BL
Lactobacillus acidophilus PBS066	DSM 24936	*C. albicans; E. faecalis; P. aeruginosa; S. aureus; E. coli*	LA
Lactobacillus reuteri PBS072	DSM 25175	*E. faecalis*	LR

Codes used in the present work was also included

Table 2 Contents of formulations of probiotics used in this study

Name	Component	Quantity (mg)
F_1	*L. acidophilus* PBS066	40.00
	L. reuteri PBS072	30.00
	Inulin	320.00
	Silica	5.00
	Talc	5.00
F_2	*L. plantarum* PBS067	12.00
	L. rhamnosus PBS070	20.00
	B. lactis PBS075	60.00
	Inulin	298.00
	Silica	5.00
	Talc	5.00
F_3	Inulin	390.00
	Silica	5.00
	Talc	5.00

sampled and the growth was evaluated by count plate technique. Experiments were performed three times with a standard error around ±10%.

Anti-microbial activity of formulates vs antagonistic microorganisms by using living cells

The antimicrobial activity of the probiotic mixtures containing lactobacilli and bifidobacteria living cells against the antagonists was evaluated by the overlay method, using the protocol described by Presti et al. [18]. A suspension of each mix F_1 and mix F_2 or placebo (mix F_3) was cultured into MRS broth until the $O.D._{600nm}$ was 0.2. Then, 50 µL of each culture was spread by forming a stripe 2 cm wide across the MRS (or cMRS) agar plates. The plates were incubated in anaerobiosis at 37 °C for 24 h. After strain growth, plates were overlaid with 10 mL of melted TSA or MYPG (3 g/L Malt extract, Difco; 3 g/L Yeast extract, Biolife; 3 g/L Bacto Peptone, Difco; 2 g/L glucose, Sigma-Aldrich; pH, 6.2) soft agar (8 g/L) media. *E. coli* ATCC 25922 and *C. albicans* ATCC 10231 colonies from freshly streaked plates were resuspended into 0.9% (w/v) saline solution at a concentration of 10^8 CFU/mL. Cell suspensions were streaked over TSA (or MYPG in the case of *C. albicans*) surface with a cotton swab. The plates were incubated at 37 °C for 24 h in aerobic conditions. The antimicrobial ability of the examined lactobacilli and bifidobacteria strains was semi-quantitatively evaluated in terms of absent (−), moderate (+) and strong (++) growth inhibition of the antagonist, depending on the dimension of the inhibition halos.

DNA extraction and manipulation

DNA from microbial cultures was extracted by the "InstaGene Matrix" (Biorad): 1 mL of culture (10^9 CFU/mL) was centrifuged at 4000 rpm and 12 °C for 15 min. DNA was extracted from the pellet following the protocol provided by the manufacturer. DNA was also extracted from tenfold dilutions of the 10^9 CFU/mL culture up to 10^2 CFU/mL to set up qPCR analysis.

DNA from microbial cultures was used as template to set up primers and PCR conditions to apply for the detection of bacteria of the formulates by qPCR. Primers were designed as follows. The two DNA regions, pre-16S rRNA and IS 16S/23S rRNA sequences [19, 20] were used to identify species-specific DNA markers. For each probiotic, the obtained sequence served as a query to perform BLAST searches against publically available nucleotide databases (http://blast.ncbi.nlm.nih.gov). We collected the top BLAST hits, with a query cover >70% and identity >80%, and aligned them with the query sequence by using Clustal Omega [21] at http://www.ebi.ac.uk/Tools/msa/clustalo/. Forward and reverse primers were manually designed with the least conserved nucleotide sequences. Selected primers were firstly tested in silico on "Primer-BLAST" at the NCBI BLAST web site (http://www.ncbi.nlm.nih.gov/tools/primer-blast) running a default BLAST search against the entire database with the designed primers as queries.

PCR analyses were performed by using the identified primers and by using 10 ng of DNA extracted from each

microbial culture. PCRs were performed with by puReTaq Ready-To-Go PCR beads (GE HealthCare Biosciences, Buckinghamshire, UK) in a 25 μL reaction according to the manufacturer's instructions. PCR cycles consisted of an initial denaturation step for 7 min at 94 °C, 35 cycles of denaturation (45 s at 94 °C), annealing (30 s at 55 °C), extension (1 min at 72 °C) and a final extension at 72 °C for 7 min.

The efficacy of amplification was verified by agarose gel electrophoresis and ethidium bromide staining. In addition, each PCR product was sequenced to verify the correspondence of the selected region. Primer synthesis and DNA sequencing were supplied by Primm, Milan, Italy.

qPCR: primers validation and standard curves

Semi-quantitative analyses of probiotics were set up using qPCR. The 10 μL qPCR mix was assembled with 5 μL of "SsoFast EvaGreen Supermix with Low ROX" (BIO-RAD) and 0.5 μL of 10 μM primer forward and 10 μM primer reverse and 4 μL of DNA template. qPCR was performed in triplicate in the ABI 7500 Real-Time PCR system (Applied Biosystems) using standard cycling conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. This qPCR program was followed by a dissociation step to verify specificity.

Tenfold dilutions of DNA extracted from microbial cultures were tested in order to verify the detection limit of the qPCR reaction and hence select an optimum number of cycles for the reaction.

Standard curves were constructed for each strains using DNA extracted from microbial cells.

Cells were prepared using tenfold dilutions ranging from 10^9 to 10^2 CFU/mL.

Pilot study design

This study consisted in a randomized, double-blind, three-arm parallel group, and placebo-controlled pilot study. It involved 60 pre-menopausal women aged between 18 and 50 years old not suffering from vaginal or urinary tract infections during the 12 months prior to the date of their enrollment in the study. All the study procedures were approved by the Independent Ethics Committee for Non-Pharmacological Clinical Investigations and were carried out according to World Medical Association's (WMA) Helsinki Declaration and its amendments (Ethical Principles for Medical Research Involving Human Subjects, adopted by the 18th WMA General Assembly Helsinki, Finland, June 1964).

All subjects provided a written informed consent before initiation of any study-related procedures. The study took place at Farcoderm Srl facilities. Farcoderm Srl is an independent testing laboratory for in vitro and in vivo safety and efficacy assessment of cosmetics, food supplements and medical devices.

Depending on a randomization plan, the 60 volunteers were divided into three groups of treatment; each subject was treated, for 14 days, respectively, with a dietary supplement containing F_1 or F_2 or placebo F_3 (Table 2). Vaginal swabs of the enrolled subjects were collected within first 4–5 cm of vagina, to take a sample of vaginal secretion, at four experimental times: the day before first intake of probiotics, after 7, 14 and 21 days from the first intake (day 21 represents seven days after the end of the treatment). Vaginal swabs were stored at −20 °C until total DNA isolation.

The tested products consisted of food supplements (capsules) containing lactobacilli and bifidobacteria (Principium Europe Srl, Solaro, MI, Italy) (Table 1). The composition of the probiotic mix F_1, F_2, and F_3 is reported above (Table 2).

DNA analysis of vaginal samples

Total DNA was extracted from the vaginal swabs by the "InstaGene Matrix" (Biorad) according to the following protocol: 120 μL were taken from each swab and centrifuged at 13,000 rpm at 10 °C for 10 min; 200 μL of "InstaGene Matrix" were added to the pellet; after an incubation at 56 °C and 1000 rpm for 30 min and 8 min at 100 °C, the solution was centrifuged at 12,000 rpm for 3 min. The supernatant containing DNA was collected and stored at −20 °C. DNA quality was verified by agarose gel electrophoresis.

DNA extracted from swabs was analyzed in triplicate, with each one of the five primer sets, with qPCR mix and qPCR protocol described before. We chose to analyze 4 μL of 1:10 dilutions of isolated DNA in order to reduce the effect of possible PCR inhibitors.

Each qPCR run contained multiple Non-Template Controls (NTC).

Statistical methods

To examine the effect of the administration of different probiotic formulations, we used a linear mixed model (LM). The different probiotics, the capsule (treatment: F_1, F_2, F_3) and the time point at four different experimental times (timepoint: t0, t7, t14, t21 days) were used as the explanatory variables. Moreover, we considered volunteers (*sample*) as random effect.

To apply generalized linear mixed model (GLMM) under Poisson-lognormal error to account for higher variation at the lower end of target abundance, MCMC.qpcr R package was used to convert Ct data in bacterial counts.

The conversion to approximate counts uses the following formula:

$$\text{Count} : E^{(\text{Ct1}-\text{Ct})}$$

where E is the efficiency of amplification and Ct1 is the number of qPCR cycles required to detect a single target molecule.

Markov Chain Monte Carlo (MCMC) algorithm implemented in the package is used to sample from the joint posterior distribution over all model parameters, in order to estimate the effects of all experimental factors on the levels of specific microbial species. GLMM was used to test whether the levels of the different microbial species in different formulation groups (F_1, F_2, F_3) differed between the baseline (t0) and the subsequent time points (t7, t14, t21 days).

The experimental design is incorporated into the following model:

$$\ln(\text{counts}) \sim \text{species} + \text{species} : \text{Formulation} + \text{species} : \text{Time} + \text{sample} + \text{species} : \text{sample} + \text{species} : \text{residual}$$

where the logarithm of bacterial counting rate is the response variable and the fixed factors are Formulation and Time (baseline and subsequent time points). The three remaining factors: sample (different subjects of the study), species:sample and species: residual are defined as random factors, accounting for the variation in quality and quantity of biological material among samples.

In particular, *lmer* package [22] *lmerTest* package [23] was used to perform linear mixed models and do statistical tests. Plots are performed using *ggplot2* package [24].

Results

Anti-microbial activity of the probiotic formulates

The anti-microbial activity of formulates F_1 and F_2 was tested against *C. albicans* and *E. coli*, as an example of microorganisms responsible for uro-genital infections, by growth inhibition with non-neutralized cell-free supernatants and by overlay assay on living cells. Both probiotics mixtures showed a strong inhibition rate against *E. coli* mediated by microbial culture supernatants during 24 h, while no influence was detected vs *C. albicans*. The inhibition degree of each probiotic formulation compared to the corresponding single strain is reported in Fig. 1. The curve of activity of formulates is in line with the expected profile from single strain performance. The inhibition capacity of the mixtures F_1 and F_2 was further investigated by direct contact of their cultures against the same microorganisms. The growth inhibition halos around the multiple colonies were coherent with the anti-microbial inhibition profiles detected for the single strains. F_1 showed a moderate activity against both *C. albicans* and *E. coli* according to a better performance of *L. acidophilus* contrary to *L. reuteri*, while a stronger inhibition capacity vs both pathogens was observed for F_2. The results are showed in Fig. 2.

Set up of DNA molecular tools for probiotics identification

To identify each probiotic, polymorphic DNA regions were identified among the pre-16S rRNA and IS 16S/23S sequences [19, 20]. BLAST analysis performed against the entire default database (nr/nt), excluding species of interest, allowed to identify the most polymorphic regions (data not shown).

For *L. rhamnosus* and *L. plantarum* the DNA marker regions were found in the 16S/23S IS, while for the other three probiotics the pre-16S partial sequence resulted more polymorphic and suitable to identify marker regions. For each selected marker, species-specific primer pairs were identified (Table 3).

For each primer combination, standard qPCR curves were constructed by using DNA of pure culture of each strain. Results are reported in Table 4, together with the R^2 value, the slope and the efficiency of the amplification. The values obtained show how the qPCR reaction set up is optimal. The R^2 values are high (>0.99) and the efficiencies are between 80 and 115%, limits necessary for a reliable standard curve [25]. The analysis performed on different culture dilutions showed a linear dynamic range to be between 10^9 CFU/mL of culture and 10^2 CFU/mL.

Evaluation of vaginal probiotic amount in the subjects of the study

The selected primers were used to estimate the presence and persistence of each probiotic species in vagina from swab samples of the enrolled subjects by qPCR. PCR analyses were performed on DNA extracted from vaginal swabs at time t0, t7, t14 and t21 days.

The qPCR analysis demonstrated that the species-specific sequences associated with the probiotics of the formulations were detected only in vaginal DNA from subjects treated with the formulations F_1 and F_2 and not with the formulation F_3. Figure 3 shows the abundance of the different probiotic species for each treatment group at the different experimental times.

The subjects treated with F_1 showed an increase in the level of both *L. acidophilus* and *L. reuteri* compared with

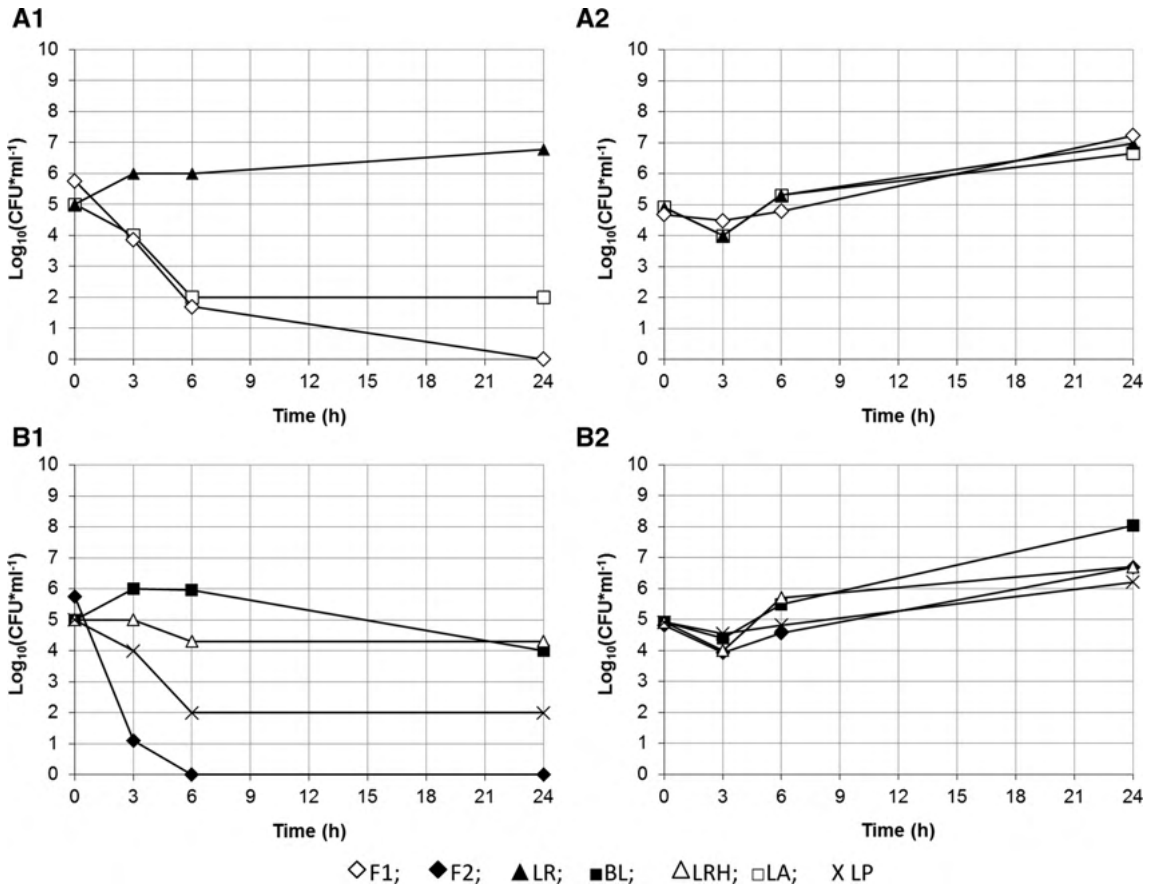

Fig. 1 Effect of non-neutralized culture supernatants of *L. acidophilus* (*square*), *L. plantarum* (*cross*), *L. rhamnosus* (*triangle*), *B. animalis subsp. lactis* (*filled square*), *L. reuteri* (*filled triangle*), mixture F_1 (*diamond*), mixture F_2 (*filled diamond*) on the growth of *E. coli* and *C. albicans*: *A1* mixture F_1 vs *E. coli*; *A2* mixture F_1 vs *C. albicans*; *B1* mixture F_2 vs *E. coli*; *B2* mixture F_2 vs *C. albicans*

F_3. This has been observed at all the times compared with the t0, including t21, 7 days after the follow-up from the last probiotics administration. The same trend was observed for *L. rhamnosus*, *L. plantarum* and *B. animalis* subsp. *lactis* in women treated with F_2 formulation. Differently, the amount of all five strains remained constant throughout the study in the vaginal DNA of women treated with the placebo F_3.

The increase of microbial cell number and the permanence of probiotic species at higher levels after the end of the oral administration (t21) indicate that these species are actually more abundant in the vaginal microbiota. This increase was observed with statistical significance (p value <0.05) since 7 days after the beginning of treatment for *L. reuteri* and *L. acidophilus* of the F_1 group and for *L. rhamnosus* and *B. animalis* subsp. *lactis* of the F_2 group. Instead, the increase of *L. plantarum* resulted statistically significant (p value <0.05) since 14 days from the beginning of the treatment. The permanence of bacteria was observed for all the species studied until the t21, 7 days from the last probiotics intake. In fact, the differences of t14 and t21 from t7 are not statistically significant (p value >0.05) for all probiotics, with the exception of *L. plantarum*.

For F_3 formulation there were not so variations in the amount of selected probiotics for the entire 21-day period.

Discussion

The microbial species that inhabit the vaginal tract play an important role in the maintenance of health and prevention of infections. In particular, the presence of high numbers of lactic acid bacteria in the vagina is often equated with an health status [26]. It appears evident that the balance between a healthy and diseased state involves an equilibrium which can depends on different factors, such as hormone levels, douching, sexual practices, as well as bacterial interactions, and host defenses [27, 28]. A way to increase the level of vaginal lactobacilli is through the use of probiotics; two ways are commonly applied: direct application through vagitories or indirect application by

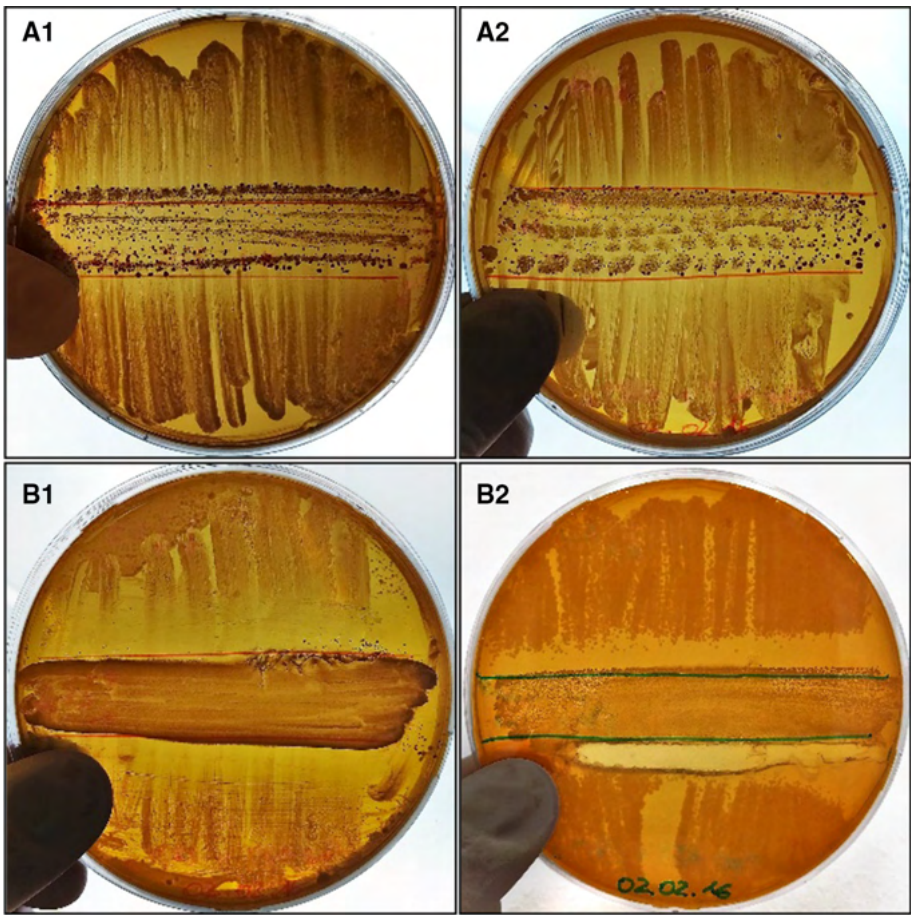

Fig. 2 Inhibition growth of *E. coli* and *C. albicans* over probiotics formulate cultures: *A1* mixture F_1 *vs E. coli*; *A2* mixture F_1 vs *C. albicans*; *B1* mixture F_2 vs *E. coli*; *B2* mixture F_2 vs *C. albicans*

Table 3 List of primers designed and used in this study

Probiotic	Primer code	Sequence (5′ → 3′)	DNA region	Amplified length (bp)
L. rhamnosus	LraF	CTAGCGGGTGCGACTTTGTT	16S/23S IS	123
	LraR	CAGCGGTTATGCGATGCGAA		
L. plantarum	Lpl2F	CATTGGAACCGAACCAGTTG	16S/23S IS	203
	Lpl2R	CGGTGTTCTCGGTTTCATTATG		
B. lactis	AnimF	GCACGGTTTTGTGGCTGG	pre16S	171
	AnimR	GACCTGGGGGACACACTG		
L. acidophilus	Lacid2F	GGGCAAATCACGAACGAGTA	pre16S	132
	Lacid2R	CTTTGTTTTCGTTCGCTTCA		
L. reuteri	Lreu2F	GTTGACGAAAGAATGAAATCCA	pre16S	118
	Lreu2R	TCATGTCGTCAATCAGATGTCA		

Table 4 Standard curves equations: y corresponds to the Cq and x the bacterial dilution in respect to 10^9 CFU (maximum concentration)

Strain	Equation	R^2	Slope	Efficiency (%)
L. rhamnosus PBS070	$y = -3.3383x + 9.3483$	0.994	−3.34	99.30
L. plantarum PBS067	$y = -3.1379x + 14.231$	0.995	−3.14	108.30
B. lactis PBS075	$y = -3.1121x + 16.418$	0.993	−3.11	109.60
L. acidophilus PBS066	$y = -3.2167x + 11.36$	0.990	−3.22	104.60
L. reuteri PBS072	$y = -3.2185x + 10.025$	0.993	−3.22	104.50

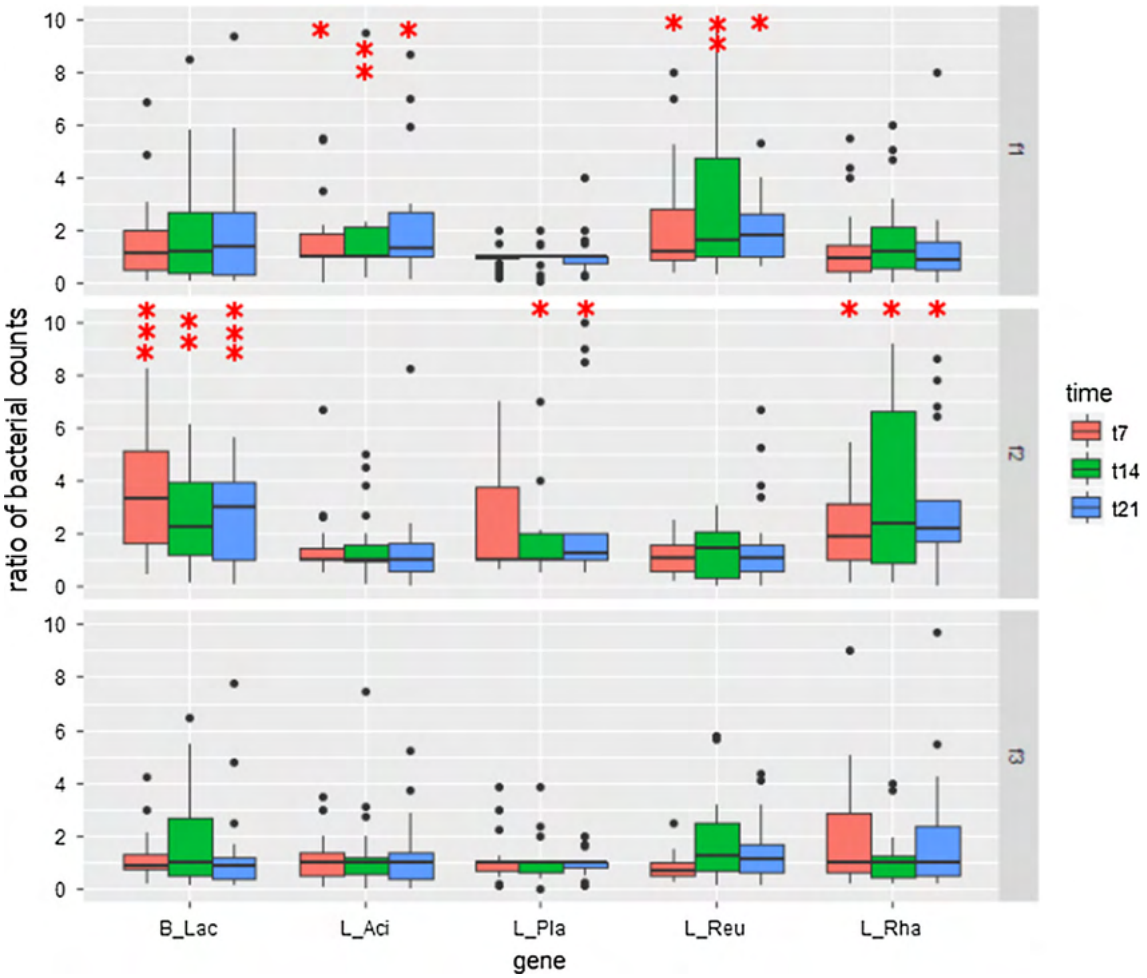

Fig. 3 Ratio of probiotics of formulations (F_1 and F_2, *vs* F_3) by qPCR of species-specific sequences at the different times of treatment vs the amount at the baseline time point, expressed as bacterial counts. Upon the *bars* is reported the statistical analysis between treatments (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$)

oral consumption of probiotics. Several probiotics have been found to both increase the overall level of vaginal lactobacilli and to aid in the treatment of bacterial vaginosis [26]. Moreover, the capacity of probiotics to exert beneficial effects in human health is recognized to be strain-specific and a multi-species formulation could take the advantage of combining a greater spectrum of activities.

In this context, the present work allowed us to assess in the vagina of 60 pre-menopausal healthy women of a pilot study, the detection of orally administered multispecies probiotic formulations showing anti-microbial properties in test in vitro. First, we tested the antagonistic capacity against *E. coli* and *C. albicans* of the formulation F_1 and F_2 through cell supernatants and through the overlay contact between probiotic formulations and pathogens. Both formulations showed a strong anti-microbial activity against *E. coli* in both the conditions, as expected by the average trend of the single strains included in the formulations, while inhibition against *C. albicans* occurred only in the overlay assay, likewise the tested single strains. This is probably due to the reliable inhibitory activity of lower pH upon the *E. coli* growth, while the same conditions are not effective against yeasts. Moreover, it is reported that bacteriocins produced by probiotics are active only at certain pH ranges but can be neutralized at different pHs; this could be the reason for not having observed any inhibitory effect on the pathogens growth by using the neutralized cell supernatants [18].

Probiotics can reach the target of vagina when orally consumed, as an alternative way to a direct local administration; so they can locally exert their effect by competition–displacement of pathogens, reducing the infection relapses and related symptoms [26, 29].

Although previous studies [30–32] had already suggested this possibility, it is not so expected and the mechanism is still unclear. The current pilot study was aimed to investigate the vaginal detection after oral

consumption of mixtures of probiotics would lead to increased levels of the consumed multispecies in the vagina of the enrolled subjects.

Our analysis showed an increased levels of all probiotics species (p value <0.05) detected in the vaginal swabs of women consuming the formulates F_1 and F_2 in comparison to women of the placebo group. The only exception was for *L. plantarum*, which was higher starting from 7 to 21 days of administration. This might suggest that: (1) *L. plantarum* is not as efficient as other probiotics, in colonizing vaginal mucosa from the intestinal region, (2) *L. plantarum* is already present at high levels in vaginal mucosa of healthy women and its abundance is not easily perturbed by the oral administration of probiotics. For this reason, *L. plantarum* might need a longer treatment or may require a higher concentration in administered capsules.

The abundance of probiotic strains in the vaginal DNA was assessed for both formulations until the last day of the experiment (t21 days), 7 days after the last intake (p value <0.05). This indicates that probiotics can actually colonize the vaginal microbiota in a short time. It would be particularly interesting to demonstrate if these can persist also after the menses. Indeed, the set-up of this study was purposely planned to match menstrual cycle of volunteers, with the first swab collected after menses and the last 21 days later, before the next menses. This was done to prevent from having blood traces in the vaginal swabs, so possibly altering the results.

The DNA markers used allowed to monitor the assessment and persistence of each probiotic species in the vaginal microbiota of healthy pre-menopausal women during the treatment and after the follow-up period. Our findings suggested that the five selected DNA markers can detect the increasing level and persistence of the studied bacteria into the vagina by using qPCR methods.

As lactobacilli and bifidobacteria have a fundamental role on the vaginal well-being, and more specifically an anti-microbial activity, we would highlighted that the anti-microbial effect detected against several pathogen microorganisms suggests that the five selected strains could similarly exert the antagonistic activity in vivo.

Conclusions

In conclusion, this study reports that the oral intake of two probiotic multi-species mixtures leads to an evident colonization of vagina of 60 volunteers of probiotic bacteria showing in vitro anti-microbial activity against pathogens involved in uro-genital infections. The adopted molecular tool represents a valid instrument to be used in future clinical trials to correlate the eventual clinical outcomes with the effective colonization in the treatment or prevention of vaginal dismicrobism and uro-genital infections.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Evaluation of the probiotic properties of new *Lactobacillus* and *Bifidobacterium* strains and their in vitro effect

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Abstract Probiotic ingestion is recommended as a preventive approach to maintain the balance of the intestinal microbiota and to enhance the human well-being. During the whole life of each individual, the gut microbiota composition could be altered by lifestyle, diet, antibiotic therapies and other stress conditions, which may lead to acute and chronic disorders. Hence, probiotics can be administered for the prevention or treatment of some disorders, including lactose malabsorption, acute diarrhoea, irritable bowel syndrome, necrotizing enterocolitis and mild forms of inflammatory bowel disease. The probiotic-mediated effect is an important issue that needs to be addressed in relation to strain-specific probiotic properties. In this work, the probiotic properties of new *Lactobacillus* and *Bifidobacterium* strains were screened, and their effects in vitro were evaluated. They were screened for probiotic properties by determining their tolerance to low pH and to bile salts, antibiotic sensitivity, antimicrobial activity and vitamin B8, B9 and B12 production, and by considering their ability to increase the antioxidant potential and to modulate the inflammatory status of systemic-miming cell lines in vitro. Three out of the examined strains presenting the most performant probiotic properties, as *Lactobacillus plantarum* PBS067, *Lactobacillus rhamnosus* PBS070 and *Bifidobacterium animalis* subsp. *lactis* PBSO75, were evaluated for their effects also on human intestinal HT-29 cell line. The obtained results support the possibility to move to another level of study, that is, the oral administration of these probiotical strains to patients with acute and chronic gut disorders, by in vivo experiments.

Keywords Probiotics · *Lactobacillus* · *Bifidobacterium* · Intestinal microbiota · Gut disorders

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Introduction

Probiotics are recognized as live microorganisms that confer a health benefit to the host when administered in adequate amounts (FAO/WHO 2001). These bacteria exert health-promoting properties including the maintenance of the gut functions and the local and systemic modulation of the host immune system. Many studies have demonstrated the clinical potential of probiotics against many diseases, such as allergic pathologies, including atopic eczema and rhinitis, diarrhoea, necrotizing enterocolitis inflammatory bowel disease and viral infection (Robles Alonso and Guarner 2013). For these reasons, there is an increasing interest in the development of adjunct or alternative therapies based on bacterial replacement, by using probiotics isolated and characterized for their specific properties (Kailasapathy and Chin 2000). Probiotics could be used as supplements to improve the functional properties of nutraceutical food products and therapeutical preparations.

Actually, the most known probiotic microorganisms are bacteria belonging to the *Lactobacillus* and *Bifidobacterium* genera (Prasad et al. 2000). However, species belonging to the genera *Lactococcus* (Kimoto et al. 1999), *Enterococcus* (Dunne et al. 1999), *Saccharomyces* (Sanders and Huis in't Veld 1999) and *Propionibacterium* (Mantere-Alhonen 1995) are also considered as probiotic microorganisms. The ability

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of lactobacilli and bifidobacteria to exert beneficial effects on the human health is a species- and strain-specific feature (Ramos et al. 2013). This explains the need for a continuous search for novel strains with probiotic potential, which could be useful to investigate their effect on different target districts or disorders.

Several important mechanisms underlying the beneficial effects of probiotics include the effects of probiotic properties on specific tissues, particularly on the intestine. Most of these mechanisms involve the modification of the gut microbiota, the competitive adherence to the mucosa and epithelium against pathogens, the strengthening of the gut epithelial barrier and the regulation of the immune system and inflammation. For this reason, the probiotic-mediated effect is an important issue that needs to be addressed in relation to strain-specific probiotic properties (Fontana et al. 2013; Collado et al. 2009).

The most efficient strains should be robust enough to survive the harsh physico-chemical conditions of the gastrointestinal tract. This includes gastric acid (Corcoran et al. 2005), bile secretion (Begley et al. 2005) and competition with the resident bacteria of the gut microbiota (Fooks and Gibson 2002). As safety requirement, the origin of the strains, their non-pathogenicity and the absence of transferable antibiotic-resistance genes should also be assessed (Saarela et al. 2000). Despite of the increasing awareness of the benefits derived from a regular intake of probiotic bacteria, the knowledge about these mechanisms is still limited. Proven modes of action include the production of substances such as bacteriocins or organic acids with antagonistic activity against potential pathogens (Servin 2004), immunomodulating attitude on the intestinal tissues (Valeur et al. 2004) or the release of essential micronutrients in the gut, such as group B vitamins (LeBlanc et al. 2011). For the gastrointestinal colonization, probiotics need to be ingested as large populations and on a daily basis (Berman et al. 2006).

By contrast, information related to the effects on human intestinal cells mediated by the action of probiotics is scarce. There is a need for further clinical studies that evaluate the mechanism of action of probiotics both in healthy and in patients with chronic diseases. During the whole life of each individual, the gut microbiota composition could be altered by lifestyle, diet, antibiotic therapies and other stress conditions, and this may lead to acute and chronic disorders (Bervoets et al. 2013; Pérez-Cobas et al. 2013).

In this prospective, another level of study is necessary before the in vivo experiments, by the evaluation of probiotial properties effects on human intestinal cell lines in vitro. In fact, in vitro evidence is particularly important considering that the EU directives tend to discourage experiments on animals.

In this work, the probiotic properties of new *Lactobacillus* and *Bifidobacterium* strains were investigated, and their biological effects on cell lines by in vitro tests were evaluated. The probiotic properties were determined by their tolerance to low pH and to bile salts, antibiotic sensitivity, vitamins B8, B9 and B12 production and antimicrobial activity, as well as their inflammation response on systemic-miming cell lines in vitro. Moreover, the probiotic properties of the most *Lactobacillus* and *Bifidobacterium* performant strains were evaluated on human intestinal cell lines, in order to acquire data to successively move to another level of study involving patients with acute and chronic gut disorders by in vivo experiments.

Materials and methods

Strains and culture conditions

This study comprised seven strains of *Lactobacillus* spp. and *Bifidobacterium* spp. supplied from a private collection. *Lactobacillus paracasei* ATCC 334 and *Bifidobacterium* sp. (supplied from University of Bologna), known for its antimicrobial properties (Aloisio et al. 2012), were also included in this work as controls.

Unless otherwise specified, *Lactobacillus* spp. strains were cultured in deMan, Rogosa and Sharpe (MRS) medium. For *Bifidobacterium* spp. strains, the MRS medium was supplemented with 0.3 g/L L-cysteine hydrochloride monohydrate (cMRS) (Sigma-Aldrich). The cultures were incubated at 37 °C under microaerophilic or anaerobic conditions using anaerobic atmosphere generation bags (Anaerogen, Oxoid). As antagonistic microorganisms for antimicrobial activity assays, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538 and *Candida albicans* ATCC 10231 were employed. With the exception of *C. albicans*, maintained in Sabouraud (Oxoid) agar (1.5 % *w*/*v* Agar Technical, Oxoid) medium, the antagonists were cultured in Tryptic Soy Agar (TSA; Oxoid) at 37 °C in aerobiosis.

Molecular identification of lactobacilli and bifidobacteria

Genomic DNA from microbial strains was extracted from 2 mL of overnight cultures by using Ultra Clean Microbial DNA Isolation Kit (Mo Bio, Carlsbad, USA). 16S rRNA gene amplification was performed with the universal primers 27 F and 1492R (Lane 1991) by a standard PCR reaction. The PCR products were then purified with NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) and sequenced. The 16S rRNA sequences of the isolated strains have been deposited in the GeneBank DataBase (accession numbers KM577182, KM577183, KM577184, KM577185, KM577186, KM577187 and KM577188).

Resistance at acidic pHs and simulated intestinal fluids

Lactobacilli and bifidobacteria strains were cultured in MRS (or in cMRS) agar at 37 °C for 24 h in anaerobiosis. Cells were collected in 0.9 % (*w*/*v*) saline solution, until the optical density at 600 nm ($O.D._{600nm}$) was 2. Cell suspensions were diluted in 50 mM of the following buffers: glycine-HCl pH 2.0, glycine-HCl pH 3.0, sodium acetate pH 4.0, sodium acetate pH 5.0 and sodium phosphate pH 7.0, as control. The residual viability was determined at 0, 30, 60, 120 and 180 min of incubation at 37 °C. Samples were serially diluted and appropriate dilutions were cultured on MRS (or cMRS) agar.

Simulated intestinal fluid (SIF) was prepared as described by Priya et al. (2011). The SIF consisted of 1 g/L of pancreatin (Sigma-Aldrich), 3 g/L of bovine bile salts (Sigma-Aldrich), 0.835 g/L of KCl (Sigma-Aldrich), 6.5 g/L of NaCl (Sigma-Aldrich), 0.22 g/L of $CaCl_2$ (Sigma-Aldrich) and 1.386 g/L of $NaHCO_3$ (Sigma-Aldrich), with a final pH of 7.5. After that, the solution was filtered through a 0.22-μm membrane. Then, 100 μL of cell suspensions at $O.D._{600nm}$ 2 was diluted in 1.9 mL of SIF and incubated for 180 min at 37 °C. As described above, at regular time intervals (0, 30, 60, 120 and 180 min), the residual viability was determined by the count plate method. Experiments were performed three times with a standard error around ±10 %.

Antibiotic resistance

The antibiotic resistance profiles of lactobacilli and bifidobacteria strains were determined by broth microdilution method in LSM broth (90 % Iso Sensitest Broth Oxoid, 10 % MRS broth) or cLSM (for bifidobacteria), according to ISO 10932:2010. The minimal inhibitory concentrations (MICs) of the following antibiotics were determined: ampicillin (range, 0.032 to 16 μg/mL), vancomycin (range, 0.25 to 128 μg/mL), gentamicin (range, 0.5 to 256 μg/mL), kanamycin (range, 2 to 1024 μg/mL), streptomycin (range, 0.5 to 256 μg/mL), erythromycin (range, 0.016 to 8 μg/mL), tetracycline (range, 0.125 to 64 μg/mL), chloramphenicol (range, 0.125 to 64 μg/mL) and clindamycin (range, 0.032 to 16 μg/mL) (Sigma-Aldrich). As control, the MICs for *L. paracasei* ATCC 334 were also determined and compared with the MIC values resulting from the interlaboratory trials (ISO 10932:2010).

The detection of *tet(W)* gene in the tetracycline-resistant strains was performed. The tetracycline resistance gene *tet(W)* in *Bifidobacterium animalis* subsp. *lactis* PBS075 strain was amplified by PCR with the specific primers TetF and TetR. The presence of the transposase gene *trp* upstream the *tet(W)* gene was investigated by using the primers TrpF and TetR (Gueimonde et al. 2010). The 50-μL reaction mixture consisted of 25 μL of GoTaq®Green (Promega), 1 μM of each primer and 0.5 ng/μL of template. The PCR product obtained by the amplification of *trp-tet*(W) region was purified with NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) and sequenced.

Assessment of the antimicrobial activity

Well diffusion agar assay using pH-neutralized and non-neutralized cell-free supernatants

A modification of the protocol of Santini et al. (2010) for lactobacilli and bifidobacteria well diffusion agar assay was used. Overnight MRS (or cMRS) cultures were centrifuged at 12,000×*g* at 4 °C for 10 min. The pHs of the supernatants were recorded and measured. An aliquot of each supernatant was neutralized to pH 7.0 with NaOH 3.0 M. The non-neutralized (NN) and the neutralized (N) supernatant fractions were filtered with 0.22-μm pore filter membranes to remove any residual bacterial cell. A single colony of the selected antagonists *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538 and *C. albicans* ATCC 10231 was inoculated into the suitable medium and allowed to grow until $O.D._{600nm}$ was 0.1, corresponding approximately at 10^6 CFU/mL.

Of each culture, 2.5 % (*v*/*v*) was inoculated into 20 mL of melted Tryptic Soy Agar (or Sabouraud agar in the case of *C. albicans*) and the plates were allowed to solidify. Five wells of 8 mm in diameter were made on each agar plate with a sterile cylinder. Of both NN and N cell culture supernatants, 100 μL was dispensed into each well. Not-inoculated MRS (100 μL) was used as control. Plates were incubated overnight at 37 °C in aerobiosis. The growth inhibition zones around the wells were measured.

Inhibitory activity of non-neutralized cell-free supernatant on the growth of antagonistic microorganisms

The antimicrobial activities previously detected were quantified by measuring the growth inhibition of *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538 and *C. albicans* ATCC 10231 in liquid cultures in the presence of the NN cell culture supernatants. Single colonies from freshly streaked plates of the antagonists were resuspended in saline solution at a concentration of 10^6 CFU/mL. The assay was performed in 96-well plates. Each well contained double concentrated TSB, 10 % (*v*/*v*) cell suspension of antagonistic strains, 25 % (*v*/*v*) of NN cell culture supernatants and distilled water up to 150 μL as final volume.

Positive controls were prepared by substituting to NN cell culture supernatants an equal volume of non-inoculated MRS medium. *Bifidobacterium* sp. strain NN supernatant was used as reference (Aloisio et al. 2012). Plates were incubated at 37 °C in aerobiosis for 24 h. At regular times, the cultures

were sampled and the growth was evaluated by count plate technique. Experiments were performed three times with a standard error around ±10 %.

Antimicrobial activity of lactobacilli and bifidobacteria strains by using living cells

The antimicrobial activity of lactobacilli and bifidobacteria living cells against the antagonists, was evaluated by the overlay method, using a modification of the protocol described by Strus et al. (2005). A single colony of each lactobacilli and bifidobacteria strain was cultured into MRS broth until the $O.D._{600nm}$ was 0.2. Then, 50 μL of each culture was spread by forming a stripe 2 cm wide across the MRS (or cMRS) agar plates. The plates were incubated in anaerobiosis at 37 °C for 24 h. After strain growth, plates were overlaid with 10 mL of melted TSA or MYPG (3 g/L malt extract, Difco; 3 g/L yeast extract, Biolife; 3 g/L Bacto Peptone, Difco; 2 g/L glucose, Sigma-Aldrich; pH, 6.2) soft agar (8 g/L) media. *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538 and *C. albicans* ATCC 10231 colonies from freshly streaked plates were resuspended into 0.9 % (*w*/*v*) saline solution at a concentration of 10^8 CFU/mL. Cell suspensions were streaked over TSA (or MYPG in the case of *C. albicans*) surface with a cotton swab. The plates were incubated at 37 °C for 24 h in aerobic conditions. The antimicrobial ability of the examined lactobacilli and bifidobacteria strains was semi-quantitatively evaluated in terms of absent (−), moderate (+) and strong (++) growth inhibition of the antagonist, depending on the dimension of the inhibition halos.

Screening of lactobacilli and bifidobacteria strains for B8, B9 and B12 vitamin production

Biotin (B8 vitamin), folic acid (B9 vitamin) and cobalamin (B12 vitamin) production was respectively assayed in the semisynthetic media: Biotin Assay Medium (Difco), Folic Acid Assay Medium (Difco) and B12 Assay Medium (Sigma-Aldrich). They possess all the nutrients for lactobacilli and bifidobacteria growth except for the vitamin of interest. A modification of the protocol described by Pompei et al. (2007) was used. The strains were cultivated in MRS (or cMRS) broth at 37 °C in anaerobiosis for 24 h. Cells were washed two times in saline solution followed by centrifugation at 12,000 rpm for 10 min, and the cell pellets were resuspended in saline solution until the $O.D._{600nm}$ was 2. Cell suspensions were inoculated (5 %*v*/*v*) into each vitamin assay medium. The cultures were incubated anaerobically at 37 °C for 48 h and sub-cultured for a maximum of seven times. At every sub-culture, the $O.D._{600nm}$ was measured.

Maintenance and growth of cell lines for in vitro tests

The experimental model was the murine embryonal fibroblast cell line BALB/c3T3, clone A31 (ATCC-CCL-163). This cell line is largely used for their capability to give standardized responses and to mimic the systemic environment of an organism; these cells are in fact used as biologic matrix alternative to in vivo tests according to ECVAM (Kinsner-Ovaskainen et al. 2009; Freire et al. 2009) and ISO guideline (ISO 10993-5). Cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) in 75-cm^2 culture flasks at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air until confluent monolayers were obtained. For biological assays, 3 T3 cells were seeded in 12-well plates at a concentration of 10^5 cells/well and incubated in 1 mL of DMEM for 24 h at 37 °C in 5 % CO_2.

For human intestinal cell line in vitro tests, the HT-29 cell line was used. HT-29 are cells from human colon presenting a complete anatomical structure with microvilli, microfilaments, large vacuolated mitochondria with dark granules and rough endoplasmic reticulum with free ribosomes, lipid droples, few primary and many secondary lysosomes. Cells were routinely grown in McCoy's 5a Medium Modified (Sigma-Aldrich) in 75-cm^2 culture flasks at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air until confluent monolayers were obtained. For biological assays, HT-29 cells were seeded in 12-well plates at a concentration of 10^5 cells/well and incubated in 1 mL of McCoy's 5a Medium Modified for 24 h at 37 °C in 5 % CO_2.

Resistance to the inflammatory stress

Inflammatory stress was induced by treating the 3 T3 or HT-29 cells with sodium dodecyl sulphate (SDS) as pro-inflammatory agent (OECD Test Guideline N.439). SDS was added into the culture medium at a final concentration of 0.05 % (*w*/*v*). Cells were incubated in 5 % CO_2 at 37 °C.

Bacterial cultures were routinely grown in MRS agar plates, and then the cells were resuspended in DMEM at a final concentration of 10^7 CFU/mL. These suspensions were added into each well. The residual cell viability at 24 h and at 5 days was evaluated by MTT assay. The cell culture supernatants were removed and collected for cytokine determination. Cells were washed with Dulbecco's phosphate buffered saline. Then, 0.05 % (*w*/*v*) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in DMEM was added into each well and incubated for 4 h at 37 °C. At the end of the incubation, the MTT medium was removed and replaced by an equal volume of MTT solubilization solution (10 %*v*/*v* Triton X-100, 0.1 N HCl in isopropanol) in order to dissolve the formazan produced by MTT reduction. After 30-min incubation on a rotary shaker, the produced formazan was measured at 570 nm with a microplate reader. The results are

expressed as A_{570} of treated cells − A_{570} untreated cells / A_{570} untreated cells × 100.

Cytokine production determination

The concentrations of interleukine 4 (IL-4) and interleukine 10 (IL-10) as anti-inflammatory cytokines and of tumour necrosis factor alpha (TNF-α) as pro-inflammatory cytokine on cell culture supernatants, collected at 24 h and 5 days, were determined by enzyme-linked immune-sorbent assay (ELISA) commercial kits (Boster Biological Technology), according to manufacturer's instructions. The sensitivity of each cytokine assay was as follows: 7.8 pg/mL for IL-4 and IL-10, and 1 pg/mL for TNF-α. The results are expressed as pg/mL and compared with a negative control, not subjected to any treatment, and a positive control, subjected to the SDS treatment previously described.

Determination of the antioxidant potential

For biological assays, 3 T3 or HT-29 cells were seeded in 12-well plates at a concentration of 10^5 cells/well and incubated in 1 mL of DMEM for 24 h at 37 °C in 5 % CO_2.

Bacterial cultures were routinely grown in MRS agar plates, and then the cells were resuspended in DMEM at a final concentration of 10^7 CFU/mL. These suspensions were added into each well. The negative control was carried out with an equal volume of DMEM without bacterial cultures. The improvement of the antioxidant ability of 3 T3 or HT-29 cells in the presence of bacterial strains was evaluated by Ferric Reducing Antioxidant Parameter (FRAP) assay, as described by Benzie and Strain (1996). Ferric to ferrous ion reduction at pH 3.6 causes a coloured ferrous-2,4,6-tripyridyl-s-triazine (TPTZ) complex. The absorbance at 595 nm of each sample was recorded after 30 min. The absorbance values are compared to a Fe(II) standard curve. Final results are expressed as Fe(II) μM.

Statistical analysis

Experiments were performed in triplicate and results elaborated as the mean±standard error of the mean of three experiments. The statistical significance was assessed by Student's *t* test. Differences were considered significant at p value <0.05.

Results

Molecular identification of the strains

The microbial strains were taxonomically characterized by 16S rRNA sequence analysis. Five *Lactobacillus* strains, *Lactobacillus acidophilus* PBS066, *Lactobacillus fermentum* PBS073, *Lactobacillus plantarum* PBS067, *Lactobacillus reuteri* PBS072 and *Lactobacillus rhamnosus* PBS070, and two *Bifidobacterium* strains, *B. animalis* subsp. *lactis* PBS075 and *Bifidobacterium longum* subsp. *longum* PBS108, were identified at the species level and deposited to the DSMZ culture collection (Table 1).

Resistance of lactobacilli and bifidobacteria strains at acidic pHs and simulated intestinal fluid

In order to evaluate the survival of lactobacilli and bifidobacteria strains at the acidic conditions, the viability of the seven strains at different acidic pHs was determined (Fig. 1). All the strains do not lose their viability when exposed at pH 3.0, 4.0 and 5.0 for 180 min. At pH 2.0, *L. reuteri* PBS072 and *L. rhamnosus* PBS070 showed the best survival rate over the 180 min of exposure. *B. lactis* PBS075 and *B. longum* PBS108 viability was slightly affected after 180 min of exposure. *L. acidophilus* PBS066, *L. plantarum* PBS067 and *L. fermentum* PBS073 viability declined at undetectable levels just after 60 min and the latter one after 120 min of exposure.

All the strains were also exposed for 180 min to the simulated intestinal fluid, containing a standard concentration of bile salts. The examined strains showed a good resistance to bile salts. The less resistant strain, *L. fermentum* PBS073, exhibited a viability drop comprised within 3 orders of magnitude (data not shown).

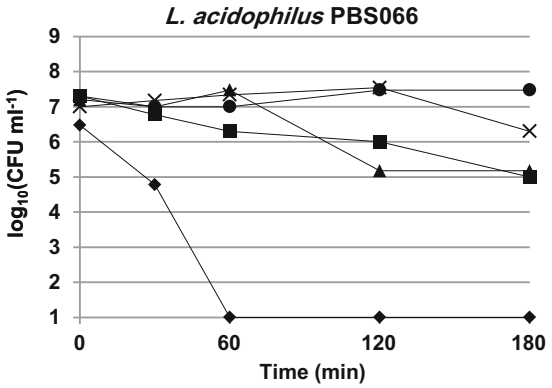
Antibiotic resistance of lactobacilli and bifidobacteria strains

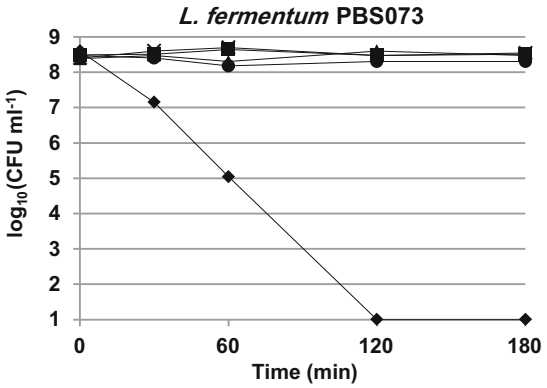
The antibiotic resistance profiles of the strains to the nine antibiotics suggested in the most recent European Food Safety Authority (EFSA) guidelines was determined. The sensitivity or resistance of the strains was assessed by comparing the obtained MIC values for each antibiotic to the reference breakpoints described for the corresponding genus or species (EFSA 2012). All the strains displayed MIC values behind or

Table 1 List of the seven lactic acid bacteria used in this study

Strain	Deposit Number	Origin
Lactobacillus acidophilus PBS066	DSM 24936	Human
Lactobacillus fermentum PBS073	DSM 25176	Human
Lactobacillus plantarum PBS067	DSM 24937	Human
Lactobacillus reuteri PBS072	DSM 25175	Human
Lactobacillus rhamnosus PBS070	DSM 25568	Human
Bifidobacterium animalis subsp. *lactis* PBS075	DSM 25566	Human
Bifidobacterium longum subsp. *longum* PBS108	DSM 25174	Human

Strains, isolated from the faeces of healthy humans, were identified by 16S rDNA gene identification


L. acidophilus PBS066
log10(CFU ml-1)
Time (min)
0
60
120
180
1
2
3
4
5
6
7
8
9


L. fermentum PBS073
log10(CFU ml-1)
Time (min)
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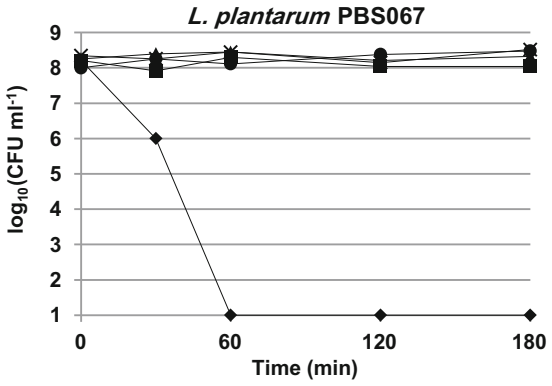
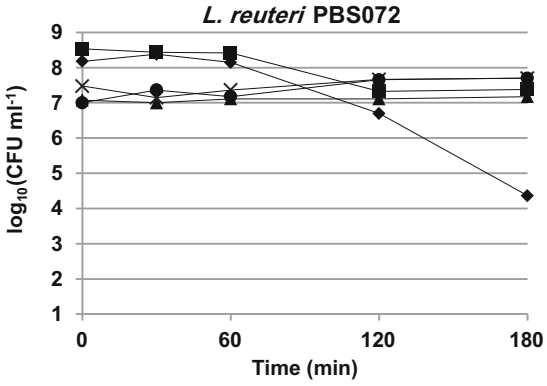
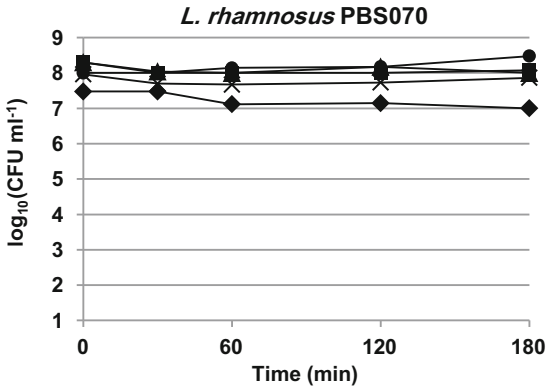
60
120
180
1
2
3
4
5
6
7
8
9


L. plantarum PBS067
log10(CFU ml-1)
Time (min)
0
60
120
180
1
2
3
4
5
6
7
8
9


L. reuteri PBS072
log10(CFU ml-1)
Time (min)
0
60
120
180
1
2
3
4
5
6
7
8
9


L. rhamnosus PBS070
log10(CFU ml-1)
Time (min)
0
60
120
180
1
2
3
4
5
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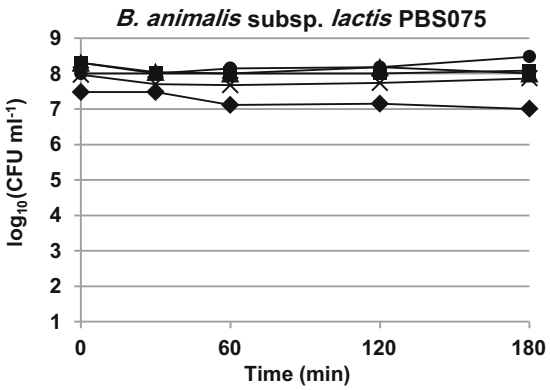
7
8
9


B. animalis subsp. lactis PBS075
log10(CFU ml-1)
Time (min)
0
60
120
180
1
2
3
4
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6
7
8
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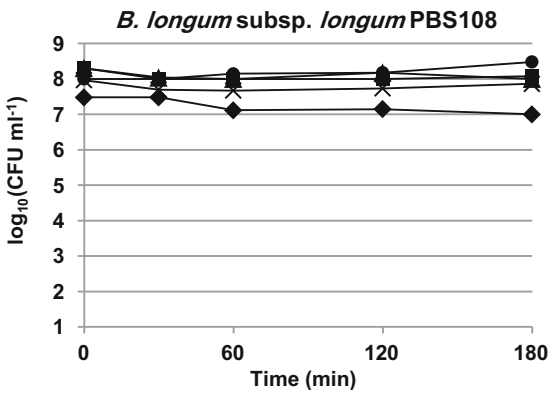
B. longum subsp. longum PBS108
log10(CFU ml-1)
Time (min)
0
60
120
180
1
2
3
4
5
6
7
8
9

◀ **Fig. 1** Effect of pH on the survival of lactobacilli and bifidobacteria strains. Cells were incubated for 3 h at 37 °C in the following buffers: 50 mM glycine buffer pH 2.0 (*diamond*) and 3.0 (*square*), 50 mM sodium acetate buffer pH 4.0 (*triangle*) and 5.0 (*cross*) and sodium phosphate buffer pH 7.0 (*circle*) as controls. The residual viability was determined at regular time intervals of 0, 30, 60, 120 and 180 min by the count plate method

close to the reference breakpoints with the exception of *B. animalis* subsp. *lactis* PBS075, whose MIC value for tetracycline was above the breakpoint established for the *Bifidobacterium* genus. The results are shown in Table 2.

The *tet(W)* gene, the most common tetracycline resistance determinant in bifidobacteria, was amplified by PCR. A second amplification with the forward primer specific for the transposase *trp* and the reverse primer specific for *tet(W)* confirmed that *tet(W)* is flanked upstream by the putative transposase gene *trp*.

Antimicrobial activity by cell culture supernatants and by cells of lactobacilli and bifidobacteria strains

Complementary approaches were combined in order to define the antimicrobial activity of lactobacilli and bifidobacteria strains against pathogens. We have chosen *Escherichia coli* ATCC 29522, *Enterococcus faecalis* ATCC 29212, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538 and *C. albicans* ATCC 10231 as the most representative pathogens able to colonize different districts of the organism.

The well diffusion agar assay provided preliminary information about the inhibitory action exerted by microbial culture supernatants.

Most of non-neutralized culture supernatants of the examined strains showed an inhibition grade on the pathogen growth, measured by the inhibition halo diameters (data not shown). Results are compared with a positive control consisting in a *Bifidobacterium* sp. strain, whose antimicrobial properties have been already described (Aloisio et al. 2012). The only exception was for *C. albicans* ATCC 10231, whose growth seemed not to be influenced by the culture supernatants. The neutralized culture supernatants did not exert any antimicrobial activity.

The non-neutralized cellular supernatants of the strains, which positively responded to the preliminary assay, were subjected to further investigations. Their antimicrobial properties were evaluated by comparing the growth of the antagonists within 24 h in the presence and in the absence of culture supernatants. Figure 2 shows the inhibition rate of the different microbial culture supernatants towards the antagonist. Some of them were more sensitive to others to the microbial culture supernatants, whereas some culture supernatants were more effective in their antimicrobial activity. *L. acidophilus* PBS066 and *L. plantarum* PBS067 strongly inhibited *Enterococcus faecalis*, *S. aureus*, *P. aeruginosa* and *Escherichia coli*.

L. rhamnosus PBS070 and *Bifidobacterium* strains PBS075 and PBS108 inhibited *P. aeruginosa* and *Escherichia coli. L. fermentum* PBS073 exerted an antimicrobial activity mainly towards *S. aureus*, *Escherichia coli* and *P. aeruginosa*, whereas *L. reuteri* PBS072 inhibited *S. aureus* and *P. aeruginosa*.

In order to verify the antimicrobial potential of cells in vivo, cultures of lactobacilli and bifidobacteria strains were overlaid by the antagonists *Escherichia coli*, *Enterococcus faecalis*, *P. aeruginosa*, *S. aureus* and *C. albicans* cultures. As shown in Fig. 3, the growth inhibition halos around the colonies confirmed the antimicrobial inhibition profile reported by the analysis of the culture supernatants. In particular, a strong growth inhibition of *C. albicans* exerted by *L. acidophilus* PBS066, *L. plantarum* PBS067, *L. rhamnosus* PBS070 and *B. longum* subsp. *longum* PBS108 could be observed.

Table 2 Antibiotic susceptibility test

	L. acidophilus PBS066		*L. fermentum* PBS073		*L. plantarum* PBS067		*L. reuteri* PBS072		*L. rhamnosus* PBS070		*B. animalis* subsp. *lactis* PBS075		*B. longum* subsp. *longum* PBS108	
Antibiotic	MIC	S/R	MIC	S/R	MIC	S/R	MIC	S/R	MIC	S/R	MIC	S/R	MIC	S/R
Ampicillin	1	S	0.5	S		S	1	S	2	S	1	S	0.25	S
Vancomycin	1	S	128	N.R.	128	N.R.	128	N.R.	128	N.R.	1	S	0.5	S
Gentamycin	4	S	1	S	2	S	1	S	4	S	32	S	64	S
Kanamycin	64	S	16	S	64	S	4	S	64	S	256	N.R.	256	N.R.
Streptomycin	8	S	8	S	32	N.R.	4	S	16	S	128	S	128	S
Erythromycin	0.25	S	0.5	S	0.25	S	0.25	S	0.125	S	0.5	S	0.032	S
Clindamycin	0.5	S	0.5	S	0.5	S	0.5	S	0.25	S	0.5	S	0.032	S
Tetracyclin	1	S	4	S	16	S	16	S	1	S	32	S	8	S
Chloramphenicol	4	S	4	S	4	S	4	S	4	S	4	S	2	S

MIC minimum inhibitory concentration (μg/mL) of the selected antibiotic, *S/R* strains are classified as sensitive (S) or resistant (R) according to EFSA breakpoint values (2012), *N.R.* the MIC determination is not required by EFSA

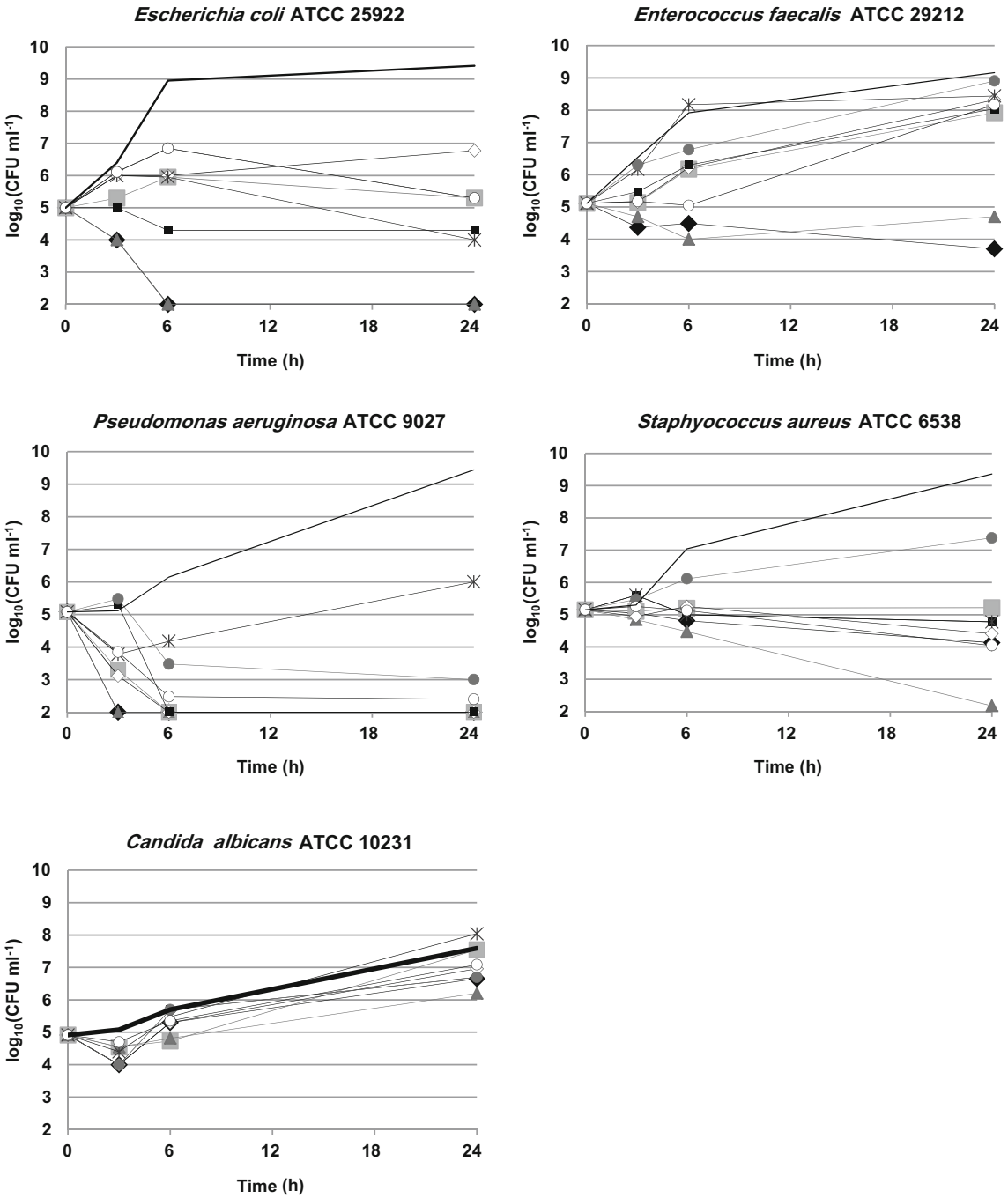

Fig. 2 Effect of non-neutralized culture supernatants of *L. acidophilus* PBS066 (*diamond*), *L. fermentum* PBS073(*square*), *L. plantarum* PBS067 (*triangle*), *L. reuteri* PBS072 (*open* and *filled diamond*), *L. rhamnosus* PBS070 (*square*), *B. animalis* subsp. *lactis* PBS075 (*cross*) and *B. longum* subsp. *longum* PBS108 (*filled circle*) on the growth of *E. coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC6538 and *C. albicans* ATCC 10231 as antagonistic strians. The non-neutralized culture supernatant of *Bifidobacterium* sp. (*open circle*), known for its antimicrobial properties (Aloisio et al. 2012), was also included. A positive growth control of each antagonist in the absence of culture supernatants was included. Samples were taken at regular time intervals of 0, 3, 6 and 24 h and the viability was determined by the count plate method

Screening for vitamin B8, B9 and B12 producers

All the strains were screened for their ability to grow on biotin (vitamin B8), folic acid (vitamin B9) or cobalamin (vitamin B12) assay media, containing all the nutrients except for biotin or folic acid or cobalamin. The examined strains were able to grow when the absent vitamin was supplemented into each medium at a final concentration of 10 μg/L. This indicates that the media composition was able to support the nutritional requirements of lactobacilli and bifidobacteria strains, with the vitamin

Fig. 3 Example of agar plate method showing strong inhibition of *Candida albicans* growth over *Lactobacillus* cultures

of interest being the limiting factor. *L. plantarum* PBS067 was able to grow on all the three vitamin assay media, while *L. fermentum* PBS073 and *L. reuteri* PBS072 were able to grow on folic acid or cobalamin vitamin assay media for at least seven sub-cultures. In the case of *L. rhamnosus* PBS070 and *B. animalis* subsp. *lactis* PBS075, the growth was observed only on B12 assay medium. *L. acidophilus* PBS066 and *B. longum* subsp. *longum* PBS108 died within four passages (data not shown).

Resistance and modulation of inflammatory stress by lactobacilli and bifidobacteria strains on a systemic-miming cell line in vitro

The model fibroblast cell line 3 T3 was treated with SDS as irritant agent for 24 h and for 5 days, in order to induce an acute and a chronic stress, respectively. The protective effect of the lactobacilli and bifidobacteria strains against the inflammatory damage was evaluated by co-culturing the cell line with the bacterial strains. The residual cell viability and inflammatory response in the presence and in the absence of microbial strains were determined. In the sole presence of SDS, the cell viability was reduced up to 50 % with respect to the negative control. The cultures of *L. plantarum* PBS067, *L. reuteri* PBS072 and *L. rhamnosus* PBS070 increased the cell viability more than 90 % both in acute and in chronic stress conditions. The beneficial effect of *B. longum* subsp. *longum* PBS108 on cell viability was more evident in acute stress condition, with an increase up to 90 %, than in chronic stress conditions. *L. fermentum* PBS073 slightly favoured cell viability just up to 60 %. In contrast, *B. animalis* subsp. *lactis* PBS075 did not exert a strong protective effect on the cells (Fig. 4).

The modulation of the anti-inflammatory response by the microbial strains on the fibroblast cells was evaluated

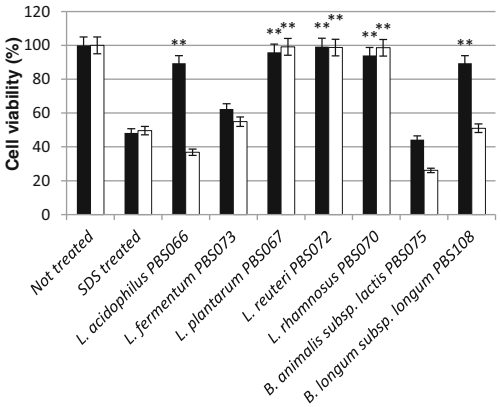

Fig. 4 Protective effect of lactobacilli and bifidobacteria strains on BALB/c3T3 cells in the presence of SDS as irritant agent after 24 h (*filled bar*) and at 5 days (*open bar*) of exposure. The negative control was represented by non-treated cells. Residual cell viability was measured by means of MTT assay. The results, expressed as percentage (%), were calculated

by determining the concentration of IL-4 and TNF-α released into the culture medium after SDS treatment. In an acute stress condition (24-h treatment with SDS), the inflammatory state determined a slight increase of IL-4 and a fourfold increase of TNF-α with respect to the untreated cells. In a chronic inflammation state (5-day treatment with SDS), the level of IL-4 was constant, whereas the TNF-α concentration further increased.

In acute inflammation condition, the cultures of *L. plantarum* PBS067, *L. reuteri* PBS072, *L. rhamnosus* PBS070 and *B. longum* subsp. *longum* PBS108 were able to further stimulate the IL-4 release. At the same time, there was a reduction of the TNF-α, whose concentration was similar to those recorded in the negative controls. *L. fermentum* PBS073 and *B. animalis* subsp. *lactis* PBS075 exerted a less significant modulatory effect (Fig. 5a). In chronic inflammation condition, *L. plantarum* PBS067, *L. reuteri* PBS072 and *L. rhamnosus* PBS070 were still able to stimulate the IL-4 release and to reduce the TNF-α concentration, at levels comparable to the negative control (Fig. 5b).

Antioxidant potential of lactobacilli and bifidobacteria strains

The antioxidant potential of 3 T3 cell line was determined by FRAP assay in the presence and in the absence of microbial strains both at 24 h and 5 days of co-culture. The results reported in Fig. 6 show that after 24 h, *L. plantarum* PBS067, *L. reuteri* PBS072, *L. rhamnosus* PBS070 and *B. animalis* subsp. *lactis* PBS075 were able to improve the antioxidant potential of the cell culture. After 5 days, only *L. plantarum* PBS067 and *L. rhamnosus* PBS070 were still able to significantly increase the antioxidant potential of the cell line.

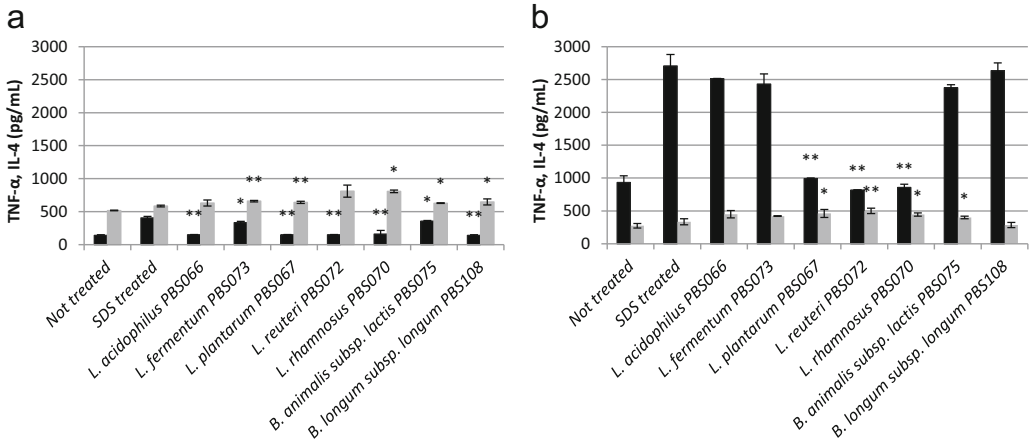

Fig. 5 Effect of lactobacilli and bifidobacteria strains on the cytokine secretion by BALB/c3T3 cells in the presence of SDS as irritant agent. The non-treated sample was included as negative control. The concentration of TNF-α (*black bar*) and IL-4 (*grey bar*) at 24 h (**a**) and at 5 days (**b**) was monitored by ELISA test. The experiments were performed three times in triplicate. The results are expressed as the means±standard deviations, *$p<0.05$ and **$p<0.01$ as compared to SDS-treated samples

Resistance and modulation of inflammatory stress by lactobacilli and bifidobacteria strains on the intestinal HT-29 cell line in vitro

On the basis of the obtained results in the preliminary study on 3 T3 cells, the most performant strains, *L. plantarum* PBS067, *L. rhamnosus* PBS070 *and B. animalis subsp. lactis* PBSO75, were selected for in vitro tests with the human intestinal HT-29 cell line.

Despite the presence of the bacterial strains does not protect the cell viability from the irritating agent, they showed a positive effect on the antioxidant potential and inflammatory status of the biological system. In an acute stress condition, the inflammatory state determined a slightly increase of IL-4 and IL-10 and an increase of TNF-α with respect to the untreated cells. In a chronic inflammation state, the level of IL-4 and IL-10 increased in a significant manner, whereas the TNF-α concentration further increased. Also, the antioxidant potential showed a significant increase especially in chronic conditions. Results are reported in the Fig. 7a, b, and Fig. 8.

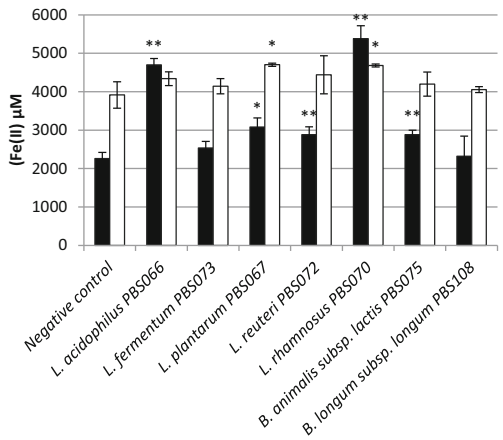

Fig. 6 Effect of lactobacilli and bifidobacteria strains on the antioxidant potential of BALB/c3T3 cells. The cell culture at 24 h (*filled bar*) and at 5 days (*open bar*) was monitored by FRAP assay. The results, expressed as µM of Fe(II), are the means±standard deviations, *$p<0.05$ and **$p<0.01$ as compared to the negative control

Discussion

The ability of lactobacilli and bifidobacteria to exert beneficial effects on the human health is a species- and strain-specific feature (Ramos et al. 2013). This explains the continuous search for novel strains with probiotic potential.

In this work, the probiotic properties of new *Lactobacillus* and *Bifidobacterium* strains were screened, and the effects of the strains on cell lines by *in vitro* tests were evaluated. Moreover, the probiotic property effects of the most *Lactobacillus* and *Bifidobacterium* performant strains were evaluated on human intestinal cell lines, in order to successively move to another level of study towards patients with acute and chronic disorders of the gut by in vivo experiments.

As fundamental requirement a probiotic strain should survive through the passage of the stomach and the duodenum. Our results showed that the viability of the examined lactobacilli and bifidobacteria strains was not affected at pH 3.0 and in presence of a standard concentration of bile salts. On the other hand, the survival at pH 2.0 was revealed to be a species-specific feature. *L. reuteri* PBS072 and *L. rhamnosus* PBS070 evidenced the highest survival rate, whereas *L. acidophilus* PBS066, *L. plantarum* PBS067 and *L. fermentum* PBS073 were the most sensitive strains. These observations are in agreement with literature data (Santini et al. 2010; Maragkoudakis et al. 2006) that evidenced a good survival of these bacteria at pH 3.0 and in presence of bile salts (Charteris et al. 1998; Jacobsen et al. 1999), but a great sensitivity to lower pH.

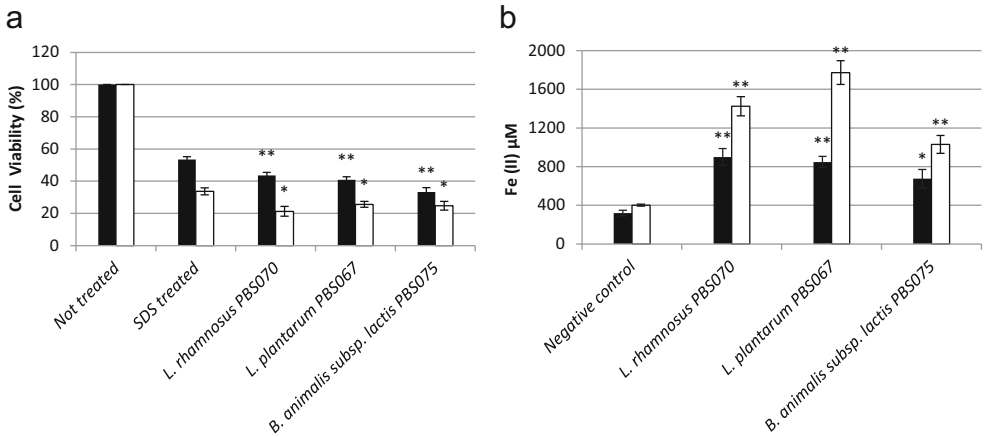

Fig. 7 Protective effect of lactobacilli and bifidobacteria strains on HT-29 cells in the presence of SDS as irritant agent after 24 h (*filled bar*) and at 5 days (*open bar*) of exposure (**a**). The negative control was represented by non-treated cells. Residual cell viability was measured by means of MTT assay. The results, expressed as percentage (%), were calculated. Effect of lactobacilli and bifidobacteria strains on the antioxidant potential of HT-29 cells (**b**). The cell culture at 24 h (*filled bar*) and at 5 days (*open bar*) was monitored by FRAP assay. The results, expressed as µM of Fe(II), are the means±standard deviations; *$p<0.05$ and **$p<0.01$ as compared to the negative control

The consumption of probiotic containing antibiotic resistance strains may pose the risk of the transfer of antibiotic resistance genes to the resident microbial community and hence to pathogenic bacteria. For this reason, the assessment of the susceptibility to antimicrobials of the strains with a standardized method was strongly required. The examined strains were sensitive to all the selected antibiotics, in agreement with the most recent EFSA guidelines (EFSA 2012). The only exception was represented by *B. animalis* subsp. *lactis* PBS075, which exhibited a MIC value of tetracycline above its breakpoint. The *tet*(W) gene, the most widespread in tetracycline resistance determinant in *B. animalis* subsp. *lactis* strains, was found to be also present in PBS075. *tet*(W) is flanked upstream by the transposase *trp* gene, which is co-trancribed in tandem (Gueimonde et al. 2010). Altough the evidence that *tet*(W) is transmissible in these bacteria have been not documented so far (Moubareck et al. 2005) (Ammor et al. 2008) (Gueimonde et al. 2010), our results suggest that *B. animalis* subsp. *lactis* PBS075 strain could not be considered an appropriate strain for future in vivo trials. As functional requirement, a probiotic strain should exert antagonistic activity against potential pathogenic bacteria. In this work, a representative selection of bacteria analogous to common human pathogens was included as antagonists. All the examined strains were capable of contrasting at different extent the growth of the antagonists. *P. aeruginosa* and *Escherichia coli* were the most sensitive microorganisms. The antibacterial activity of cell cultural supernatants is

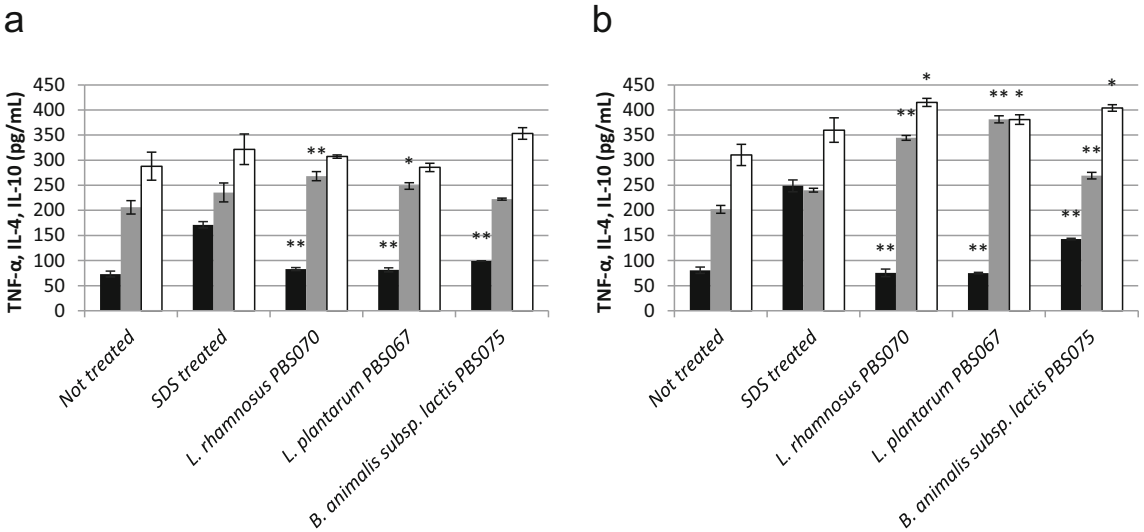

Fig. 8 Effect of lactobacilli and bifidobacteria strains on the cytokine secretion by HT-29 cells in the presence of SDS as irritant agent. The non-treated sample was included as negative control. The concentration of TNF-α (*black bar*), IL-4 (*grey bar*) and IL-10 (*white bar*) at 24 h (**a**) and at 5 days (**b**) was monitored by ELISA test. The experiments were performed three times in triplicate. The results are expressed as the means ±standard deviations; *$p<0.05$ and **$p<0.01$ as compared to SDS-treated samples

conventionally attributed to the production of organic acids, diacetyl, bacteriocins or hydrogen peroxide (Servin 2004). Preliminary investigations about the nature of the antimicrobial activities of the examined strains were performed. The lack of pathogen inhibition of the pH neutralized culture supernatants revealed that the antimicrobial activity mainly resulted from the production of acidic metabolites, such as lactic acid, rather than excreted bacteriocins. The higher amount of lactic acid produced by the homofermentative bacteria with respect to the heterofermentative ones could explain why *L. acidophilus* PBS066 and *L. plantarum* PBS067 exhibited a broader inhibition activity than all the other examined strains. None of the non-neutralized culture supernatants was able to inhibit the growth of *C. albicans*. Interestingly, when *L. acidophilus* PBS066, *L. fermentum* PBS073, *L. plantarum* PBS067, *L. rhamnosus* PBS070 and *B. longum* subsp. *longum* PBS108 cells were exposed to *C. albicans*, a remarkable inhibition occurred. This effect could be may be due to an inducible production of some antimicrobial metabolites with antifungal properties.

Vitamin production is not a common characteristic of the species, but a single probiotic strain property. For example, as observed by Pompei et al. (2007) despite the presence in *B. longum* NCC2705 genome of the entire pathway for folic acid production, only few *B. longum* strains were able to grow in the absence of this vitamin. A screening for biotin, folic acid and cobalamin producers by our strains revealed that only *L. plantarum* PBS067 was able to synthesize the three examined vitamins, while *L. fermentum* PBS073 and *L. reuteri* PBS072 folic acid and cobalamin. It was already demonstrated that some strains of *L. plantarum* (Sybesma et al. 2003) and *L. reuteri* (Santos et al. 2008) are able to produce significant amount of folate. To our knowledge, no *L. fermentum* strains have been characterized as folic acid producers so far, but the completely sequenced *L. fermentum* IFO 3956 possessed the entire biosynthetic cluster (Rossi et al. 2011). Cobalamin is the only vitamin that is exclusively synthetized by microorganisms. The analysis of recently sequenced lactobacilli genomes suggested that cobalamin biosynthesis is common among different *Lactobacillus* species (Capozzi et al. 2012). This observation is in agreement with our screening, which showed the ability of five strains out of seven to grow in the absence of B12 vitamin.

Our strains were also characterized for their ability to interact with the host's cells by in vitro experiments. First, a systemic model as the murine fibroblast BALB/c 3T3a cell line was used in this study in order to evaluate the possible different effects. The examined strains were able to modulate the release of IL-4 and TNF-α by the 3 T3 cells in vitro. Three of the seven strains revealed to have the potential to neutralize the response caused by an irritant agent, by inducing higher production of the anti-inflammatory cytokine IL-4, thus lowering the TNF-α production. For instance, a comparative study of the immunomodulatory properties of *B. longum* evidenced a different ability of the various strains to diverge the immune response towards a Th1 or a Th2 pathway (Medina et al. 2007). The dendritic cells differently responded to *L. plantarum* strains inducing the release of different amounts of IL-10 and IL-12 (Meijerink et al. 2010). It should be noted that the immune profile obtained in co-culture assays with bacteria and immune cells was demonstrated to be predictive of their in vivo immunomodulatory activities (Foligne et al. 2007). Moreover, it is demonstrated that probiotic bacteria contribute in the reduction of oxidative damage in humans (Amaretti et al. 2013; Lin and Yen 1999; Spyropoulos et al. 2011). The oxidation in living organisms, essential for energy production, leads to the accumulation of harmful reactive species, which induce oxidative damage. This plays a significant role in the pathogenesis of several human diseases such as cancer, emphysema, cirrhosis and atherosclerosis (Kryston et al. 2011). Results on the examined strains showed that some of them, such as *L. plantarum* PBS067, *L. rhamnosus* PBS070 and *B. longum* subsp. *longum* PBS108, seemed to have a stronger effect on the antioxidative potential of the cell line. FRAP assay employed in this study provided information on the efficacy of the antioxidants present in the sample (Benzie and Strain 1996; Lin and Yen 1999).

Many studies have demonstrated the clinical potential of probiotics against many diseases, such as necrotizing enterocolitis, inflammatory bowel disease and colon cancer (Robles Alonso and Guarner 2013). In this study, three most performant strains, *L. plantarum* PBS067, *L. rhamnosus* PBS070 *and B. animalis subsp. lactis* PBSO75, were selected for in vitro tests with a human intestinal cell line. Data showed that the trend of action of HT-29 cells was quite similar to the 3 T3 cell one and the examined strains were able to modulate the release of IL-4, IL-10 and TNF-α and to increase the antioxidant potential. This allows to move to another level of study, that is, the administration of the most probiotical performant strains to patients with acute and chronic disorders of the gut by in vivo experiments.

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Immunity
Article

Tryptophan Catabolites from Microbiota Engage Aryl Hydrocarbon Receptor and Balance Mucosal Reactivity via Interleukin-22

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SUMMARY

Endogenous tryptophan (Trp) metabolites have an important role in mammalian gut immune homeostasis, yet the potential contribution of Trp metabolites from resident microbiota has never been addressed experimentally. Here, we describe a metabolic pathway whereby Trp metabolites from the microbiota balance mucosal reactivity in mice. Switching from sugar to Trp as an energy source (e.g., under conditions of unrestricted Trp availability), highly adaptive lactobacilli are expanded and produce an aryl hydrocarbon receptor (AhR) ligand—indole-3-aldehyde—that contributes to AhR-dependent *Il22* transcription. The resulting IL-22-dependent balanced mucosal response allows for survival of mixed microbial communities yet provides colonization resistance to the fungus *Candida albicans* and mucosal protection from inflammation. Thus, the microbiota-AhR axis might represent an important strategy pursued by coevolutive commensalism for fine tuning host mucosal reactivity contingent on Trp catabolism.

INTRODUCTION

Indoleamine 2,3-dioxygenase 1 (IDO1), a "metabolic" enzyme conserved through the past 600 million years of evolution, degrades tryptophan (Trp), suppresses inflammation, and is involved in mammals in the peripheral generation of regulatory T (Treg) cells, an event requiring the combined effects of Trp starvation and a series of endogenous Trp catabolites collectively known as "kynurenines" (Puccetti and Grohmann, 2007). In particular, L-kynurenine, the first breakdown product in the IDO1-dependent Trp degradation pathway, activates the aryl hydrocarbon receptor (AhR) in lymphoid tissues (Opitz et al., 2011) and promotes Treg cell development (Fallarino et al., 2006; Mezrich et al., 2010). In the gastrointestinal tract, diet-derived AhR ligands promote local IL-22 production (Lee et al., 2012) by innate lymphoid cells (ILCs) (Qiu et al., 2012), now referred to as group 3 ILCs (ILC3s) (Spits et al., 2013). Metabolomic analysis has revealed that gut bacteria impact host metabolism and immunity through a variety of chemically different metabolites, including amino acid metabolites (Wikoff et al., 2009). In particular, dietary lack of Trp impairs intestinal immunity in mice and alters the gut microbial community (Hashimoto et al., 2012), suggesting that mucosal homeostasis is a multifactorial phenomenon of which Trp metabolism is an important regulatory component. However, the source and nature of any such AhR ligands, any impact of microbial dysbiosis on AhR- and IL-22-driven mucosal reactivity, and whether AhR activation by microbiota-derived metabolites also occurs have all been unclear.

Candida albicans is a commensal of the orogastrointestinal tract, vagina, and skin (Romani, 2011). Owing to microbial dysbiosis or defects in the innate and adaptive immune systems, the fungus shifts from commensalism to parasitism, and it might cause severe infections, such as the oropharyngeal, vulvovaginal, and chronic mucocutaneous forms of human candidiasis. In combination with IL-17A, IL-22 mediates a pivotal innate antifungal resistance in mice (De Luca et al., 2010) and humans (Puel et al., 2010). These cytokines are regulated by the intestinal microbiota (Ivanov et al., 2009; Satoh-Takayama et al., 2008). This could not only explain the high risk of mucosal candidiasis after antibiotic therapy in humans (Sobel, 2007) and gnotobiotic mice (Balish et al., 1993) but also suggest an interplay between Trp catabolism by microbiota, the host's own pathway of metabolite production, and AhR-orchestrated mucosal function.

Here, we used *Ido1*$^{-/-}$ mice and dietary Trp feeding to investigate any links among Trp catabolism in the host, activation of the AhR-IL-22 axis, and the role of microbiota in mucosal immune homeostasis in the gut. By correlating changes in metabolite profiles with microbiota metagenomic composition, we have defined a functional node by which lactobacilli contribute to host-microbial symbiosis and homeostasis at mucosal surfaces.

RESULTS

The AhR-IL-22 Axis Provides Resistance to Mucosal Candidiasis in *Ido1*$^{-/-}$ Mice

To examine any effects of host-extrinsic Trp catabolism on AhR-dependent IL-22 production and colonization resistance to *Candida*, we analyzed *Ido1*$^{-/-}$ or *Ahr*$^{-/-}$ mice with or without

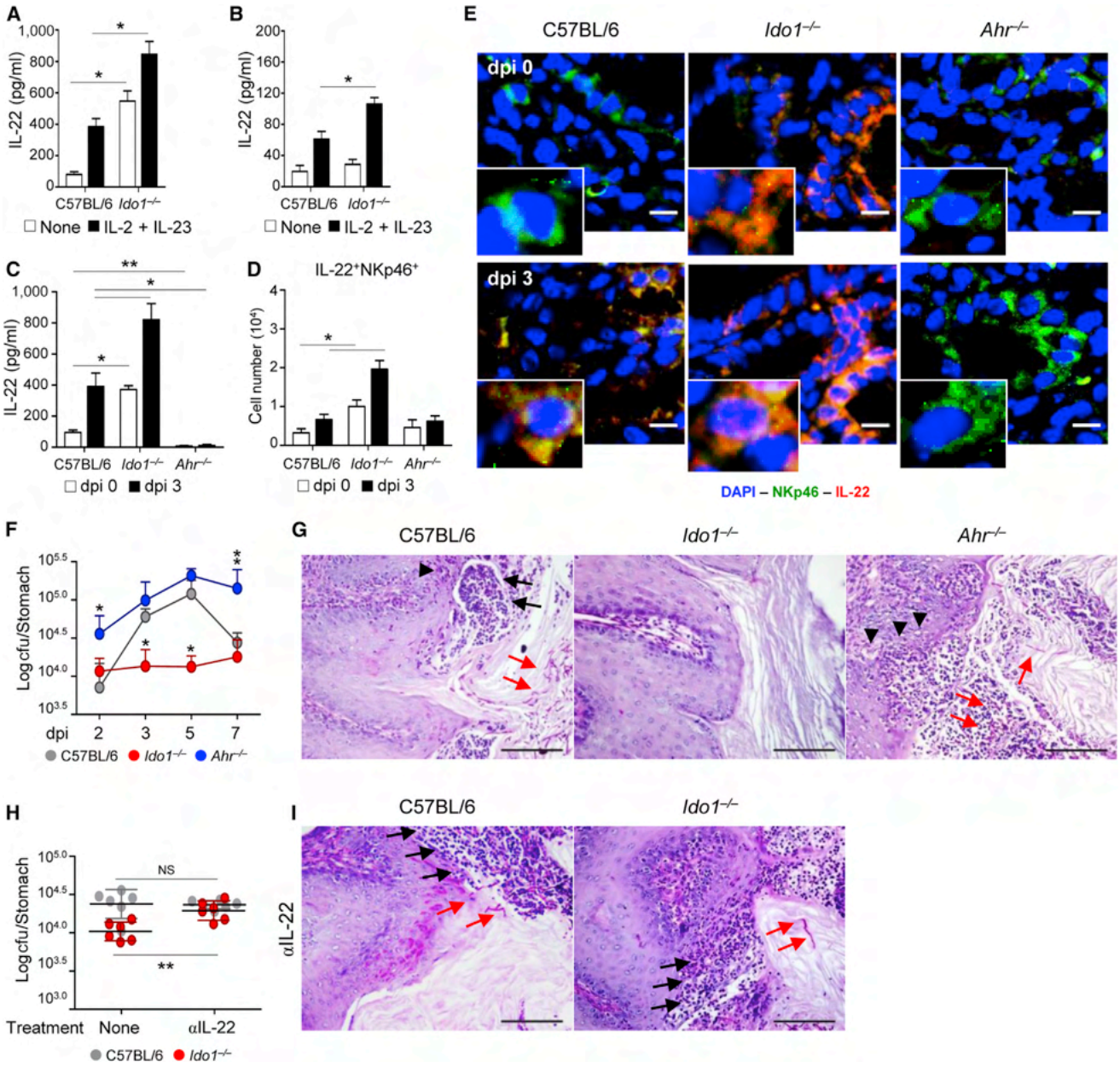


Figure 1. The AhR-IL-22 Axis Provides Antifungal Resistance in *Ido1*$^{-/-}$ Mice

(A and B) IL-22 production (ELISA) by PPs (A) and purified PP-NKp46^{+} cells (B) stimulated with IL-2 and IL-23 in vitro.

(C) IL-22 production (ELISA) in vivo in the stomach of mice naive infected with *Candida albicans* intragastrically.

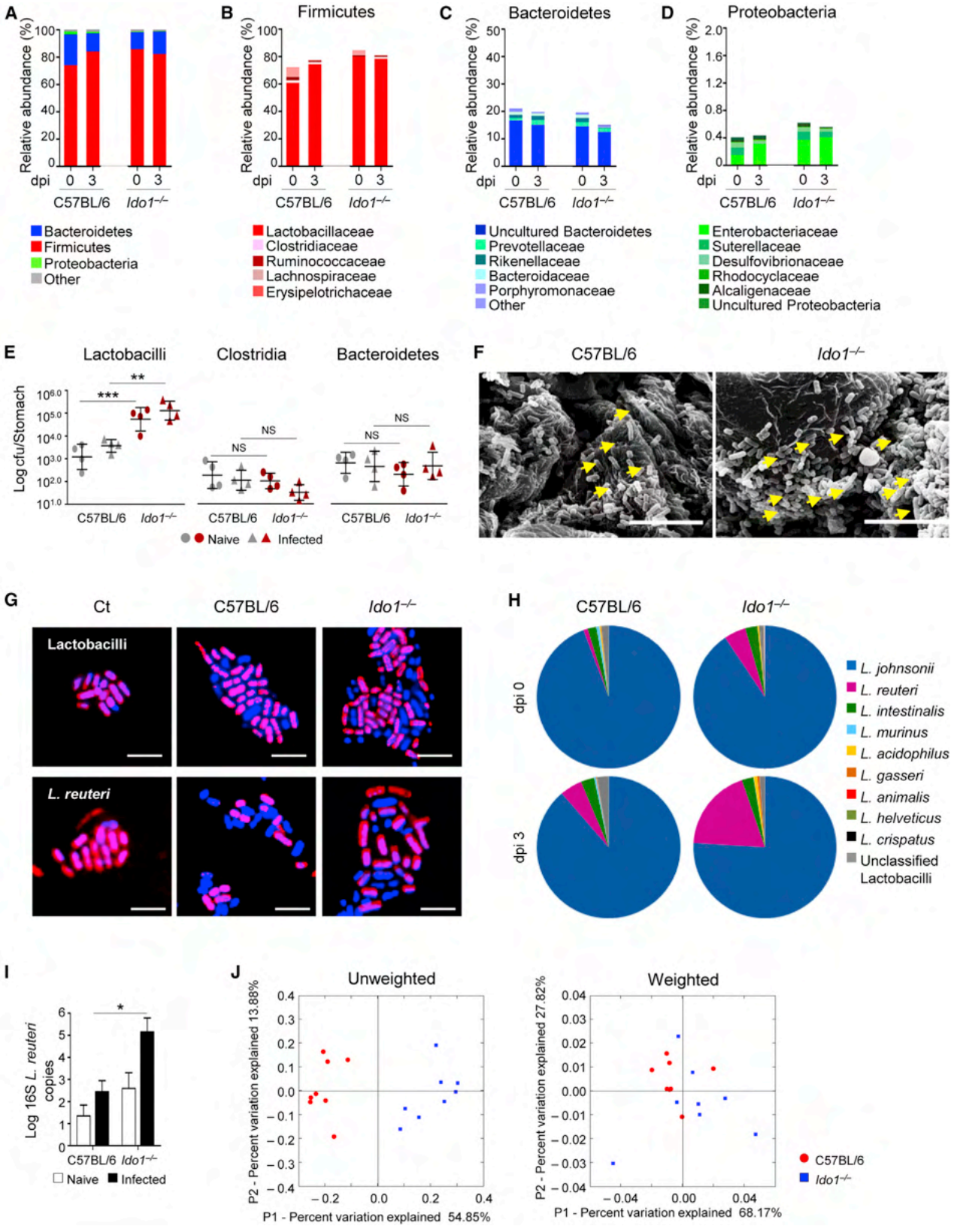
(D and E) Number of NKp46^{+} IL-22^{+} cells (D) and immunostaining with anti-NKp46-FITC and anti-iL-22-PE antibody (E) in the stomach at different days post-infection. Cell nuclei were stained with DAPI (in blue). Scale bars represent 20 μm (dpi 0, naive mice).

(F and G) Colony-forming units (cfu) in the stomach (F) and stomach histology (3 dpi) (G). Red arrows indicate fungi, black arrows indicate inflammatory cells, and arrowheads indicate epithelial ulceration in sections stained with periodic acid Schiff. Images were acquired with a 40× objective. Scale bars represent 100 μm.

(H and I) Fungal growth (H) and stomach histology (I) (3 dpi) of infected mice treated with IL-22 mAb neutralizing antibody. Red arrows indicate fungi, and black arrows indicate inflammatory cells. Scale bars represent 100 μm. "None" refers to isotype control antibody.

Results shown are compiled data from four independent experiments (means ± SD) or a representative image. All error bars represent the SD of samples within a group. *$p < 0.05$, **$p < 0.01$. "NS" stands for not significant. Control littermates of the genetically deficient mice—occasionally tested in parallel with wild-type (WT) hosts in these and subsequent experiments—showed a pattern of reactivity indistinguishable from the latter. See also Figure S1.

infection. Higher amounts of IL-22 were found in Peyer's patches (PPs) (Figure 1A), in NKp46^{+} cells from PPs (Figure 1B), and in the stomachs of naive (0 days postinfection [dpi]) *Ido1*$^{-/-}$ mice than in their wild-type (WT) counterparts (Figure 1C). After oral infection with *C. albicans*, the amounts of IL-22 were still higher in the stomach, a target organ in candidiasis, of *Ido1*$^{-/-}$ mice



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(Figure 1C) and correlated with the expansion of NKp46^{+} cells producing IL-22, as revealed by cell number (Figure 1D) and immunofluorescence staining (Figure 1E, 3 dpi). IL-22-producing NKp46^{+}NK1.1^{lo} cells, expressing lineage-specific transcripts (Figure S1A, available online), also expanded in the PPs (Figure S1B). IL-17F and IL-10, but not IL-17A (Figure S1C), were also higher in the stomach (Figure S1C), esophagus (Figure S1D), and ileum (Figure S1E) of *Ido1*$^{-/-}$ mice. Fewer signs pathognomonic for infection were observed in those mice, as revealed by restricted fungal growth (Figure 1F) and low-grade histopathology in the stomach (Figure 1G).

In this setting, dependency of the anticandidal resistance on functional AhR and IL-22 production was demonstrated by the detrimental effects of genetic AhR deficiency (Figures 1F and 1G), which would per se negate IL-22 production (Figure 1C) and ILC3 expansion (Figures 1D and 1E), and by IL-22 neutralization experiments in infected *Ido1*$^{-/-}$ mice (Figures 1H and 1I and Figure S1F). Overall, these data suggest that sources of AhR ligands—other than host kynurenines—can influence or even enhance mucosal reactivity to *Candida*.

Lactobacilli Expand in the Stomachs of IDO1-Deficient Mice

Of the dietary Trp that is not used in protein synthesis, 99% is metabolized in mammals by IDO1 (Puccetti and Grohmann, 2007).Thus, increased Trp availability in the gut of *Ido1*$^{-/-}$ mice could alter both the qualitative composition and the metabolic pathways in the local mixed microbial community. We searched for qualitative and/or quantitative changes in microbial communities from fecal samples and the stomach—whose metagenomic profile is far less characterized than that of feces—by barcoded pyrosequencing of 16S rRNA. Upon analyzing a total of 620,369 reads with a median length of 365.4 bp, we found that WT and *Ido1*$^{-/-}$ mice, either uninfected or infected with *Candida*, displayed similar relative abundances of major bacterial phyla in the stomach (Firmicutes predominated, and Bacteroidetes and Proteobacteria were the next most abundant) (Figure 2A). Both types of mice shared most taxa at the family levels within Firmicutes (e.g., Lactobacillaceae, Clostridiaceae, Ruminococcaceae, Lachnospiracae, and Erysipelotrichaceae) (Figure 2B), Bacteroidetes (e.g., uncultured Bacteroidetes, Prevotellaceae, Rikenellaceae, Bacteroidaceae, Porphyromonaceae, and others) (Figure 2C), and Proteobacteria (e.g., Enterobacteriaceae, Suterellaceae, Desulfovibrionaceae, Rhodocyclaceae, Alcaligenaceae, and uncultured Proteobacteriae) (Figure 2D) (Tables S1–S4). However, closer examination of the relative abundance of taxa in Firmicutes revealed noticeable differences between *Ido1*$^{-/-}$ and WT mice. Lactobacilli predominated over Clostridia in *Ido1*$^{-/-}$ mice, whether naive or after infection (Figures 2B and 2E). Scanning electron microscopy (Figure 2F) and fluorescence in situ hybridization (FISH) (Figure 2G) confirmed that *Lactobacillus* morphotypes were abundant in the stomach, and lactobacilli could be identified in gastric fluids from *Ido1*$^{-/-}$ mice. Lactobacilli were also expanded by infection in the esophagus (10^4/g versus 10^2/g; Figure S2A) and ileum (10^6/g versus 10^5/g; Figure S2B) of *Ido1*$^{-/-}$ mice.

Distinct species of the *Lactobacillus* genus are present in the murine intestinal tract and are grouped in several taxa on the basis of, among other parameters, resistance or susceptibility to vancomycin (Peña et al., 2004). We found striking differences at the level of lactobacilli species between *Ido1*$^{-/-}$ and WT mice, either naive or carrying infection. *L. johnsonii*, a member of the vancomycin-sensitive *acidophilus* group, found as a commensal in humans (Pridmore et al., 2004) and abundantly present in rodents (Jenq et al., 2012), was most abundant in both types of mice, either naive or infected (Figure 2H and Figure S2C). In contrast, vancomycin-resistant *L. reuteri*—a model gut symbiont in vertebrates as a result of host-driven evolution (Walter et al., 2011)—was poorly represented in naive WT mice but expanded upon infection to a degree comparable to that seen in naive *Ido1*$^{-/-}$ mice, in which a further striking increase was observed after infection (Figure 2H and Figure S2C). FISH analysis with a *L. reuteri*-specific fluorescent probe (Figure 2G), unreactive to other *Lactobacillus* species in gastric fluids (Figure S2D), and quantitative PCR on amplifying 16S rRNA genes (Figure 2I) confirmed the expansion of *L. reuteri* in the stomachs of *Ido1*$^{-/-}$ mice. In addition, FISH analysis showed copresence of *L. johnsonii*, but not *L. acidophilus* (Figure S2E). Microbial profiling of fecal samples showed that *L. reuteri* was also highly represented in those mice (Figure S2F).

Differences within lactobacilli in the stomach (Figure 2J) and feces (Figure S2G) of *Ido1*$^{-/-}$ and WT mice were confirmed by unweighted and weighted UniFrac distance analysis, which takes into account the presence or absence and evolutionary relatedness of operational taxonomical units (OTUs), and by subsequent principal-coordinate analysis, the first principal coordinate (P1) of which explained most of the variance in each

Figure 2. Bacterial 16S-rRNA-Based Analysis of the Stomach Microbiota of WT and *Ido1*$^{-/-}$ Mice

(A–D) Relative abundance of major bacterial phyla (A) and bacterial taxa (B–D) in the stomach microbiota from C57BL/6 and *Ido1*$^{-/-}$ littermates, either uninfected or at 3 dpi.

(E) Counts of bacteria in the stomach of naive or infected (3 dpi) mice. $^{**}p < 0.01$, $^{***}p < 0.005$. "NS" stands for not significant. Results shown are compiled data from four independent experiments (means ± SD).

(F) Stomach SEM of infected mice. Arrows indicate the presence of bacilli of *L. reuteri*-like morphologies (short, pleomorphic cells with round edges). Scale bars represent 10 μm.

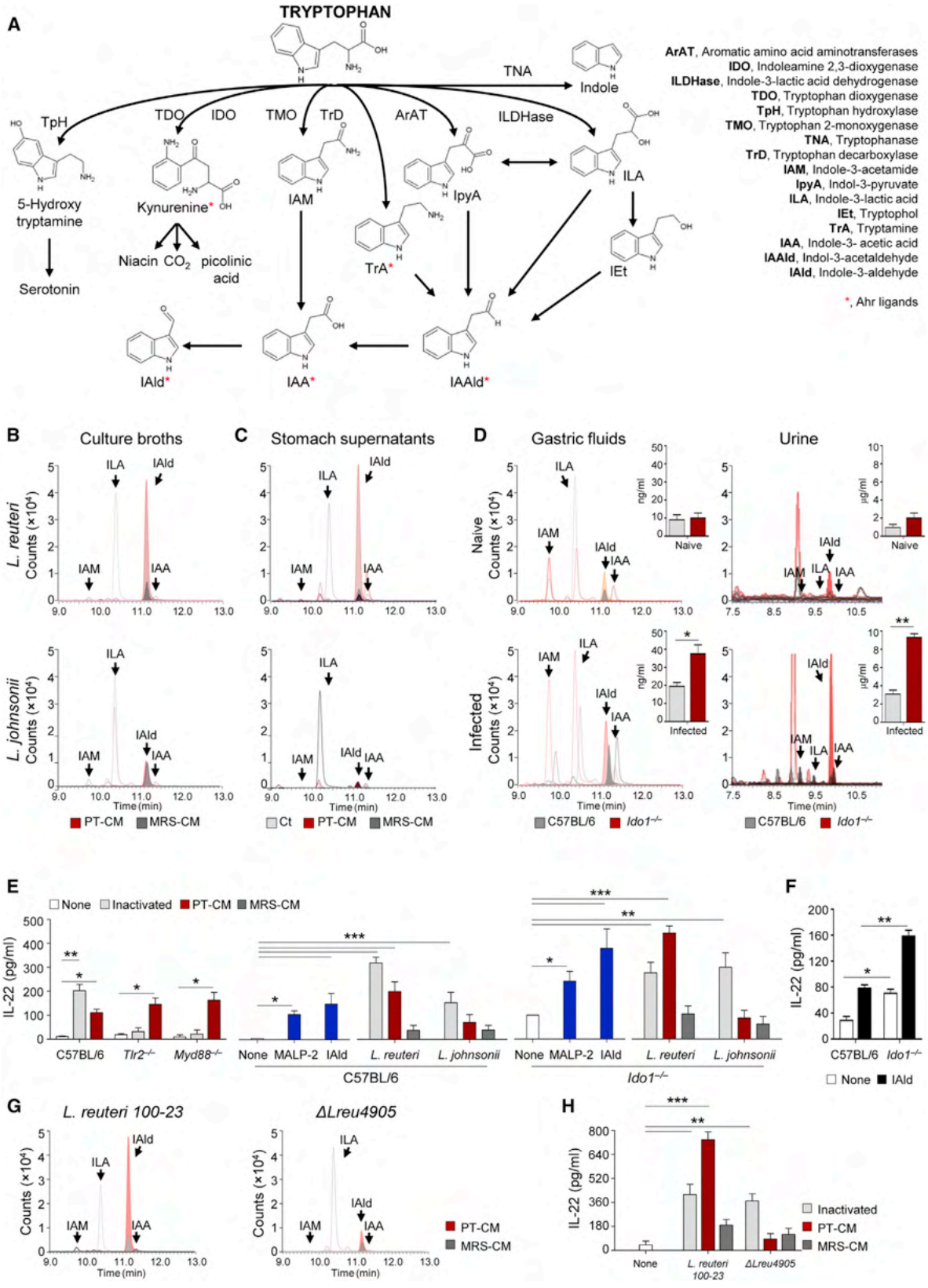
(G) Identification of lactobacilli and *L. reuteri* (red) in the gastric fluids of mice by FISH analysis using bacterial-specific probes. Scale bars represent 10 μm. "Ct" stands for pure cultures of bacteria. Bacteria counterstained with DAPI are in blue. Images are representative of two experiments.

(H) The taxonomic distribution of the most abundant *Lactobacillus* species is shown. Species names were assigned to organizational taxonomic units (OTUs) with the use of the k-nearest-neighbor classifier SeqMatch from the Ribosomal Database Project.

(I) *L. reuteri* in the stomach by quantitative PCR analysis of 16S rRNA. Data are represented as means ± SD. $^{*}p < 0.05$.

(J) Lactobacilli communities in the stomach from individual mice (each point) are clustered according to principal-coordinate analysis of unweighted and weighted UniFrac distances. Percentages of variation explained by plotted principal coordinates P1 and P2 are indicated on the x and y axes, respectively.

Related data are in Figures S2 and S3.



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sample between WT and *Ido1*$^{-/-}$ mice and the second principal coordinate (P2) of which showed a high degree of similarity among all samples. In accordance with the ability of lactobacilli to inhibit autochthonous uncultivable segmented filamentous bacteria (SFB) (Fuentes et al., 2008), SFB, abundantly present in the distal ilea of naive *Ido1*$^{-/-}$ mice, were markedly decreased in these mice by *Candida* infection (Figures S3A and S3B). The microbial composition and lactobacilli species in the stomachs of C57BL/6 mice bred at Charles River Laboratories (Calco) was similar to that of C57BL/6 mice bred at The Jackson Laboratory (Bar Harbor) (Figure S3C), thus ruling out the existence of variation from colony to colony. Therefore, lactobacilli are the dominant microbiota in the mouse stomach (Tannock et al., 1982), and IDO1 deficiency is permissive for *L. reuteri* to establish a niche there.

L. reuteri Is Selectively Expanded as a Result of Unrestricted Availability of Trp

Lactobacilli are nutritionally fastidious anaerobes and rely on the availability of easy fermentable sugars, amino acids, vitamins, and nucleotides. In vivo expression technology has shown that lactobacilli might undergo gut-specific gene expression in order to adapt (Frese et al., 2011). Both *L. johnsonii* (Denou et al., 2007) and *L. reuteri* (Frese et al., 2011; Walter et al., 2011) are very active transcriptionally in the stomach, where distinct nutritional adaptations provide niche differentiation that allows cohabitation by the two strains in the mouse forestomach (Tannock et al., 2012). Because lactobacilli are typically auxotrophic for several amino acids and are capable, under carbohydrate-starvation conditions, of catabolizing L-Trp to bioactive indole derivatives (Gummallaa and Broadbent, 1999), we reasoned that under conditions of high levels of Trp, lactobacilli might switch from sugar to Trp as an energy source. We measured Trp levels in *Ido1*$^{-/-}$ and WT mice and assessed the consequences of exposure to Trp, over that range, on the growth and metabolic activity of *L. reuteri* and *L. johnsonii*. We found that Trp levels were much higher in *Ido1*$^{-/-}$ mice than in WT mice and that levels of L-kynurenines were considerably lower (Figure S4A). Exposure to concentrations of Trp in the range of *Ido1*$^{-/-}$ mice (0.4–0.6 mM) greatly promoted growth of *L. reuteri*, but not of *L. johnsonii*, whose growth was, in fact, inhibited (Figure S4B). Growth of *L. reuteri* was also promoted in the presence of epithelial cells in which *Ido1* expression had been inhibited by siRNA technology (Figure S4C), suggesting that an increased Trp availability in *Ido1*$^{-/-}$ mice would selectively expand *L. reuteri* while promoting alternate pathways of Trp degradation in the population being expanded.

Lactobacilli Produce Indole-3-Aldehyde, a Trp-Indole Derivative

A variety of indole derivatives act as AhR ligands (Chung and Gadupudi, 2011), which are generated through catabolism of dietary Trp along the "indole pathway" (Figure 3A) by mostly anaerobic intestinal bacteria with tryptophanase activity (Wikoff et al., 2009). We employed targeted metabolomics to detect indole derivatives both in vivo during infection and in vitro in conditioned media (CM) from *L. reuteri* and *L. johnsonii*, which, recovered from the stomach of *Ido1*$^{-/-}$ mice, were grown in the presence of 0.4–0.6 mM Trp with (MRS broth) or without (peptone-tryptone water) carbohydrates. Metabolic profiling revealed that, of the different putative metabolites, indole-3-aldehyde (IAld), a molecule with AhR ligand activity (Figure S4D), was abundantly produced by *L. reuteri* and only poorly by *L. johnsonii* in the presence of Trp under carbohydrate-starvation conditions (Figure 3B). IAld was also detected in ex vivo cultures of the stomachs exposed to *L. reuteri*, but not *L. johnsonii* (Figure 3C). In infection, IAld was detected in the gastric fluids and urine, where excretion of specific metabolites reflects pathways in Trp metabolism (Figure 3D), and it was maximally produced in *Ido1*$^{-/-}$ mice (Figure 3D insets). IAld was not produced by Clostridia or indole-positive bacteria with tryptophanase activity (Figure S4E), nor were indole levels higher in *Ido1*$^{-/-}$ mice than in WT mice (Figure S4F). Overall, the bulk of these data demonstrate that IAld-producing lactobacilli (rather than tryptophanase-competent organisms or Clostridia) are mostly expanded and utilize Trp in *Ido1*$^{-/-}$ mice.

IAld is a metabolite derived from Trp via several pathways (Figure 3A). The indole pyruvate (IPyA) route is one of the main pathways for IAld synthesis from Trp (Trp → IPyA → IAld) and is catalyzed by the aromatic amino acid aminotransferase (ArAT), a key enzyme phylogenetically conserved in many bacterial species, including lactobacilli, but not Clostridia spp., belonging in the clusters IV and XIVa, which are dominant in the murine gut (Atarashi et al., 2011) (Figure S4G). Although transamination of Trp belongs in minor pathways of amino acid metabolism, ArAT plays a major role in the conversion of aromatic amino acids to aroma compounds by lactobacilli (Rijnen et al., 1999). We assessed whether any enzyme with ArAT-type activity is involved in IAld production by *L. reuteri*. We found that *Lreu23DRAFT_4905*, encoding an ArAT-related aminotransferase (Figure S5A) (http://jgi.doe.gov/), was maximally expressed in *L. reuteri* under conditions of high Trp levels and low carbohydrate levels (Figure S5B). We were also able to confirm the crucial role of this aminotransferase in Trp utilization

Figure 3. Lactobacilli Induce IL-22 via IAld, a Trp-Indole Derivative

(A) The Trp metabolic pathways, enzymes, and molecular structures of Trp-indole derivatives. Asterisks indicate metabolites with AhR agonistic activity.

(B–D) Peak levels of IAld in broth cultures (peptone-tryptone water [PT-CM] versus MRS [MRS-CM]) (B), supernatants from ex vivo stomach exposed to lactobacilli (C), and gastric fluids and urine (D) from naive or infected (3 dpi) mice (insets show quantification; data represent means ± SD). Abbreviations are as follows: IAM, indole-3-acetamide; IAA, indole-3-acetic acid; and ILA, indole-3-lactic acid.

(E and F) IL-22 levels (ELISA) in ex vivo stomach cultured with MALP-2, IAld, UV-inactivated lactobacilli, and supernatants of lactobacilli grown in PT-CM or MRS-CM for 24 hr (E) and supernatants of purified PP-NKp46^{+} cells stimulated in vitro with IAld (F).

(G) Peak levels of IAld in PT-CM and MRS-CM of *L. reuteri* 100-23 (WT) and its *ΔLreu4905* mutant.

(H) IL-22 levels in ex vivo stomach exposed to the two *Lactobacillus* strains. Data are representative (metabolomics) of or compiled (IL-22 assay) from five experiments and represent the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Related data are in Figures S4 and S5.

by *L. reuteri* by generating an *araT* mutant (referred to as *ΔLreu4905*) of *L. reuteri* 100-23, a highly adaptive strain from the murine gut (Walter et al., 2011). The mutant was still capable of growing in the presence of externally added Trp (Figure S5C) yet was unable to produce IAld (Figure 3G). Importantly, at variance with *L. reuteri*, *araT* in *L. johnsonii* FI9785 (National Center for Biotechnology Information [NCBI] GI 268319536) (53% identity, $p = 1 \times 10^{-137}$, by a translated nucleotide query in BLASTx [http://www.blast.ncbi.nlm.nih.gov/]) was not upregulated in high-Trp growing conditions (Figure S5B). In line with the defective growth of *L. johnsonii* under high Trp levels (Figure S4B), the lack of the pyruvate dehydrogenase complex and other enzymes required for conversion of pyruvate to acetaldehyde in this strain (Boekhorst et al., 2004), and the role of sugar, more than amino acid, digestion in affecting its gut persistence (Denou et al., 2008), this finding suggests that *L. reuteri*, more than *L. johnsonii*, is equipped with the appropriate metabolic machinery for Trp utilization in the stomach.

Lactobacilli Induce IL-22 via IAld

To investigate the role of IAld in AhR-dependent IL-22 production in infection, we measured IL-22 production in ex vivo cultures of gastric specimens exposed to a physiologically relevant concentration of IAld or to supernatants of *L. reuteri* or *L. johnsonii* grown in the presence of Trp. Owing to the ability of Toll-like receptor 2 (TLR2) to regulate IL-22 production (Crellin et al., 2010), we also used inactivated lactobacilli and MALP-2, a TLR2 agonist, as stimuli. The results indicated a dual mechanism of IL-22 production, i.e., through direct microbial recognition via TLR2 and MyD88 (mostly exploited by *L. johnsonii* in WT mice) and via IAld (mostly exploited by *L. reuteri* in *Ido1*$^{-/-}$ mice) (Figure 3E). Importantly, IAld also induced IL-22 by purified NKp46^{+} cells (Figure 3F). Lactobacilli-induced IL-22 occurred in severe combined immunodeficient (SCID) mice and in IL-17A-deficient mice (Figure S5D), i.e., through innate immune mechanisms and independently of IL-17A. Cytokines other than IL-22 were induced by other intestinal bacteria (Figure S5E). Not only was the *araT* mutant (*ΔLreu4905*) described above unable to produce IAld (Figure 2G), but it was also incapable of producing IL-22 (Figure 2H). Thus, lactobacilli, but not other intestinal bacteria, induce IL-22 via IAld in *Ido1*$^{-/-}$ mice.

Dietary Trp Affects Amounts of IAld and IL-22 in the Stomach

To investigate whether lactobacilli expand and produce IAld under physiologically relevant conditions, we evaluated resistance to candidiasis in conventional mice fed a Trp-enriched (Trp^{+}) or Trp-low (Trp^{-}) diet for 4 weeks and treated with ampicillin that promotes a long-lasting depletion of lactobacilli, among others, in the ileum and cecum (Jenq et al., 2012; Ubeda et al., 2010) (minimum inhibitory concentration [MIC] ranges of *L. reuteri* and *L. johnsonii* isolated from the murine stomach were 0.15–2.50 and 0.25–1.00 μg/ml, respectively, on susceptibility testing). We found that ampicillin and chronic restriction of Trp both decreased antifungal resistance in WT and *Ido1*$^{-/-}$ mice, whereas in WT mice, Trp feeding increased antifungal resistance, which was negated by ampicillin (Figure 4A) (incidentally, psoriatic-like lesions were observed in Trp-fed *Ido1*$^{-/-}$ mice; Figure S6A). The degree of resistance correlated with the local expression of *Lreu23DRAFT_4905* (Figure 4B), IAld (Figure 4C), IL-22 (Figure 4D), and IL-22-producing ILC3s (Figure 4E) in the stomach; all were higher in Trp-fed WT mice and lower in mice treated with ampicillin or on a low-Trp diet. Scanning electron microscopy (Figure 4F) and FISH (Figure 4G) analyses confirmed a decreased lactobacilli content in the stomach after ampicillin treatment and chronic restriction of Trp and the selective expansion of *L. reuteri* upon Trp feeding. Ampicillin did not expand other commensals, such as Clostridia, in the stomach (Figure S6B), nor did it eliminate SFB from the ileum (Figure S6C). Accordingly, treatment with vancomycin, which inhibits Th17-inducing SFB in the ileum (Ivanov et al., 2009), as well as Bacteroidetes from the cecum (Ubeda et al., 2010), slightly decreased antifungal resistance in WT, but not in *Ido1*$^{-/-}$, mice (Figure S6D), a finding pointing to a minor role for SFB and Bacteroidetes in providing antifungal resistance under conditions of high amounts of Trp. We could confirm that, within the species-dependent susceptibility of lactobacilli to vancomycin (Peña et al., 2004), *L. johnsonii* from the mouse stomach was susceptible (MIC range of 1.50–3.00 μg/ml) and *L. reuteri* was resistant (MIC > 256 μg/ml). Thus, these data support the observation that gut amounts of Trp impact gut ecology (Hashimoto et al., 2012) and, specifically, that vancomycin-resistant but ampicillin-sensitive *L. reuteri* expand in the stomach upon Trp feeding and affect local immune homeostasis.

Lactobacilli Exert Species-Specific Probiotic Effects in Candidiasis

We next assessed the species-specific probiotic effects of lactobacilli in vivo by employing several experimental approaches that did not intentionally include lactobacilli-free mice, because these animals also lack SFB (Tannock and Archibald, 1984). We assessed antifungal resistance in germ-free (GF) mice, whose stomach is a target organ in candidiasis (Balish et al., 1993), and conventional mice after gut decontamination by prolonged antibiotic treatment. We reconstituted mice with *L. reuteri* 100-23, its *ΔLreu4905* mutant, and murine *L. johnsonii*. Although equally capable of colonizing mice (Figure S7A), *L. reuteri* 100-23 fully restored antifungal resistance in GF mice, an effect only minimally afforded by the *L. reuteri* mutant or *L. johnsonii*, as indicated by the restrained fungal growth (Figure 5A), high levels of IAld in the stomach (Figure 5B) and urine (ranges: 0.4–0.5 μg/ml in naive GF mice, 1.1–1.5 μg/ml in infected GF mice, 5.8–8.7 μg/ml in GF mice + *L. reuteri* 100-23, 1.2–1.7 μg/ml in GF mice + *ΔLreu4905* mutant, and 0.9–1.4 μg/ml in GF mice + *L. johnsonii*), IL-22 production (Figure 5C), increased numbers of IL-22^{+}NKp46^{+} cells (Figure S7B), and a visible reduction of the enlarged cecum (Figure S7C). *L. reuteri* 100-23, but much less its mutant, similarly provided antifungal resistance (Figure 5D) and mucosal protection (Figure S7D) in antibiotic-treated mice, an effect that was dependent on IL-22 and AhR. As in the in vitro setting, the efficacy of lactobacilli in vivo was independent of IL-17A and adaptive immunity (Figure S7E). These findings indicate that lactobacilli promote anticandidal resistance in vivo via host innate mechanisms converging on the AhR-IL-22 axis, although alternative and/or additional mechanisms of protection are also plausible (Table S5).

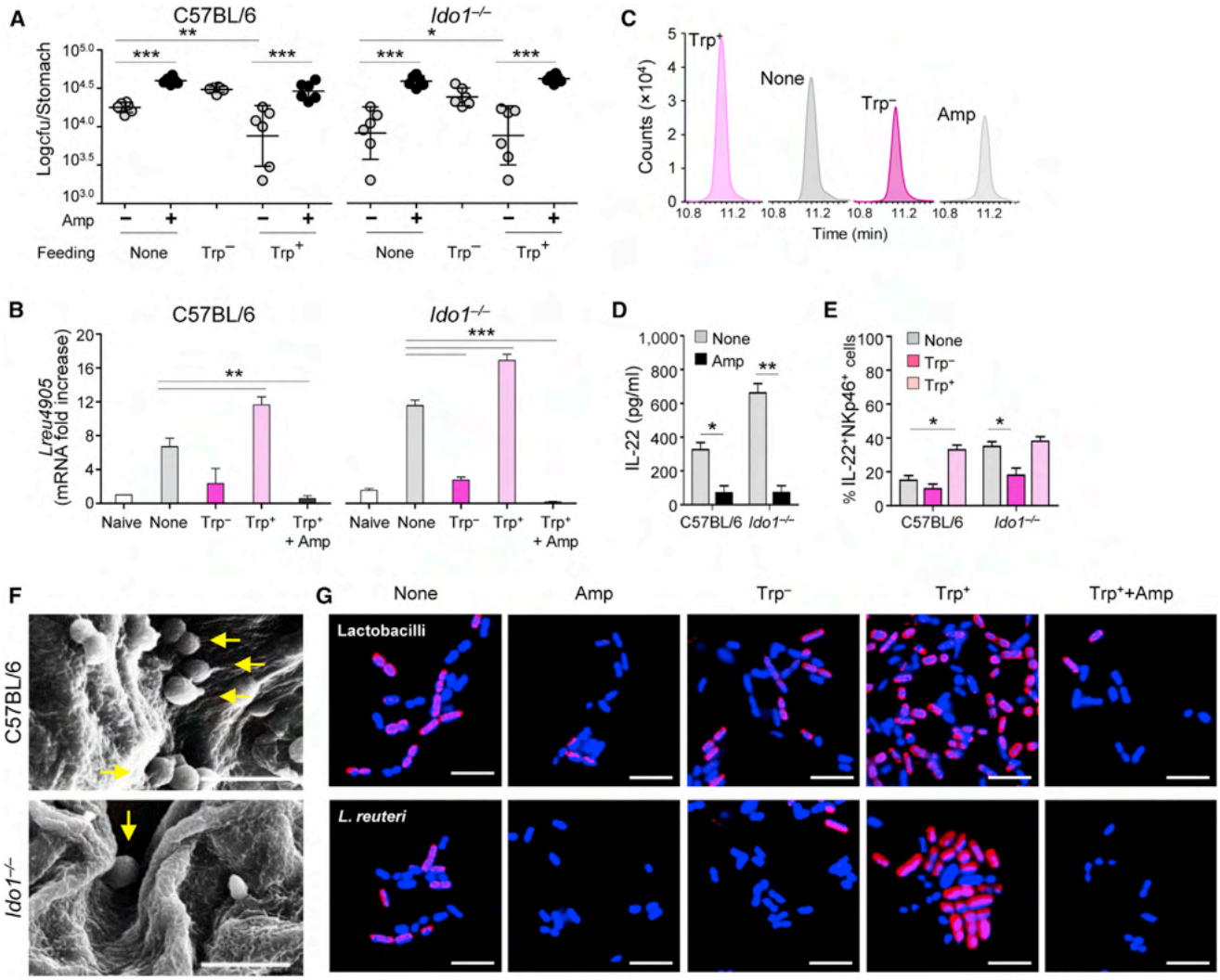

Figure 4. Dietary Trp Affects Levels of IAld and IL-22 in the Stomach

(A–E) Mice were fed a standard ("none"), low (Trp^-), or enriched (Trp^+) Trp diet for 4 weeks, treated or not with ampicillin ("Amp"), infected with *C. albicans*, and assessed for fungal growth (A), relative *Lreu_4905* expression normalized with 16S rRNA (B), peak levels of IAld (C), IL-22 production (D), and percentage of ILC3s in the stomach (3 dpi) (E). Data represent means ± SD.

(F) Scanning electron microscopy of the stomach after Amp treatment (3 dpi). Scale bars represent 10 μm. Arrows indicate *C. albicans* cells. Data are representative (SEM) of or compiled from four experiments.

(G) Identification of lactobacilli and *L. reuteri* (red) in the gastric fluids of treated mice by FISH analysis. Bacteria counterstained with DAPI are in blue. Scale bars represent 10 μm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data are representative of two experiments.

Related data are in Figure S6.

Lactobacilli Exert Organ-Specific Probiotic Effects in Candidiasis

"Normal" vaginal flora typically shows a predominance of several *Lactobacillus* species, which are believed to promote a healthy vaginal milieu by providing numerical dominance and by other mechanisms (Lamont et al., 2011). We assessed whether and which *Lactobacillus* species would act through the Ahr-IL-22 axis involving IAld in murine vaginal candidiasis. Lactobacilli and in particular *L. acidophilus*, a constituent of the human vaginal microbiota, were present in the vagina (Figure 6A) (about 10^5 to 10^3 cfu/g of vagina, *Ido1*$^{-/-}$ versus WT mice, by quantitative cultures in selected media), as detected by FISH (Figure 6A) and 16S rRNA PCR (Figure 6B). *L. acidophilus*, recovered from the vagina of *Ido1*$^{-/-}$ mice, induced IL-22 (Figure 6C) and produced IAld in vitro (Figure 6D) and in vivo upon administration to GF mice (Figure 6E). IAld was also detected in the vaginal fluids of conventional mice after the infection (Figure 6F) (ranges: 12.3–15.7 ng/ml in naive WT mice, 17.6–22.5 ng/ml in naive *Ido1*$^{-/-}$ mice, 38.4–45.7 ng/ml in infected WT mice, and 59.6–71.4 ng/ml in infected *Ido1*$^{-/-}$ mice), resistance to which involved expression of antimicrobial-peptide-encoding genes (Figure 6G), occurred via IL-22 and AhR (Figure 6H), and was negated in *Ahr*$^{-/-}$ mice or by IL-22 blockade (Figure 6I). Therefore, *L. acidophilus* exploits the IL-22-AhR axis to provide local resistance to *Candida* in the vagina, but not in the stomach, thus explaining why healthy women remain

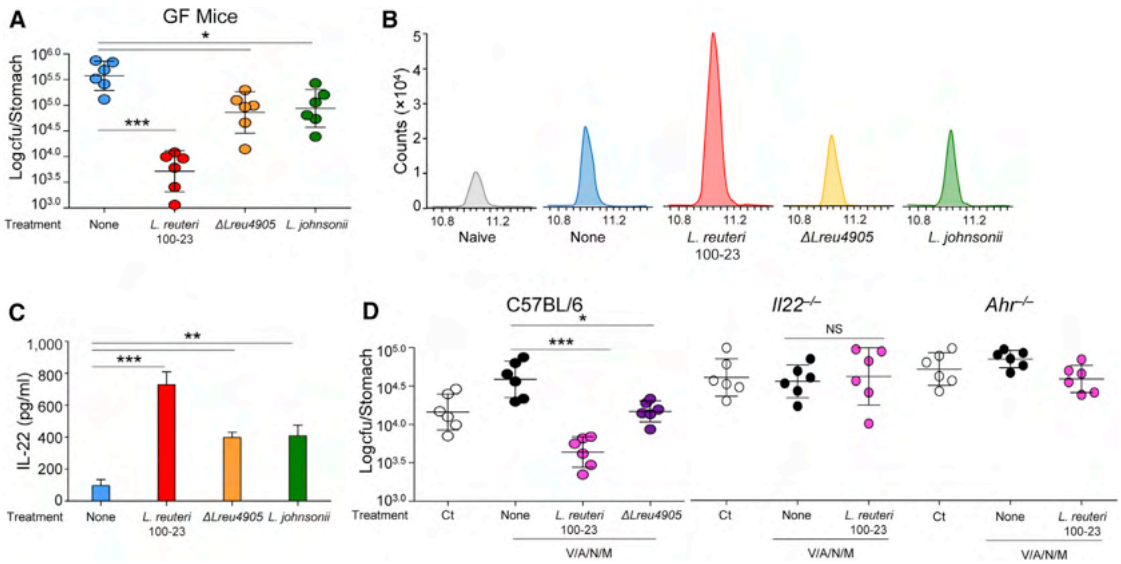

Figure 5. Probiotic Lactobacilli Exert Species-Specific Effects in Candidiasis

(A–C) Fungal growth (A), IAld (B), and IL-22 (C) levels in the stomachs (3 dpi) of GF mice untreated ("none") or monoassociated by gavage with *L. reuteri* 100-23, its *ΔLreu4905* mutant, and *L. johnsonii* before the *C. albicans* intragastric infection.

(D) Fungal growth in the stomach of conventional mice untreated ("none") or monoassociated with *L. reuteri* 100-23 or its *ΔLreu4905* mutant and infected with *C. albicans*. Mice were treated with vancomycin, neomycin, ampicillin, and metronidazole ("V/A/N/M") for 4 weeks. "Ct" indicates the control mice, which were infected only. Fecal counts of lactobacilli and Gram staining revealed colonization at necroscopy. Data are compiled from three experiments and represent the mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. "NS" stands for not significant.

Related data are in Figure S7.

entirely asymptomatic despite being colonized by *Candida* spp. (Sobel, 2007).

IAld Administration Provides Antifungal Resistance and Mucosal Protection

The ability of IAld to act as an AhR ligand and to promote IL-22 production led us to assess whether IAld could be exploited to provide antifungal resistance and mucosal protection from inflammation. We administered IAld to WT and *Ahr*$^{-/-}$ mice with mucosal candidiasis or with dextran sodium sulfate (DSS)-induced colitis, a model in which AhR signaling inhibits inflammation via IL-22 (Monteleone et al., 2011). IAld restored antifungal resistance (Figure 7A) and IL-22 production (Figure 7B) upon infection, ameliorated colitis (Figures 7C and 7D), and induced IL-22 production by colonic NKp46^{+} cells (Figure 7E) in WT mice. These effects were not seen in *Ahr*$^{-/-}$ mice (Figures 7A–7E). Considering that defective Th1 and Treg cell adaptive immune responses—required for clearing the fungus and preventing infection-associated chronic inflammation (De Luca et al., 2010)—are observed in *Ido1*$^{-/-}$ mice (Figure S8), these data clearly indicate that the AhR agonistic activity of IAld could be exploited to provide homeostasis and microbial symbiosis at mucosal surfaces in conditions of impaired adaptive immunity.

DISCUSSION

Our study discloses a signaling pathway that links the bacterial-fungal population dynamics with the mammalian host at mucosal surfaces. A microbial Trp metabolic pathway appeared to be evolved to preserve immune physiology at mucosal surfaces via recruitment of host cells competent for AhR-regulated *Il22* transcription. The IL-22-regulated mucosal response allows for survival of mixed microbial communities yet provides colonization resistance to *C. albicans*. Infections caused by opportunistic fungi have traditionally been viewed as the gross result of a pathogenic automatism, which makes a weakened host more vulnerable to microbial insults. Our study reveals that fungal interaction with the host is more complex than previously appreciated because it includes a triad interaction with indigenous bacteria.

It is known that lactobacilli promote specific immune and metabolic processes in a strain-specific manner (Tannock, 2004; van Baarlen et al., 2011) and that "metabolic products of the *lactobacillus* population in the stomach could influence the physiology of the adjacent tissues of the host" (Tannock, 2004). The stomach favors the colonization of acid-resistant lactobacilli and is a normal habitat of various fungal taxa in rodents and humans, where yeasts are associated with the mucin layer covering the secreting epithelial cells (Karczewska et al., 2009; Scupham et al., 2006; Tannock and Savage, 1974). Within the stomach, lactobacilli are known to promote resistance to colonization by the fungus (Noverr and Huffnagle, 2004; Savage, 1969) and to be antagonized by *C. albicans* during postantibiotic recolonization and gastritis (Mason et al., 2012). Decreased lactobacilli content is indeed associated with sustained gastric colonization by the fungus in wasting nude mice (Brown and Balish, 1978) or mice fed a purified diet (Yamaguchi et al., 2005). Thus, a functional interplay between *C. albicans* and lactobacilli does occur, a disturbance of which might affect microbial symbiosis and *C. albicans* mutualism. Although the mechanisms of protection might include the production of other mediators (Voltan et al., 2008) and direct microbial antagonism by lactobacilli (Noverr and Huffnagle, 2004) and IAld (Table S5), both affecting

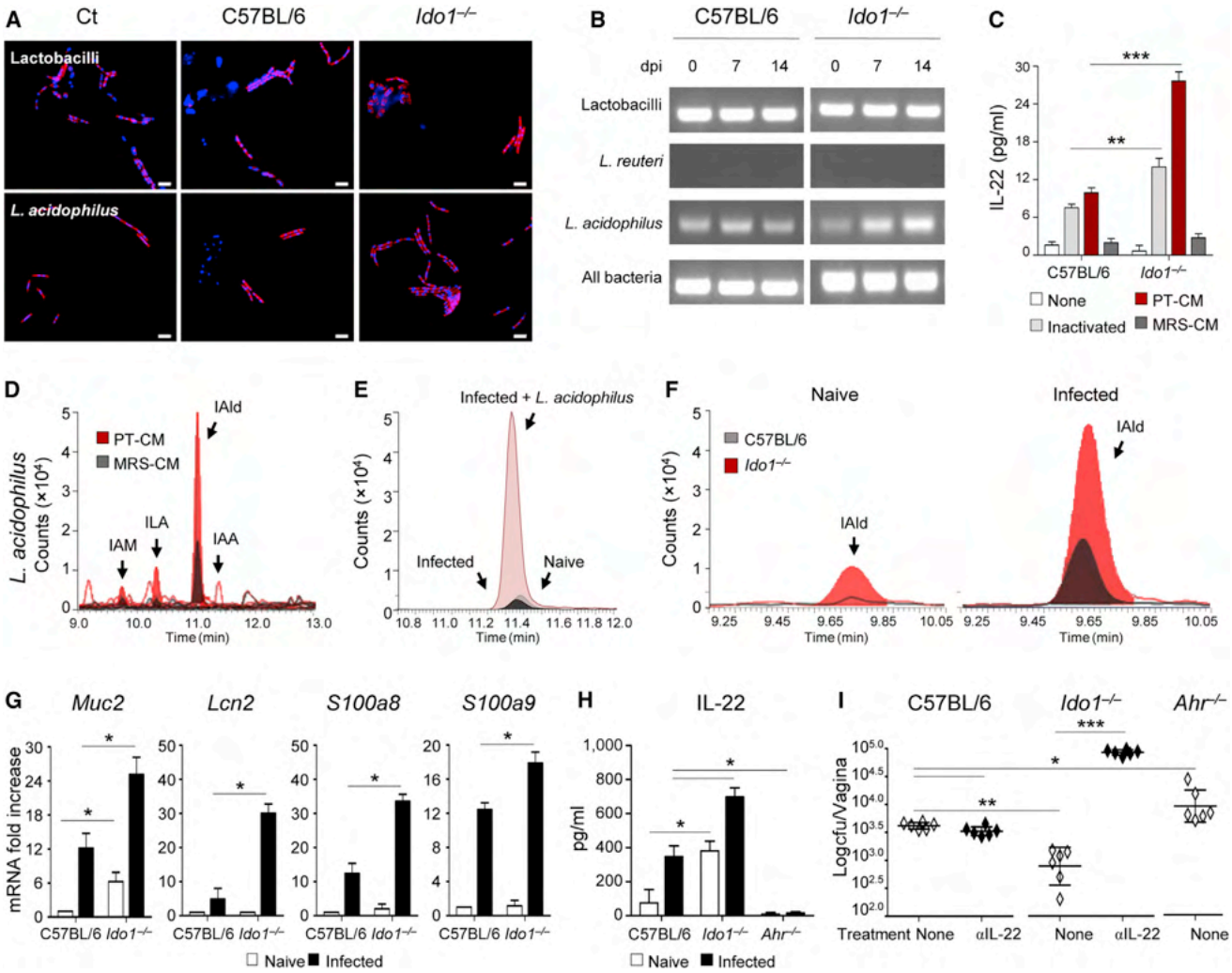

Figure 6. *L. acidophilus* Provides Protection in Vaginal Candidiasis through the AhR-IL-22 Axis

(A and B) Detection of lactobacilli and *L. acidophilus* by FISH analysis (red bacteria) (A) and 16S rRNA RT-PCR (B) in the vagina of mice intravaginally infected with *C. albicans* blastospores (3 dpi). "Ct" stands for pure cultures of bacteria. Bacteria counterstained with DAPI are in blue. Scale bars represent 10 μm.

(C) IL-22 levels in ex vivo vaginal tissue exposed to inactivated *L. acidophilus* or *L. acidophilus* grown in PT-CM or MRS-CM. "None" indicates PT alone. $^{**}p < 0.01$, $^{***}p < 0.001$.

(D–F) Peak levels of IAld in supernatants of *L. acidophilus* grown in PT-CM versus MRS-CM (D), vaginal fluids of GF mice, naive or infected and/or mono-associated with *L. acidophilus* (E), and naive or infected conventional mice at 3 dpi (F). Abbreviations are as follows: IAM, indole-3-acetamide; IAA, indole-3-acetic acid; and ILA, indole-3-lactic acid.

(G–I) Expression of antimicrobial-peptide-encoding genes (G), IL-22 levels (H), and fungal growth (I) in the vagina of mice treated with mAb neutralizing IL-22 or isotype control antibody ("none").

Data are representative (FISH and RT-PCR) of or compiled from three experiments and represented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Candida morphology, our study might help explain how the interaction between commensal fungi and the host's immune system via local microbiota determines their position from commensals to pathogens and why this position can change continuously (Romani, 2011).

Through the activation of local metabolic pathways involving AhR and converging on IL-22, lactobacilli mediated mucosal antifungal resistance that was contingent upon the host metabolic environment, involved microbial adaptation, and was modulated by diet. In mice, autochthonous SFB are responsible for Th17 accumulation and IL-22 production by Th17 cells in the gut (Ivanov et al., 2009), but it has been unclear whether and which signals from the microbiota selectively direct the production of IL-22 by ILC3s (Lee et al., 2012). A substantial reduction in IL-22^{+}NKp46^{+} pool size was observed in the gut upon depletion of lactobacilli, whereas monoassociation with *L. reuteri* increased IL-22 production by NKp46^{+} cells in GF mice, thus revealing that certain *Lactobacillus* species stimulate ILC3s for IL-22 production, an effect carried out in a strain-specific manner, as typically occurs (Tannock, 2004; van Baarlen et al., 2011).

Although lactobacilli share with Gram-positive bacteria the ability to stimulate IL-22 production via TLR2, the metabolic pathway of IL-22 production via AhR stimulation by IAld

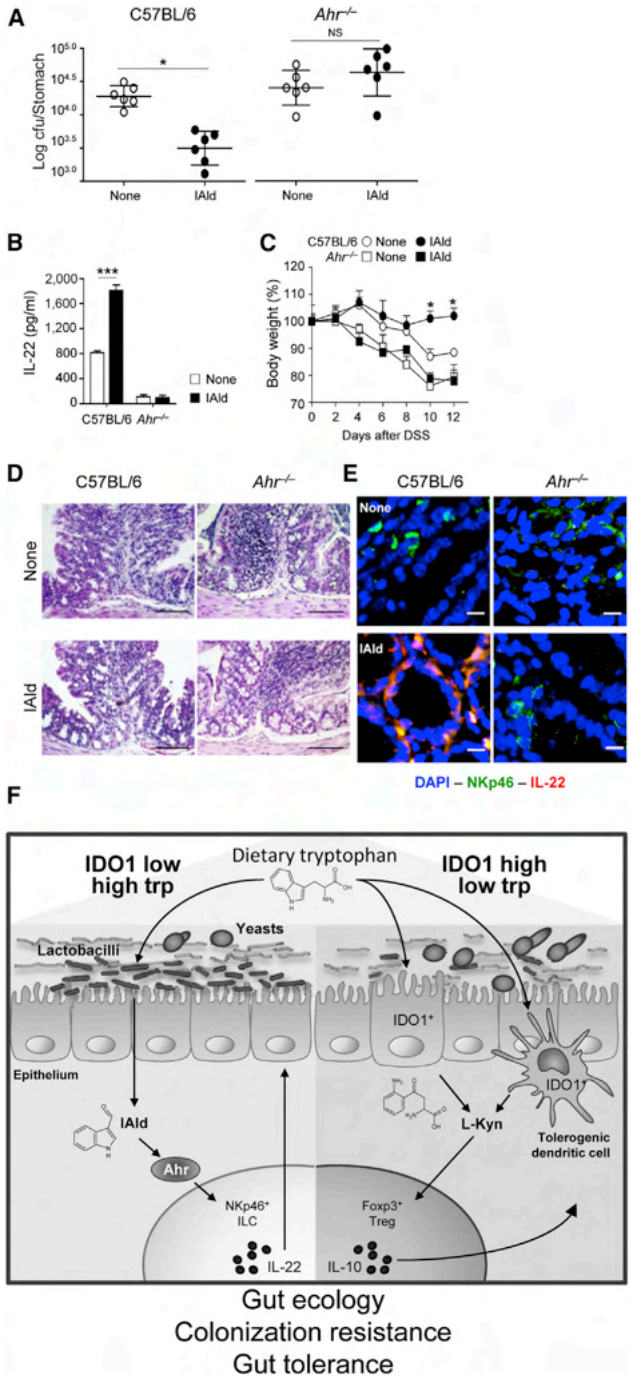

Figure 7. IAld Protects against Candidiasis and Colitis

(A and B) Fungal growth (A) and IL-22 production (B) in untreated ("none") or IAld-treated *C. albicans*-infected mice. Error bars represent the SD of samples within a group. *$p < 0.05$, ***$p < 0.001$. "NS" stands for not significant.

(C–E) Percentage body weight (C), colon histopathology (hematoxylin and eosin; scale bars represent 200 μm) (D), and detection of colonic NKp46$^+$IL-22$^+$ cells by immunofluorescence (E) in DSS-treated mice that received vehicle alone ("none") or IAld intragastrically (day 12 after DSS challenge). Scale bars represent 20 μm. Data are representative (histology and immunofluorescence) of or compiled from three experiments and represented as mean ± SD. Error bars represent the SD of samples within a group.

(F) Proposed model for the role of IDO1 in the host-microbiota symbiotic relationship. Besides the immunomodulatory activity of the host's own kynurenine production, by regulating the local amino acid levels and consequently the size and metabolic activity of gut microbiota, IDO1 might be a key molecule in directing the host-microbiota symbiotic relationship and its integration within the innate and adaptive immune systems of vertebrate hosts.

occurred as a result of their microbial adaptation. Lactobacilli are phylogenetically closely related by their small genomes and common metabolic pathways for sugar fermentation and lactic acid production, and they occupy a diverse set of ecological niches as a result of considerable genetic adaptation (Frese et al., 2011). Both *L. johnsonii* (Denou et al., 2007) and *L. reuteri* (Walter et al., 2011) are transcriptionally very active in the mouse forestomach, where carbohydrate partitioning provides niche differentiation that allows cohabitation by the two strains (Tannock et al., 2012). We found in this study that, in addition to sugar, amino acid partitioning might also crucially affect cohabitation of the strains in the stomach, where amino acid digestion is crucial for *L. reuteri* expansion. Accordingly, *L. reuteri* not only expanded but also expressed *Lreu23-DRAFT_4905*, encoding ArAT, in the presence of Trp. Thus, *L. reuteri*, more than *L. johnsonii*, is competent for Trp utilization in the stomach. Similar to *L. reuteri*, *L. acidophilus* is competent for Trp utilization in the vagina, and together these findings highlight the organ-specific dependency of the host-microbiota interaction at the mucosal surfaces.

The species-specific effects of lactobacilli on *C. albicans* colonization might offer a plausible explanation for the organ tropism of mucosal candidiasis in mice and humans, for the susceptibility to infection in specific clinical settings, and for the variable and inconsistent effects of probiotic administration in human candidiasis. Because lactobacilli are greatly reduced in the neonatal period (Sjögren et al., 2009) and by stress (Lutgendorff et al., 2008; Tannock and Savage, 1974), our data are consistent with the occurrence of neonatal and vaginal candidiasis, clinical conditions under which the empirical use of lactobacilli as probiotics to prevent infection has long been advocated (Falagas et al., 2006; Manzoni et al., 2006) but never mechanistically explained. Moreover, the very high diversity of *Lactobacillus* spp. in the human vagina (Human Microbiome Project Consortium, 2012) might offer a plausible explanation for the idiopathic recurrence of vaginal candidiasis in the relative absence of recognized risk factors (Sobel, 2007). We also anticipate that responsiveness to the different lactobacilli probiotic species is determined not only by the characteristics of the consumed strains but also by microbial adaptation, metabolic, and nutritional status of the host. On a translational level, considering that probiotic *L. reuteri* of human origin also produced IAld upon growing on Trp (data not shown), these findings imply that Trp supplementation in the context of antibiotic coverage might optimize antifungal and probiotic therapy and, most likely, immune physiology (Li et al., 2011). We also found that IAld could substitute for probiotics in protecting and maintaining mucosal integrity during infectious or chemical damage, a finding pointing to the possible use of IAld as a supportive therapy during flora manipulation and intestinal dysbiosis. In this regard, loss of overall diversity and expansion of lactobacilli has been described in murine and human recipients of

allogeneic bone marrow transplantation, whereas reintroducing the predominant species of *Lactobacillus* induced significant protection against graft-versus-host disease (Jenq et al., 2012). Considering the reciprocal influence between the fungal community in the gut and local levels of inflammation (Iliev et al., 2012), IAld might represent a prototypical candidate capable of multitasking effectively in transplanted recipients and patients with mucosal inflammatory diseases. The flipside will be the generation of potentially harmful proinflammatory effects of IL-22, such as psoriasis in susceptible hosts.

In conclusion, although the enzyme Trp 2,3-dioxygenase (Opitz et al., 2011), mainly expressed in the liver, regulates Trp concentrations after nutritional Trp uptake under normal circumstances, the high amounts of IDO1 expression at mucosal sites during immune activation (Dai and Zhu, 2010) point to IDO1 as the dominant enzyme regulating the local amino acid nutrient levels, the size and metabolic activity of gut microbiota, and, owing to the host's own immunomodulatory activity via L-kynurenine production, mucosal immune reactivity. Thus, these data qualify IDO1 as a key molecule in dictating host-microbiota symbiotic relationships and their integration within the adaptive immunity of vertebrate hosts (Figure 7F).

EXPERIMENTAL PROCEDURES

A detailed description of the experimental procedures used in this paper can be found in the Supplemental Information.

Mice, Infections, Colitis, and Treatments

C57BL/6 mice, 8–10 weeks old, were purchased from The Jackson Laboratory and Charles River Laboratories. Breeder pairs of *Ido1*$^{-/-}$ mice were purchased from The Jackson Laboratory. Homozygous SCID mice on a BALB/c background and *Tlr2*$^{-/-}$, *Myd88*$^{-/-}$, *Ahr*$^{-/-}$, *Il17a*$^{-/-}$ mice on a C57BL/6 background were bred under specific pathogen-free conditions at the Animal Facility of Perugia University. Mice were housed in specific pathogen-free barrier facilities at the Animal Facility of Perugia University. Female 8-week-old GF C3H/Orl mice were purchased from The Jackson Laboratory and housed in isolators and fed an irradiated standard rodent chow diet (Mucedola). For infections and colitis, see Supplemental Information. GF mice were monoassociated with a single gavage (gastrointestinal candidiasis) or intravaginal injection (vaginal candidiasis) of 10^9 cfu of lactobacilli 1 day before infection. For Trp supplementation, mice were fed the control diet supplemented with 1g/kg Trp. The composition of the diets and their final Trp levels are listed in Table S6. For metagenomic analysis, littermates from *Ido1*$^{+/-}$ × *Ido1*$^{+/-}$ crosses, kept in the same cage, were compared. Control littermates were included in individual experiments so that nonspecific effects of each genetic manipulation could be checked for, but none were found. See also Supplemental Information.

Microbiota Analysis by 16S rRNA Sequencing

Bacterial DNA from gastric fluids and feces of eight mice from each group was extracted with the QIAamp DNA Mini Kit (QIAgen). For each sample, 16S rRNA genes were amplified with a composite forward primer and a reverse primer containing a unique 12-base barcode used for tagging each PCR product. For each sample, five replicate PCR products were combined and purified with Ampure XP magnetic purification beads (Agencourt Bioscience), and products were quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). All libraries were subjected to quality control with the DNA 1000 Agilent Bioanalyzer (Agilent Technologies). Two master DNA libraries were generated from the purified products in equimolar ratios to final concentrations of 35.6 and 33.9 ng/μl. The pooled products were sequenced with a Roche 454 GS Titanium pyrosequencer at the BMR Genomics genome sequencer center at the University of Padova.

Data Analysis and Sequence Classification

A total of 620,369 reads with a median length of 365.4 bp were obtained for all samples in both libraries. For making phylogenetic assignments, reads were clustered against a high-quality seed library with the use of CD-HIT 454 with a 97% similarity cutoff. Species names were assigned to OTUs with Ribosomal Database Project SeqMatch (http://rdp.cme.msu.edu/seqmatch/seqmatch.jsp). Weighted and unweighted UniFrac distances were calculated and principal-coordinate analyses were performed with QIIME software.

Quantification of Gut Microbiota by Culture Method and by Qualitative and Quantitative PCR

See Supplemental Information.

Bacterial Strains and Cultures

Lactobacilli were isolated from the stomach of *Ido1*$^{-/-}$ infected mice, as previously described (Roach et al., 1977). The isolates were identified as lactobacilli on the basis of Gram-stain morphology and by the API 50 CHG system (BioMérieux Italia). For obtaining lactobacilli from the murine stomach, gastric fluids were plated on Man-Rogosa-Sharpe (MRS) agar plates and were incubated anaerobically at 37°C for at least 3 days, after which visible colonies were selected and cultured anaerobically in MRS broth at 37°C. DNA was extracted from isolated colonies as described in the Supplemental Information. The obtained sequences were subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) for the identification of *Lactobacillus* species. Murine *L. intestinalis*, *murinus*, *gasseri*, *animalis*, *helveticus*, and *crispatus* were purchased from Leibniz Institut DSMZ-German Collection of Microorganisms and Cell Cultures (Brunswick). *E. coli*, Clostridia, Enterococci, and Bacteroidetes were obtained from mice specimens derived from the gastrointestinal tract and were isolated with the use of selective media.

Generation of the *L. reuteri ΔLreu4905* Mutant

The aromatic amino acid aminotransferase nucleotide sequences of *L. reuteri* DSM 20016 (NCBI GI 148530277, *Lreu_0044*) and JCM 1112 (NCBI GI 183223999, *LAR_0041*) were used for identifying a homologous gene in the genome of *L. reuteri* 100-23 (http://www.jgi.dow.gov/). The locus of the gene encoding the putative aromatic amino acid aminotransferase and flanking nucleotide sequences in *L. reuteri* 100-23 were analyzed with the BLASTx program against the NCBI databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The gene coding for aminotransferase class I and II (*Lreu23DRAFT_4905*, referred to as *Lreu4905*) in *L. reuteri* 100-23 was truncated with the use of pJRS233 according to a deletion strategy described earlier (see Supplemental Information for references). Transformants were grown in mMRS-erythromycin broth at 42–44°C for 80 generations for the selection of single-crossover mutants.

Immunohistochemistry and FISH

Organs were removed and fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned at 5 μm. Sections were then rehydrated, and after antigen retrieval in Citrate Buffer (10 mM, pH6), sections were blocked with 5% BSA in PBS and stained with PE anti-iL-22 and FITC-anti-NKp46 (eBioscience). mAbs were incubated overnight at 4°C. Images were acquired with a fluorescence microscope (BX51 Olympus) with a 40× objective and the analySIS image processing software (Olympus). DAPI (Molecular Probes) was used for counterstaining tissues and detecting nuclei. FISH analysis with fluorescently labeled specific probes was done in gastric and vaginal fluids (see Supplemental Information). Images were acquired with a fluorescence microscope (BX51 Olympus) and the analySIS image processing software (Olympus).

HPLC-HRMS Analysis

For metabolomic analysis, HPLC-HRMS (high-performance liquid chromatography-high-resolution mass spectrometry) was performed with both the 1290 Infinity HPLC coupled to a 6540 Q-ToF mass spectrometer equipped with a Jet Stream ESI interface (Agilent Technologies) and the Ultimate 3000 HPLC coupled to a LTQ-Orbitrap mass spectrometer through an ESI interface (Thermo Scientific) (see Supplemental Information).

Statistical Analysis

Data are expressed as mean ± SD. Horizontal bars indicate the means. For multiple comparisons, p values were calculated by a one-way ANOVA (Bonferroni's post hoc test). For single comparison, p values were calculated by a two-tailed Student's t test. The data reported are either from one representative experiment out of three to five independent experiments or compiled from three to five experiments.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, eight figures, and eight tables and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.08.003.

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