ORIGINAL ARTICLE

The social structure of microbial community involved in colonization resistance

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It is well established that host-associated microbial communities can interfere with the colonization and establishment of microbes of foreign origins, a phenomenon often referred to as bacterial interference or colonization resistance. However, due to the complexity of the indigenous microbiota, it has been extremely difficult to elucidate the community colonization resistance mechanisms and identify the bacterial species involved. In a recent study, we have established an *in vitro* mice oral microbial community (O-mix) and demonstrated its colonization resistance against an *Escherichia coli* strain of mice gut origin. In this study, we further analyzed the community structure of the O-mix by using a dilution/regrowth approach and identified the bacterial species involved in colonization resistance against *E. coli*. Our results revealed that, within the O-mix there were three different types of bacterial species forming unique social structure. They act as 'Sensor', 'Mediator' and 'Killer', respectively, and have coordinated roles in initiating the antagonistic action and preventing the integration of *E. coli*. The functional role of each identified bacterial species was further confirmed by *E. coli*-specific responsiveness of the synthetic communities composed of different combination of the identified players. The study reveals for the first time the sophisticated structural and functional organization of a colonization resistance pathway within a microbial community. Furthermore, our results emphasize the importance of 'Facilitation' or positive interactions in the development of community-level functions, such as colonization resistance.
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Introduction

The association with commensal microbiota is a general feature for virtually all animals, including humans (Ley *et al.*, 2008a,b; Robinson *et al.*, 2010a,b). These indigenous microbial communities co-evolve with their hosts and have crucial roles in host development and health, from nutrient acquisition in termites to the establishment of the mucosal immune system in mice and energy metabolism in humans (Hongoh *et al.*, 2003; Backhed *et al.*, 2004; Leslie, 2012).

Among the proposed beneficial effects, the ability of the indigenous community to resist the invasion and colonization by exogenous organisms, termed colonization resistance, has been regarded as one of the major functions of the host-associated microbiota (Brook, 1999; Reid *et al.*, 2001; Falagas *et al.*, 2008). It has been extensively documented in invertebrate- as well as vertebrate-associated commensal microbial communities (Veivers *et al.*, 1982; Guarner and Malagelada, 2003; Dillon *et al.*, 2005). The notion that colonization resistance could be a crucial component of host defense against pathogens and may be manipulated to the host's advantage makes it the subject of constant investigation for the past few decades (Freter, 1956; Lloyd *et al.*, 1977; Van der Waaij and Van der Waaij, 1990; Rolfe, 1997).

Several mechanisms have been proposed to explain the colonization resistance observed in host-associated microbiota, including stimulating the host-immune response against invaders (Corthesy *et al.*, 2007), competition for substrates and host-binding sites (Chan *et al.*, 1985), generating a micro-environment that is inhibitory to potential competitors (Bernet-Camard *et al.*, 1997), and the production of antibiotic substances (Vandenbergh, 1993). Recent work further suggested that community structure of indigenous microbiota likely has a significant role in its colonization resistance (Robinson *et al.*, 2010b). However, due to the complexity of the indigenous microbiota, these hypotheses have scarcely been proven, and it has been extremely difficult to elucidate the community invasion resistance mechanisms by identifying the bacteria species involved and revealing the associated complex processes at the molecular level.

Recently, we established an *in vitro* oral microbial community (O-mix) derived from mice oral cavity (He *et al.*, 2010a). Although it was not a defined community, the O-mix contained consistent microbial diversity of more than 10 bacteria species, as revealed by PCR-denaturing gradient gel electrophoresis (DGGE), when being recovered from frozen stock. The same microbial profile could be maintained up to 72 h in suspension culture when fresh medium was provided every 24 h (He *et al.*, 2010a). The O-mix displayed a striking ability to restrict the integration of an *E. coli* strain of mice gut origin as well as *E. coli* reference strain MG1655, a phenomenon indicative of the colonization resistance within oral microbial community (He *et al.*, 2010a,b). Further investigation revealed that the O-mix was able to detect the presence of *E. coli* by recognizing its surface lipopolysaccharides (LPS), and respond by producing H_2O_2, a bactericidal agent that is more effective in killing *E. coli* cells than various oral isolates tested (He *et al.*, 2010b).

Based on these results, we hypothesized that the presence of *E. coli* could trigger the activation of a colonization resistance pathway within the O-mix. The pathway might involve multiple bacterial species that have coordinated roles in preventing the colonization of *E. coli*. In this study, we employed PCR-DGGE analysis to track and compare the microbial profiles of the communities derived from differentially diluted O-mix. By correlating major shift in microbial profiles with the changes in communities' responsivity to *E. coli*, we sought to investigate the underlying pathway and identify the key players involved in the colonization resistance of the O-mix against the integration of bacteria of foreign origin.

Materials and methods

Bacterial strains and growth conditions

Mice oral isolates, including *Staphylococcus saprophyticus* MO-100, *Streptococcus sanguinis* MO-101 and *Streptococcus infantis* MO-102 (He *et al.*, 2010a), as well as *Escherichia coli* reference strain (MG1655) (He *et al.*, 2010b) were cultivated in brain heart infusion (BHI) broth supplemented with hemin ($5\,\mu g\,ml^{-1}$), vitamin K ($0.5\,\mu g\,ml^{-1}$), sucrose (0.1%), mannose (0.1%) and glucose (0.1%). Cultures were incubated at $37\,^{\circ}C$ under anaerobic conditions (nitrogen 85%, carbon dioxide 5% and hydrogen 10%). When needed, kanamycin ($50\,\mu g\,ml^{-1}$) was added to the medium for selecting *E. coli* MG1655.

The cultivated mice oral microbiota (O-mix) were recovered from a labarotory stock that was described in a previous study (He *et al.*, 2010a). Briefly, $50\,\mu l$ of BHI-cultivated O-mix stock was inoculated into 5 ml of supplemented BHI broth. The cultures were incubated at $37\,^{\circ}C$ under anaerobic conditions for $\sim 16\,h$ until the exponential growth phase was reached, which was determined by the community growth curve measured by optical density reading.

Community dilution assay

The community dilution assay was performed as previously described (Franklin and Mills, 2006) with modifications. Briefly, an overnight culture of the O-mix ($\sim 10^9$ colony-forming units (c.f.u.) per ml) was subjected to serial dilution (10-fold) from 10^0 to 10^{-8}, using fresh supplemented BHI as the diluent. The corresponding O-mix subcultures for each dilution were incubated at $37\,^{\circ}C$ under anaerobic condition. Each subculture was harvested and stocks were made when an OD_{600} of 1 ($\sim 10^9$ c.f.u. per ml) was reached. The resulting sub-communities were referred to as Community 10^0, Community 10^{-1}, etc. up to Community 10^{-8}. Fifty microliters of each O-mix sub-community stock was inoculated into 2 ml fresh BHI and incubated at $37\,^{\circ}C$ under anaerobic conditions until the exponential growth phase was reached. 1.5 ml of each regrown community was collected by centrifugation at $14\,000 \times g$ for 1 min and the cell pellets were used for DNA extraction and PCR-DGGE analysis as described below. The remainders of the subcultures were harvested, resuspended in fresh BHI to a final OD_{600} of 0.1 ($\sim 10^8$ c.f.u. ml^{-1}) and were used for testing their hydrogen peroxide (H_2O_2) production.

Hydrogen peroxide (H_2O_2) production assay on agar plates

An overnight culture of *E. coli* reference strain (MG1655) was harvested and resuspended in fresh BHI medium to a final OD_{600} of 0.01 ($\sim 10^7$ c.f.u. ml^{-1}). $10\,\mu l$ of resuspended *E. coli* culture was mixed with equal volumes ($10\,\mu l$) of resuspended O-mix subcultures (OD_{600} of 0.1), resulting in a 1:10 *E. coli*-to-O-mix ratio and used for the H_2O_2 production assay.

The H_2O_2 production assay was performed as previously described (He *et al.*, 2012). Briefly, spots of $5\,\mu l$ of horseradish peroxide ($1\,ml\,mg^{-1}$; Thermo Scientific, Rockford, IL, USA) were added onto BHI agar plate containing $1\,mg\,ml^{-1}$ leuco crystal violet (Acros Organics, Fair Lawn, NJ, USA). After the liquid was absorbed into the agar, $10\,\mu l$ of the different mixtures was inoculated on top of the spots containing peroxidase. Plates were incubated at $37\,^{\circ}C$ under anaerobic conditions for 18 h, and the development of purple color on/around the colonies as indication of H_2O_2 production was monitored. Three independent replicates were performed and a representative result was shown.

PCR-DGGE analysis

PCR-DGGE was employed to track and compare the profiles of microbial communities derived from community dilution assay. Total genomic DNA of

bacterial samples from the communities regrown from differentially diluted original O-mix was isolated using the MasterPure DNA Purification Kit (EPICENTRE), DNA quality and quantity were measured by a spectrophotometer at 260 nm and 280 nm (Spectronic Genesys, Spectronic Instruments, Inc., Rochester, NY, USA).

Amplification of bacterial 16S ribosomal RNA genes by PCR was carried out as described previously (Wang *et al.*, 2012). Briefly, the universal primer set, Bac1 (5′-CGCCCGCCGCGCCCCGCGC CCGTCCCGCCGCCCCCGCCCGACTACGTGCCAGC AGCC–3′) (Sheffield *et al.*, 1989) and Bac2 (5′-GGA CTACCAGGGTATCTAATCC-3′) was used to amplify an $\sim$300-bp internal fragment of the 16S ribosomal RNA gene. Each 50 µl PCR contains 100 ng purified genomic DNA, 40 pmol of each primer, 200 µM of each dNTP, 4.0 mM $MgCl_2$, 5 µl 10 × PCR buffer, and 2.5 U Taq DNA polymerase (Invitrogen). Cycling conditions were 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min, with a final extension period of 5 min at 72 °C. The resulting PCR products were evaluated by 1% agarose gel electrophoresis.

Polyacrylamide gels at an 8% concentration were prepared with a denaturing urea/formamide gradient between 40% [containing 2.8 M urea and 16% (vol/vol) formamide] and 70% [containing 4.9 M urea and 28% (vol/vol) formamide]. Approximately 300 ng of the PCR product were applied per well. The gels were submerged in 1 × TAE buffer (40 mM Tris base, 40 mM glacial acetic acid, 1 mM ethylene-diaminetetraacetic acid) and the PCR products were separated by electrophoresis for 17 h at 58 °C using a fixed voltage of 60 V in the Bio-Rad DCode System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After electrophoresis, gels were rinsed and stained for 15 min in 1 × TAE buffer containing $0.5 \mu g \, ml^{-1}$ ethidium bromide, followed by 10 min of de-staining in 1 × TAE buffer. DGGE profile images were digitally recorded using the Molecular Imager Gel Documentation system (Bio-Rad Laboratories). Three independent biological replicates were performed and a representative gel image was shown.

Identification of candidate bacterial species involved in colonization resistance

To identify major bacterial species whose absence as a result of community dilution could correlate with the changes in communities' indigenous H_2O_2 production profiles as well as their responsiveness to the presence of *E. coli*, DGGE gel images derived from each sub-community were compared. PCR bands present in communities with lower dilution factors, while missing from cultures with higher dilution factors were excised from the DGGE gels, eluted into 20 µl sterile dH_2O as preciously described (He *et al.*, 2010a) and re-amplified with the Bac1/Bac2 universal primers. The resulting PCR products were purified and sequenced at the UCLA

Sequencing and Genotyping Core Facility. The obtained partial 16S rRNA gene sequences (about 300 bp) were used to BLAST search against the HOMD (http://www.homd.org) and NCBI (http://www.ncbi.nlm.nih.gov) databases. Sequences with 98%–100% identity to those deposited in the public domain databases were considered to be positive identification of taxa.

Isolation and identification of bacterial species producing hydrogen peroxide

Hydrogen peroxide production assay revealed that the regrowth of the most diluted O-mix was still able to produce H_2O_2 (Figure 1), suggesting that hydrogen peroxide producing species was one of the most abundant bacteria within the O-mix. To isolate H_2O_2 producer, the 10^{-7} community derived from original O-mix *via* dilution was subjected to serial dilution and the diluted samples were plated onto BHI agar plates. Plates were incubated at 37 °C under anaerobic condition until distinct colonies appeared. Multiple colonies were picked and cultivated in BHI medium until turbid. Hydrogen peroxide production assay on BHI plate was performed as previously described to determine bacterial H_2O_2-producing capability. For isolates that were able to produce H_2O_2, their identities were determined by 16S sequencing as previous described (He *et al.*, 2010a).

Construction of synthetic oral microbial communities

Isolated mice oral bacterial species from previous studies, including *S. saprophyticus* MO-100, *S. sanguinis* MO-101 and *S. infantis* MO-102 (He *et al.*, 2010a,b) were used to construct synthetic oral microbial communities. Overnight cultures of individual oral isolates were harvested and resuspended in fresh BHI to an OD_{600} of 0.01. Synthetic communities with distinct microbial compositions were constructed by combining equal volumes of the selected resuspended bacterial cultures.
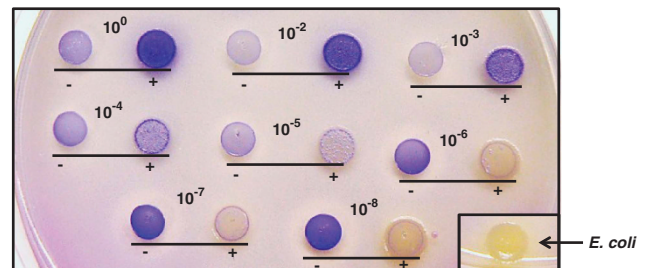

Figure 1 H_2O_2 production of oral communities when challenged with *E. coli*. Oral communities derived from the dilution (10^0 to 10^{-8})/regrowth of original O-mix were challenged with (+) or incubated without (−) *E. coli* (in a 10:1 oral cell-to-*E. coli* ratio) and the mixtures were spotted onto BHI agar plates containing leuco crystal violet and horseradish peroxidase; blue color development reflecting the H_2O_2 production was monitored after overnight incubation at 37 °C. Three independent replicates were performed and a representative result is shown.

The cultures were incubated at 37 °C under anaerobic condition for 16 h to allow the community to reach equilibrium indicated by the stable abundance of each bacterial species as determined by PCR-DGGE analysis. The cells in the synthetic community were harvested, and resuspended in fresh BHI medium to a final OD_{600} of 0.1 before being used for the following Community Invasion Resistance assay.

Community invasion resistance assay
Synthetic communities with distinct microbial composition (OD_{600} of 0.01, with $\sim 10^7$ c.f.u. per ml bacterial cells) were mixed with an equal volume of *E. coli* MG1655 (OD_{600} of 0.001, with $\sim 10^6$ c.f.u. per ml), resulting in a 1:10 *E. coli*-to-synthetic community ratio. Co-cultures were incubated at 37 °C under anaerobic condition, and the viability of *E. coli* as well as the whole community was monitored periodically by plating the cells onto selective (with Kan 50 μg ml^{-1}) and non-selective BHI plates. Plates were incubated at 37 °C for 2 days before colonies were counted. Two independent experiments were performed in triplicates and the average value ± standard deviation (s.d.) was shown.

Quantitative measurement of H_2O_2 concentration in bacterial culture
The H_2O_2 concentration in bacterial co-cultures was measured as previously described (He *et al.*, 2012). Briefly, 100 μl of bacterial co-culture was subjected to centrifugation at 14 000 × g for 3 min. 20 μl of supernatant or a 1:2 serial dilution of supernatant with PBS was mixed with 20 μl of horseradish peroxidase (0.5 mg ml^{-1}) and 50 μl buffer solution (0.1 mg ml^{-1} of 3,3′,5,5′-tetramethylbenzidine, 0.05 M phosphate citrate buffer at pH 5.0). The mixture was incubated at room temperature for 1 min, and 100 μl of 2 M H_2SO_4 was added to stop the reaction. Color intensity was measured at 420 nm using a microplate reader. Three independent experiments were performed and the average value ± s.d. is shown.

Two-chamber assay
The two-chamber assay was performed as previously described (He *et al.*, 2010a) with minor modification. Briefly, 2 ml of BHI containing $\sim 2 \times 10^7$ total bacterial cells of different combination of oral bacterial isolates with equal number cells of each species, was inoculated into the bottom chamber of a 12-well plate containing a 0.4 μm PET membrane insert (Millipore, Billerica, MA, USA); while 0.7 ml of bacterial mixture containing *E. coli* MG1655 (with $\sim 10^6$ c.f.u. per ml) alone, or *E. coli* with different oral isolates (with $\sim 10^7$ c.f.u. per ml for each oral isolate) was added to the top chamber. The viability of *E. coli* as well as the total bacterial count within the upper and lower chamber was

monitored as previously described (He *et al.*, 2010b). Briefly, culture samples were taken periodically, vortexed for 1 min, subjected to serial dilution and plated onto selective plates. Plates were incubated for 3 days at 37 °C under anaerobic condition before colonies were counted. Two independent experiments were performed in duplicates, and average value ± s.d. was presented.

Statistical analysis
Significance of differences between average values was analyzed by Student's *t*-tests.

Results

Communities derived from dilution/regrowth of the original O-mix displayed distinct response patterns when challenged with E. coli
Recently, we demonstrated *in vitro* that the cultivable mice oral microbial community (O-mix) displayed colonization resistance and responded to the presence of the Gram-negative gut isolate *E. coli* by elevating its H_2O_2 production (He *et al.*, 2010b). To further investigate whether the observed colonization resistance required a specific community structure and the involvement of particular bacterial species within the O-mix, we applied a dilution/regrowth approach to test the effect of changes in community structure on its response to the presence of *E. coli.* The method was based on the premise that dilution of a relatively diverse community would remove less abundant species, resulting in communities with different species richness, and has been employed in studying community composition and structure (Franklin *et al.*, 2001). The result of H_2O_2 production on agar plates showed that, the original O-mix community produced very low amounts of H_2O_2 when grown on BHI plates, while co-incubation with *E. coli* triggered a dramatic increase in H_2O_2 production as indicated by the development of dark blue color (Figure 1). This spike in H_2O_2 production was accompanied by a drastic (>90%) reduction in viability of *E. coli* within the mix (data not shown). Interestingly, compared with the original O-mix, the communities derived from dilution/regrowth displayed altered H_2O_2-production profile in the absence, as well as in the presence of *E. coli*.

Specifically, when the original O-mix was titrated below 10^{-4}, the regrown communities failed to respond to the presence of *E. coli* with elevated H_2O_2 level. Meanwhile, the regrown communities derived from 10^{-6} (and lower) dilution of the O-mix displayed drastically increased H_2O_2 level even in the absence of *E. coli,* as reflected by the increased intensity of purple color (Figure 1). However, these regrown communities failed to achieve a similar inhibition effect against *E. coli* as demonstrated by the diminished hydrogen peroxide level when co-incubated with *E. coli* (Figure 1), as well as increased survival rate (>90%) of *E. coli*, compared

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with 10% when *E. coli* was co-incubated with original O-mix for 24 h (data not shown).

Identification of candidate players involved in community colonization resistance against *E. coli*

The H_2O_2 production assay revealed the differential response of the serially diluted/regrown communities to the presence of *E. coli* (Figure 1). The apparent transition to an 'inversion' of the response to *E. coli* was associated with a reduction in species diversity in the oral communities, suggesting the involvement of different bacterial species with distinct roles in colonization resistance via regulated H_2O_2 production. In an effort to identify these players, microbial profiles of dilution-defined oral communities were obtained by PCR-DGGE analysis (Figure 2), the 'banding patterns' representing the bacterial community diversity were compared and the unique 16S DNA fragments were sequenced to identify the corresponding bacterial species. All the identified species had been isolated from mice oral cavity in our previous studies (He *et al.*, 2010a,b).

Our data revealed that when O-mix was titrated to 10^{-4}, the absence of certain bacterial species including *Staphylococcus saprophyticus*, was concurrent with the reduced community response to the presence of *E. coli*. Meanwhile, the absence of *Streptococcus infantis* from community 10^{-6} was coincident with the community's spontaneous high-level H_2O_2 production (Figure 2). The results implicated the involvement of these bacterial species in the resistance pathway. Furthermore, H_2O_2 has been shown to be responsible for killing invading *E. coli*, which makes H_2O_2-producing bacteria one of the important players in this pathway (He *et al.*, 2010b). As community 10^{-7} comprised less bacterial species while still containing H_2O_2 producers, colony isolation coupled with the H_2O_2 production assay was used to identify the H_2O_2 producer within the community 10^{-7}, the result revealed that the H_2O_2-producing *Streptococcus sanguinis* was the most abundant species within the communities 10^{-7} and 10^{-8}. This was corroborated by our DGGE data showing that the band with the highest density within these communities was *S. sanguinis* (Figure 2).

Synthetic oral community comprised of identified players was sufficient to exert colonization resistance against *E. coli*

Based on the concurrence of the removal of specific bacteria species from communities derived from dilution and the corresponding community functional shift, we identified three potential players that were likely to be involved in the pathway leading to the resistance against *E. coli*. The candidates were searched against the panel of isolated oral bacterial species from mice oral cavity. All three candidates, *S. saprophyticus*, *S. infantis* and *S. sanguinis*, had been isolated during our previous studies (He *et al.*, 2010a,b). In an effort to investigate if the identified bacterial species were required and sufficient to elicit the colonization resistance when challenged with *E. coli*, we established synthetic communities composed of different combination of three identified potential players and tested their responsivity to the presence of *E. coli*, whose initial inoculum was about 10% of the total cell numbers within synthetic communities.

Results showed that, when *E. coli* was added to mono-species communities, no significant inhibition effect was observed (Figure 3). There was a two- to fivefold increase in the percentage of viable count of *E. coli* in respect to the total viable cells within the co-culture during the 2-day period. Similar outcome was observed when synthetic duo-species community containing *S. infantis* and *S. sanguinis* was challenged with *E. coli*. However, when co-cultivated with a two-species community composed of *S. saprophyticus*/*S. infantis*, or *S. saprophyticus*/*S. sanguinis*, a two- to threefold reduction in the percentage of viable *E. coli* was observed. Most strikingly, when *E. coli* encountered the synthetic community composed of all three species, it suffered drastic viability loss, its proportion relative to the total bacteria count dropped from around 10% to less than 0.05% after 48 h of co-cultivation, while no significant change in the total bacterial count was apparent (data not shown).
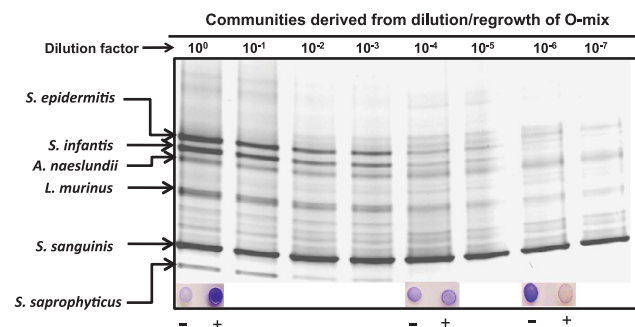

Figure 2 PCR-DGGE analysis of microbial communities derived from dilution/regrowth of original O-mix. Original O-mix was subjected to serial dilution (10^{-0}–10^{-7})/regrowth to establish new bacterial communities. The microbial profile of each derived community was assessed by PCR-DGGE. The major bacterial species within O-mix were identified as indicated by the arrows. Inlet shows the H_2O_2 production of the specific communities in response to the absence (−) and presence (+) of *E. coli*. Three independent biological replicates were performed and a representative gel image is shown.

S. saprophyticus initiates the colonization resistance pathway

Our previous study showed that when *E. coli* was co-cultivated with the O-mix, it suffered drastic reduction in viability. However, when *E. coli* and O-mix cells were separated in a two-chamber vessel with a 0.4-μm pore size membrane, no inhibition
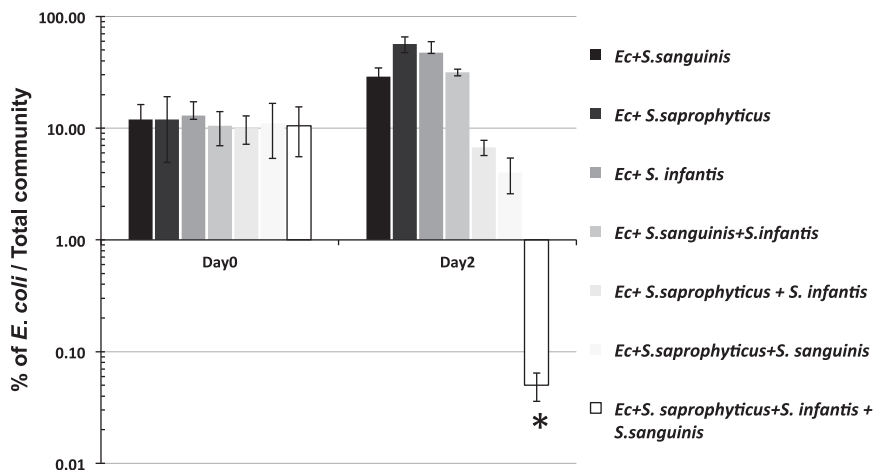
Figure 3 Colonization resistance of synthetic communities against *E. coli* (Ec). *E. coli* was co-cultivated with synthetic communities comprised different combination of identified bacterial species in a 1:10 ratio, and its viability was monitored after 48 h incubation at 37 °C under anaerobic condition. Two independent experiments were performed in triplicates and the average value ± s.d. is shown. The asterisk indicates that the value of that specific experimental setup is significantly lower than that of other setups (Student's *t*-test *P*-value < 0.05).

was observed even though they shared the same culture medium (He *et al.*, 2010b). Meanwhile, *E. coli* LPS mutants displayed much reduced ability in triggering the production of H_2O_2 by the O-mix, which was the main factor responsible for killing *E. coli* (He *et al.*, 2010b). These data indicated that certain bacterial species within the O-mix could potentially detect the presence of *E. coli* via cell–cell contact (possibly through direct contact with *E. coli* LPS) and initiate the resistance response (He *et al.*, 2010b). In an effort to determine if such a role can be carried out by one of the identified players, two-chamber assay was applied to study the possible contact-dependent activation of the resistance against *E. coli* by a synthetic oral community composed of *S. saprophyticus*, *S. infantis* and *S. sanguinis*. When *E. coli* (in the upper chamber) was physically separated from the rest of the synthetic community (in the low chamber) by a 0.4-μm pore size membrane while sharing the same culturing medium, no inhibition was observed, which confirmed our recent data that cell–cell contact might be required for triggering the response (He *et al.*, 2010b). To test which oral isolate could have the role in sensing *E. coli* and initiating the response, each one of the three oral isolates was co-cultured with *E. coli* in the upper chamber, whereas other community members were grown in the lower chamber. Our result showed that a drastic reduction in *E. coli* viability was monitored only when *E. coli* was co-incubated with *S. saprophyticus* in the upper chamber (Figure 4). As co-cultivation with *S. saprophyticus* alone did not cause any growth inhibition in *E. coli* (Figure 3), the co-incubation of the two species in the upper chamber might induce the production of diffusible signal(s), which could relay the response to the rest of the community members in the lower chamber, and eventually result in the production of H_2O_2.

S. infantis inhibits S. sanguinis H_2O_2 production and responds to the diffusible signal(s) derived from E. coli-*activated S. saprophyticus*

Our result suggested that *S. saprophyticus* might be able to sense the presence of *E. coli* via cell–cell contact and activate the resistance pathway by producing diffusible signal(s) (Figure 4). To further determine the role of *S. infantis* in the resistance pathway, we tested the effect of spent medium of *E. coli*, *S. saprophyticus* monoculture or their dual-species culture on the H_2O_2 production of either *S. sanguinis*, or its co-culture with *S. infantis*. The co-cultivation with *S. infantis* highly suppressed the H_2O_2 production of *S. sanguinis* (reduced from 0.58 ± 0.10 mM to 0.05 ± 0.01 mM), while the addition of the spent medium of *S. saprophyticus* and *E. coli* co-culture not only rescued the repression but also resulted in a significantly higher H_2O_2 level (0.97 ± 0.09 mM) within the community (Table 1).

H_2O_2 production of synthetic oral communities in response to the presence of E. coli

Quantitative measurement of H_2O_2 production revealed that H_2O_2 can only be detected in synthetic communities containing H_2O_2-producing *S. sanguinis*. The H_2O_2 level in *S. sanguinis* monoculture grown under community growth condition was determined to be ~0.62 mM, a concentration below the minimum inhibitory concentration (0.8 mM) against *E. coli* (He *et al.*, 2010b). The inclusion of *S. saprophyticus* in the community did not affect the H_2O_2 level. However, when these two cultures were challenged with *E. coli* (in a 10:1 ratio) for 24 h, there was a significant reduction in the H_2O_2 level detected within the community. Furthermore, when *S. infantis* was used to establish two-species community with *S. sanguinis*, or tri-species community with *S. sanguinis/S. saprophyticus*, the H_2O_2 level
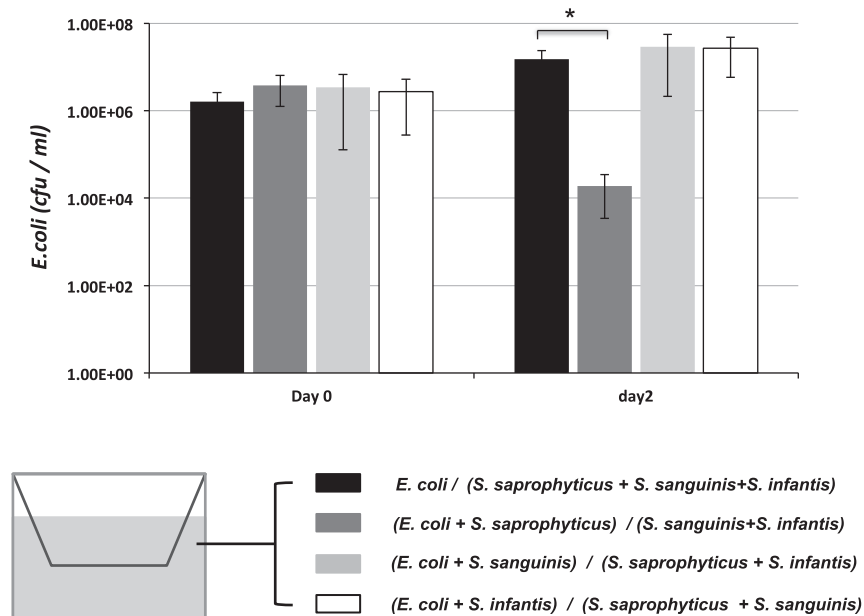
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Figure 4 Survival of *E. coli* within synthetic oral communities in two-chamber assay. *E. coli* alone, or mixed with different oral isolates was cultured in the upper chamber of the two-chamber system, while the rest of the members of the synthetic communities were inoculated to the lower chamber. Viability of *E. coli* in the upper chamber in each setup (represented by different bars) was monitored after 48 h. Two independent experiments were performed in duplicates, and average value ± s.d. is presented. The asterisk indicates significant differences between two values (Student's *t*-test *P*-value <0.05).

Table 1 Quantitative measurement of H_2O_2 production of synthetic communities under different treatments

Community	Added spent medium			
		E. coli	S. saprophyticus	S. saprophyticus + E. coli
S. sanguinis	0.58 ± 0.10	0.65 ± 0.10	0.72 ± 0.12	0.57 ± 0.09
S. sanguinis + S. infantis	0.05 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.97 ± 0.09[a]

Three replicates were performed for each assay. The values represent the H_2O_2 production (mM) of synthetic communities under different treatments. Average values ± s.d. are shown.
[a]Indicates significant differences between the indicated value and the value obtained from same community without adding spent medium (Student's *t*-test *P*-value <0.05).

dropped to ∼0.02 mM (Table 2). Most strikingly, when the synthetic community contained all three bacterial species, it demonstrated the most drastic response to the presence of *E. coli* by increasing its H_2O_2 level almost 40-fold, reaching to about 1 mM (Table 2), a concentration that could exert significant inhibitory effect on *E. coli* (He *et al.*, 2010b). It is worth noting that, although no H_2O_2 could be detected in duo-species community comprised of *S. saprophyticus* and *S. infantis*, either in the presence or absence of *E. coli* (Table 2), it still exerted certain inhibitory effect toward *E. coli* (Figure 3), suggesting the involvement of mechanism(s) other than the production of H_2O_2 in inhibiting *E. coli*.

Discussion

Colonization resistance is an intrinsic property of ecological communities and has significant roles in protecting the residential niches and maintaining the stability of established communities. This phenomenon has been well documented in a variety of ecosystems, ranging from grassland, marine ecosystem to host-associated microbiota (Brook, 1999; Stachowicz *et al.*, 1999; Dillon *et al.*, 2005; Thomsen *et al.*, 2006).

The past decade has witnessed an increasing interest in studying the ecology of host-associated microbiota. As one of the potential beneficial effects conferred by these indigenous microbial communities, colonization resistance has been implicated in protecting host against pathogens and has received great attention (Rolfe, 1997; Brook, 1999; Adlerberth *et al.*, 2000; Fons *et al.*, 2000; Reid *et al.*, 2001; Guarner and Malagelada, 2003; Reid *et al.*, 2003; Alain, 2004; Dillon *et al.*, 2005; Falagas *et al.*, 2008). However, due to the complexity of host-associated indigenous microbial community, it is very difficult to identify the bacterial species responsible for colonization resistance and to

Table 2 Quantitative measurement of H_2O_2 production in synthetic communities 24 h after challenged with and without *E. coli*

Synthetic communities	H_2O_2 production (mM) without E. coli	H_2O_2 production with E. coli
S. sanguinis	0.62 ± 0.08	0.19 ± 0.05
S. saprophyticus	ND	ND
S. infantis	ND	ND
S. sanguinis + S. infantis	0.02 ± 0.01	0.03 ± 0.01
S. saprophyticus + S. infantis	ND	ND
S. saprophyticus + S. sanguinis	0.54 ± 0.05	0.17 ± 0.08
S. saprophyticus + S. infantis + S. sanguinis	0.03 ± 0.01	1.17 ± 0.14[a]

Abbreviation: ND, not detected.
Three replicates were performed for each assay. Average values ± s.d. are shown.
[a]Significant differences between the indicated value and value obtained from same community without challenged with *E. coli* (Student's *t*-test *P*-value < 0.05).

Figure 5 Tentative model of colonization resistance pathway of the O-mix against integration of *E. coli*. Green, orange and black balls represent *S. saprophyticus, S. infantis* and *S. sanguinis,* respectively.

elucidate the mechanisms involved. In this study, using an established *in vitro* microbial community (O-mix) derived from the mice oral cavity, we have revealed one of its important colonization resistance mechanisms by identifying the key bacterial species in a pathway involved in regulated H_2O_2 production to prevent *E. coli* integration (Figure 5).

This particular colonization resistance pathway is comprised of three key bacterial species: *S. saprophyticus* (the 'Sensor'), *S. infantis* (the 'Mediator') and *S. sanguinis* (the 'Killer') (Figure 5). In the absence of foreign invader (for example, *E. coli*), *S. infantis* represses *S. sanguinis'* capacity to produce H_2O_2, resulting in minimal H_2O_2 level within the community (Tables 1 and 2). When encountering *E. coli*, *S. saprophyticus* acts as the 'Sensor' due to its ability to detect the presence of *E. coli* (Figure 4) and initiates the invasion resistance response by producing diffusible signal(s) (Figure 4; Table 1). The signal(s) could relay the information to *S. infantis* (Table 1), which not only alleviates its suppression on *S. sanguinis'* H_2O_2 production but also stimulates the Killer's H_2O_2-producing capability (Table 1). The resultant increased H_2O_2 level exerts inhibitory effect on the invading *E. coli* (Figure 5).

A few lines of evidence suggested *S. saprophyticus* might sense the presence of *E. coli* via direct cell–cell contact. First of all, two-chamber assay showed that only when co-incubating with *S. saprophyticus* in the upper chamber, while the rest of the two members of the synthetic community remained in the lower chamber, did *E. coli* suffer drastic inhibition (Figure 4). As co-cultivation of *S. saprophyticus* with *E. coli* alone did not result in the inhibition of *E. coli* (Figure 3), the result suggested that the interaction between *E. coli* and *S. saprophyticus* was the initial step in initiating the response, while the inhibition of *E. coli* was mainly
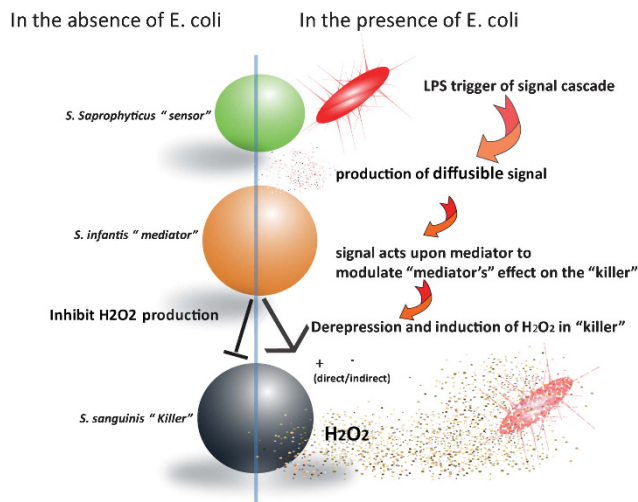
due to the production of H_2O_2 by other players in the same pathway. Meanwhile, the membrane used for separating the two chambers has a pore size of 0.4 μm, which should be permeable to most, if not all, of the diffusible factors. If the initial triggering event were due to the released metabolites or secretary substances by *E. coli*, we would expect to observe similar inhibition regardless of which community member was co-incubated with *E. coli* in the upper chamber. These results, together with our previous studies showing *E. coli* LPS was required for triggering the resistance response in the O-mix (He *et al.*, 2010b), indicated a direct physical contact between *E. coli* and the 'Sensor' in triggering the response. Furthermore, the ability of the spent medium of *E. coli/S. saprophyticus* co-culture to induce H_2O_2 production of the community (Table 1), as well as the observed relay of the inhibition event even when *E. coli* and *S. saprophyticus* were physically separated from the rest of the community members in the two-chamber assay (Figure 4) were indicative of the diffusible nature of the signal(s) produced by *S. saprophyticus* upon physical contact with *E. coli*.

Our data also showed that a two-species community comprised *S. saprophyticus* and *S. infantis*, but missing H_2O_2-producing *S. sanguinis* still exerted inhibitory effect against *E. coli*, although at a much reduced level, suggesting other potential mechanisms might also contribute to the observed resistance against *E. coli* (Figure 3). All three isolates or their close relatives have been identified in mice oral cavities from a separate study using 454-pyrose-quecning analysis (Chun *et al.*, 2010), however, their physiological or biological functions have not been investigated. Our synthetic community data demonstrated that the *E. coli*-induced colonization resistance required the participation of these three

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players, and missing any one of them resulted in a greatly reduced colonization resistance against the integration of *E. coli*.

Ecological theory suggests that species-rich communities are more resistant to invasion than species-poor communities, this negative invasibility–diversity relationship have been documented in a wide range of community types, including animals, plants, marine invertebrates, protists and bacteria (Case, 1990; McGrady-Steed *et al.*, 1997; Stachowicz *et al.*, 1999; Hodgson *et al.*, 2002; Cook *et al.*, 2006; Thomsen *et al.*, 2006). It has been proposed that, the observed invasibility–diversity relationship in established ecological communities is due to three non-mutually exclusive mechanisms, including dominance, niche complementarity and positive interactions (Bruno *et al.*, 2003). Our synthetic community data are consistent with those from numerous community ecology studies (Case, 1990; McGrady-Steed *et al.*, 1997; Stachowicz *et al.*, 1999; Hodgson *et al.*, 2002; Cook *et al.*, 2006; Thomsen *et al.*, 2006), further confirming the importance of species diversity or richness within the community in developing colonization resistance. However, the observed negative diversity–invasibility relationship is not simply because more diverse communities have higher probability of containing an oral bacterial species that exerts particularly high colonization resistance against *E. coli* (dominance), but rather it emphasized on the importance of 'Facilitation', or positive species interactions, in the colonization resistance of mice oral community. Facilitation are encounters between organisms that benefit at least one of the participants and cause harm to neither (Bruno *et al.*, 2003). It is an important community-level process that can be found in many ecological communities, particularly bacterial ecosystems, and could potentially increase colonization resistance against invasion and contribute to community stability (Hodgson *et al.*, 2002; Burmolle *et al.*, 2006). Within established microbial communities, different types of residential bacterial species are often involved in synergistic metabolism, or engaged in cooperative beneficial interactions, which have been shown to have striking influence on the survival of the communities (Kuramitsu *et al.*, 2007). Within our synthetic community, the colonization resistance is neither dependent on one particular species nor manifested by simple additive inhibitory effect of each player. Instead, it requires a complex pathway with multiple players, including *S. saprophyticus* (the 'Sensor'), *S. infantis* (the 'Mediator') and *S. sanguinis* (the 'Killer'), which are actively engaged in information exchange and relay, eventually resulting in the production of an antimicrobial compound to prevent the integration of foreign bacterium.

Within the *in vitro* mice oral community, multiple Streptococci with H_2O_2-producing capability have been identified (He *et al.*, 2010a), the replacement of *S. sanguinis* with another H_2O_2 producer, *S. gordonii*,

resulted in a similar *E. coli*-specific response of the synthetic community (data not shown), suggesting the functional redundancy within the O-mix. This result is consistent with the observation that even for communities with relatively low biodiversity, functional redundancy could have an important role in the stability of microbial communities (Franklin *et al.*, 2001; Franklin and Mills, 2006). Because of the limitation of cultivation-dependent methods, the microbial profile within the O-mix in this study could not fully represent microbial diversity of the original mice oral community. Thus, we cannot rule out the possibility that multiple sensors or mediators participate in the colonization resistance pathway.

Over the last decade, we have begun to appreciate the fact that the residential bacteria are often engaged in extensive interactions, which could result in many new physiological and biological functions that cannot be observed with individual species (Kuramitsu *et al.*, 2007). The introduction of the term 'Sociomicrobiology' (Parsek and Greenberg, 2005) further emphasizes the significance of the community level, or group behavior observed in microbial communities. However, due to their complex nature, our knowledge of the structures within most of the studied microbial communities is often limited to their residential bacterial profiles or simplified one-on-one interactions, while lacking more detailed understanding of the community organizations. Our study revealed a sophisticated colonization resistance pathway involving multiple bacterial species that have differential roles in preventing the integration of bacterium of foreign origin. Although the molecular details of this pathway require further investigation, our result clearly demonstrates the presence of sophisticated community structures and their important roles in specific community-level functions, such as colonization resistance.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

References

Adlerberth I, Cerquetti M, Poliane I, Wold A, Collignon A. (2000). Mechanisms of colonisation and colonisation resistance of the digestive tract part 1: bacteria/host interactions. *Microb Ecol Health Dis* **12**: 223–239.

Alain LS. (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiol Rev* **28**: 405–440.

Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A *et al.* (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proc Nati Acad Sci USA* **101**: 15718–15723.

Bernet-Camard MF, Lievin V, Brassart D, Neeser JR, Servin AL, Hudault S. (1997). The human Lactobacillus acidophilus strain LA1 secretes a nonbacteriocin antibacterial substance(s) active *in vitro* and *in vivo*. *Appl Environ Microbiol* **63**: 2747–2753.

Brook I. (1999). Bacterial interference. *Crit Rev Microbiol* **25**: 155–172.

Bruno JF, Stachowicz JJ, Bertness MD. (2003). Inclusion of facilitation into ecological theory. *Trends Ecol Evol* **18**: 119–125.

Burmolle M, Webb JS, Rao D, Hansen LH, Sorensen SJ, Kjelleberg S. (2006). Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. *Appl Environ Microbiol* **72**: 3916–3923.

Case TJ. (1990). Invasion resistance arises in strongly interacting species-rich model competition communities. *Proc Nati Acad Sci USA* **87**: 9610–9614.

Chan RC, Reid G, Irvin RT, Bruce AW, Costerton JW. (1985). Competitive exclusion of uropathogens from human uroepithelial cells by Lactobacillus whole cells and cell wall fragments. *Infect Immun* **47**: 84–89.

Chun J, Kim K, Lee JH, Choi Y. (2010). The analysis of oral microbial communities of wild-type and toll-like receptor 2-deficient mice using a 454 GS FLX Titanium pyrosequencer. *BMC Microbiol* **10**: 101.

Cook KL, Garland JL, Layton AC, Dionisi HM, Levine LH, Sayler GS. (2006). Effect of microbial species richness on community stability and community function in a model plant-based wastewater processing system. *Microb Ecol* **52**: 725–737.

Corthesy B, Gaskins HR, Mercenier A. (2007). Cross-talk between probiotic bacteria and the host immune system. *J Nutr* **137**: 781S–790S.

Dillon RJ, Vennard CT, Buckling A, Charnley AK. (2005). Diversity of locust gut bacteria protects against pathogen invasion. *Ecol Lett* **8**: 1291–1298.

Falagas ME, Rafailidis PI, Makris GC. (2008). Bacterial interference for the prevention and treatment of infections. *Int J Antimicrob Agents* **31**: 518–522.

Fons M, Gomez A, Karjalainen T. (2000). Mechanisms of colonisation and colonisation resistance of the digestive tract part 2: bacteria/bacteria interactions. *Microb Ecol Health Dis* **12**: 240–246.

Franklin R, Mills A. (2006). Structural and functional responses of a sewage microbial community to dilution-induced reductions in diversity. *Microb Ecol* **52**: 280–288.

Franklin RB, Garland JL, Bolster CH, Mills AL. (2001). Impact of dilution on microbial community structure and functional potential: comparison of numerical simulations and batch culture experiments. *Appl Environ Microbiol* **67**: 702–712.

Freter R. (1956). Experimental enteric Shigella and Vibrio infections in mice and guinea pigs. *J Exp Med* **104**: 411–418.

Guarner F, Malagelada JR. (2003). Gut flora in health and disease. *Lancet* **361**: 512–519.

He X, Hu W, Kaplan C, Guo L, Shi W, Lux R. (2012). Adherence to streptococci facilitates fusobacterium nucleatum integration into an oral microbial community. *Microb Ecol* **63**: 532–542.

He X, Tian Y, Guo L, Ano T, Lux R, Zusman D *et al.* (2010a). *In vitro* communities derived from oral and gut microbial floras inhibit the growth of bacteria of foreign origins. *Microb Ecol* **60**: 665–676.

He X, Tian Y, Guo L, Ano T, Lux R, Zusman D *et al.* (2010b). Oral-derived bacterial flora defends its domain by recognizing and killing intruders—A molecular analysis using Escherichia coli as a model intestinal bacterium. *Microb Ecol* **60**: 655–664.

Hodgson DJ, Rainey PB, Buckling A. (2002). Mechanisms linking diversity, productivity and invasibility in experimental bacterial communities. *Proc R Soc Lond. Series B: Biological Sciences* **269**: 2277–2283.

Hongoh Y, Ohkuma M, Kudo T. (2003). Molecular analysis of bacterial microbiota in the gut of the termite Reticulitermes speratus (Isoptera; Rhinotermitidae). *FEMS Microbiol Ecol* **44**: 231–242.

Kuramitsu HK, He X, Lux R, Anderson MH, Shi W. (2007). Interspecies interactions within oral microbial communities. *Microbiol Mol Biol Rev* **71**: 653.

Leslie M. (2012). Gut microbes keep rare immune cells in Line. *Science* **335**: 1428.

Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS *et al.* (2008a). Evolution of mammals and their gut microbes. *Science* **320**: 1647–1651.

Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI. (2008b). Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat Rev Micro* **6**: 776–788.

Lloyd AB, Cumming RB, Kent RD. (1977). Prevention of Salmonella typhihurium infection in poultry by pretreatment of chickens and poults with intestinal extracts. *Aust Vet J* **53**: 82–87.

McGrady-Steed J, Harris PM, Morin PJ. (1997). Biodiversity regulates ecosystem predictability. *Nature* **390**: 162–165.

Parsek MR, Greenberg EP. (2005). Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol* **13**: 27–33.

Reid G, Howard J, Gan BS. (2001). Can bacterial interference prevent infection? *Trends Microbiol* **9**: 424–428.

Reid G, Jass J, Sebulsky MT, McCormick JK. (2003). Potential uses of probiotics in clinical practice. *Clin Microbiol Rev* **16**: 658–672.

Robinson C, Schloss P, Ramos Y, Raffa K, Handelsman J. (2010a). Robustness of the bacterial community in the cabbage white butterfly larval midgut. *Microb Ecol* **59**: 199–211.

Robinson C, Bohannan BJM, Yong VB. (2010b). From structure to function: the ecology of host-associated microbial communities. *Microbiol Mole Biol Rev* **74**: 453–476.

Rolfe RD. (1997). Colonization resistance. In: Mackie RI, White BA, Isaacson RE (eds). *Gastrointestinal Microbiology*. Chapman and Hall: New York, NY, pp 501–535.

Sheffield VC, Cox DR, Lerman LS, Myers RM. (1989). Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc Natl Acad Sci USA* **86**: 232–236.

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Stachowicz JJ, Whitlatch RB, Osman RW. (1999). Species diversity and invasion resistance in a marine ecosystem. *Science* **286**: 1577–1579.

Thomsen MA, D'antonio CM, Suttle KB, Sousa WP. (2006). Ecological resistance, seed density and their interactions determine patterns of invasion in a California coastal grassland. *Ecol Lett* **9**: 160–170.

Van Der Waaij D, Van Der Waaij BD. (1990). The colonization resistance of the digestive tract in different animal species and in man; a comparative study. *Epidemiol Infect* **105**: 237–243.

Vandenbergh PA. (1993). Lactic acid bacteria, their metabolic products and interference with microbial growth. *FEMS Microbiol Rev* **12**: 221–237.

Veivers PC, O'Brien RW, Slaytor M. (1982). Role of bacteria in maintaining the redox potential in the hindgut of termites and preventing entry of foreign bacteria. *J Insect Physiol* **28**: 947–951.

Wang R, Kaplan A, Guo L, Shi W, Lux R, He X. (2012). The influence of iron availability on human salivary microbial community composition. *Microb Ecol* **64**: 152–161.