HLA-B27 and Human β2-Microglobulin Affect the Gut Microbiota of Transgenic Rats

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Abstract

The HLA-B27 gene is a major risk factor for clinical diseases including ankylosing spondylitis, acute anterior uveitis, reactive arthritis, and psoriatic arthritis, but its mechanism of risk enhancement is not completely understood. The gut microbiome has recently been shown to influence several HLA-linked diseases. However, the role of HLA-B27 in shaping the gut microbiome has not been previously investigated. In this study, we characterize the differences in the gut microbiota mediated by the presence of the HLA-B27 gene. We identified differences in the cecal microbiota of Lewis rats transgenic for HLA-B27 and human β2-microglobulin (hβ2m), compared with wild-type Lewis rats, using biome representational in situ karyotyping (BRISK) and 16S rRNA gene sequencing. 16S sequencing revealed significant differences between transgenic animals and wild type animals by principal coordinates analysis. Further analysis of the data set revealed an increase in Prevotella spp. and a decrease in Rikenellaceae relative abundance in the transgenic animals compared to the wild type animals. By BRISK analysis, species-specific differences included an increase in Bacteroides vulgatus abundance in HLA-B27/ hβ2m and hβ2m compared to wild type rats. The finding that HLA-B27 is associated with altered cecal microbiota has not been shown before and can potentially provide a better understanding of the clinical diseases associated with this gene.

Introduction

The role of the gut microbiota in diseased and healthy states has become increasingly apparent, as evidenced by its importance in a number of conditions including metabolic syndrome, type 1 diabetes, multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease [1–7]. Many of these diseases are also influenced by the genes of the major histocompatibility complex (MHC), otherwise known as human leukocyte antigens (HLA) in humans. In many conditions, the HLA alleles confer greater disease risk than other genetic factors identified by genome wide association studies [8]. Ankylosing spondylitis (AS), a potentially disabling condition that results in axial and sometimes peripheral arthritis is highly associated with an MHC class I molecule, HLA-B27, with ~ 90% of patients with AS carrying the HLA-B27 allele. The mechanism by which HLA-B27 predisposes to disease is not well understood, although several hypotheses have been postulated [9–11]. Our study tests the hypothesis that HLA-B27 alters the repertoire of the gut microbiome [11], which may be one mechanism by which it predisposes to disease.

Studies in both humans and rodents support this hypothesis [12]. Bacteria including Shigella, Salmonella, Yersinia, and Campylobacter are well-characterized triggers for HLA-B27-associated reactive arthritis [13–16]. Cross reactivity between monoclonal antibodies to HLA B27 and Gram-negative bacteria...
has been reported [14,17,18]. There is also sequence homology between a nitrogenase from Klebsiella and HLA-B27, although the significance of this finding has been debated [13,19,20]. Raising HLA-B27 transgenic rats in a germ-free environment prevented gut and joint inflammation that otherwise spontaneously occurs in these rats [21]. Subsequent studies showed that mono-association of HLA-B27 transgenic rats with Bacteroides vulgatus in a germ-free environment was sufficient to re-establish colitis in association of HLA-B27 transgenic rats with 
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It is plausible that a dysregulated immune response in HLA-B27 
disease-prone HLA-B27 transgenic rats exhibit a variety of 
dendritic cells abnormalities [30,31] including disrupted trafficking 
of dendritic cells from the gut to the mesenteric lymph nodes [32]. 
There are various types of animal models of HLA-B27 disease. 
The HLA-B27 transgenic rats on a Lewis background created by 
crossing the 21–3 and 283–2 strains express high copy numbers of 
the HLA-B27 subtype B27:05 and human beta 2 microglobulin 
(hβ2m) and have a high penetrance and severity of arthritis in the 
absence of any gastrointestinal inflammation (Table 1) [33,34]. 
Whereas expression of either transgene alone does not cause 
disease, expression of both together causes a disease similar to 
human spondyloarthritis. This Lewis transgenic rat line (21– 
3x283–2) was chosen for the majority of our studies because 70% 
develop severe arthritis by 6 months, but the absence of colitis 
allowed us to avoid the confounding variable of gut inflammation 
contributing to the changes seen in the gut microbiota. Recent 
advances in both sequencing technology and our knowledge of the 
gut microbiome in various mammals including the rat have 
allowed us for the first time to characterize the differences in the 
gut microbiota associated with the expression of HLA-B27 and 
hβ2m.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the 
recommendations in the Guide for the Care and Use of 
Laboratory Animals of the National Institutes of Health (NIH). 
The protocols were approved by the Institutional Animal Care 
and Use Committee at the University of Texas Southwestern in 
Dallas, Texas, or the National Institute of Arthritis, Musculoskel-
etal and Skin Diseases (NIAMS) at the NIH.

Animals and sample preparation

The transgenic rat lines bred on a Lewis background expressing 
human HLA-B27 and hβ2m by crossing the 21–3x283–2 lines 
have been described previously [33]. Male 21–3 rats develop 
epididymo-orchitis, peripheral arthritis, and tail spondylitis 
[33,35]. None of the males of any of these genotypes develop 
inflammatory bowel disease [33]. Three main cohorts between 
67–70 days old were studied (Table 2): (1) 6 nontransgenic Lewis 
male rats co-housed with 7 HLA-B27/hβ2m rats (21–3x283–2 F1 
males), and 3 age-matched nontransgenic Lewis males housed 
separately; (2) 6 nontransgenic Lewis males co-housed with 6 age-
matched HLA-B27/hβ2m (21–3x283–3) F1 males; (3) 4 female 
and 4 male rats of three genotypes: HLA-B27/hβ2m (21–3x283– 
2 F1), hβ2m (283–2), and nontransgenic, all housed separately by 
genotype and gender. All of the above rats were produced and 
housed at the University of Texas Southwestern animal facility 
under specific pathogen free (spf) conditions. We also investigated 
a cohort of Lewis rats between 60–180 days old, produced and 
housed at the NIH in which 28 HLA-B7/ hβ2m rats (line 120–4) 
were compared to 26 wild type and 28 HLA-B27/hβ2m rats (line 
33–3) [36]. All of the 33–3 rats had evidence of colon and cecum 
inflammation histologically, although the younger animals (8 week 
old) were clinically normal. The HLA-B7 and wild type animals in 
this control cohort were phenotypically and histologically normal 
(Tables 1 and 2).

All specimens from cohorts 1–3 were collected at 67–70 days in 
age, prior to the onset of arthritis. At this age, rats are expected to 
have an adult repertoire of gut microbiota, but will not develop 
arthritis for at least another 40 days, so that we could distinguish 
genotypic effects from phenotypic effects. Whole cecum samples 
were collected from the first cohort, and cecal luminal contents 
and cecal mucosal specimens were separately collected from the 
other two cohorts as well as the control cohort. Following 
euthanasia, the cecum was removed under sterile conditions and 
either the intact cecum (including contents) or the cecal mucosa 
and cecal contents separately were snap frozen in sterile microfuge 
tubes. Genomic DNA was then extracted from the samples using a 
Qiagen DNAeasy kit and then sequenced using biome represen-

| Table 1. HLA-B27/hβ2m transgenic rat lines. |
|--------------------------------------------|
| Line | Transgene locus zygosy | Transgene scopies |
|------|------------------------|-------------------|
|      |                        | HLA-B27 | hβ2m | HLA-B7 |
| 21–3 | hemi-                  | 20      | 15   |       |
|      | homo-                  | 40      | 30   |       |
| 283–2| hemi-                  | 0       | 35   |       |
|      | homo-                  | 0       | 70   |       |
| 21–3x283–2 | hemi- x hemi- | 20 | 50 |       |
| 33–3 | homo-                  | 55      | 66   |       |
| 120–4| homo-                  | -       | 10   | 52    |

Adapted from [35].

doi:10.1371/journal.pone.0105684.t001
A portion of the 16S rRNA gene was amplified using the 515–806 primers as specified by the EMP (http://www.earthmicrobiome.org/) and sequenced on the Illumina MiSeq platform [38–42]. Data were quality-filtered using QIIME (Quantitative Insights Into Microbial Ecology) [40]. QIIME was used for alignment using Infernal [43], a stochastic context-free grammar aligner, clustering of sequences into operational taxonomic units using uclust [44], phylogenetic reconstruction using the reference + de novo protocol (given the high incidence of likely new clusters), and taxonomy assignment with the RDP classifier [45]. Alpha and beta diversity analyses and visualizations were also performed [46–48]. Statistical significance was assessed using parametric and nonparametric approaches including false discovery rate (FDR) corrections by the Benjamini-Hochberg method.

### 16S rRNA gene amplification, taxonomic and diversity analysis

A portion of the 16S rRNA gene was amplified using the 515–806 primers as specified by the EMP (http://www.earthmicrobiome.org/) and sequenced on the Illumina MiSeq platform [38–42]. Data were quality-filtered using QIIME (Quantitative Insights Into Microbial Ecology) [40]. QIIME was used for alignment using Infernal [43], a stochastic context-free grammar aligner, clustering of sequences into operational taxonomic units using uclust [44], phylogenetic reconstruction using the reference + de novo protocol (given the high incidence of likely new clusters), and taxonomy assignment with the RDP classifier [45]. Alpha and beta diversity analyses and visualizations were also performed [46–48]. Statistical significance was assessed using parametric and nonparametric approaches including false discovery rate (FDR) corrections by the Benjamini-Hochberg method.

### Quantitative and semi-quantitative PCR

Confirmation of bacterial species differences between the transgenic and wild type animals was obtained using both traditional PCR and then quantified by real time PCR (RT-PCR) using the ΔΔCt relative quantification method as described previously using the 16S rRNA gene as a reference gene [49]. Primer sequences, annealing temperatures, target genes, and product sizes are listed in Table S1.

### Results

#### Expression of the HLA-B27 transgene is associated with alterations in the gut microbiota by 16S rRNA gene sequencing

Using 16S rRNA amplicon sequencing, there were statistically significant differences in bacterial sequences between transgenic and wild-type animals in both cecal lumen and cecal mucosa samples shown by principal coordinates analysis using UniFrac distances [50] (Figure 1a, 1b), with p-values shown in Table 3 by PERMANOVA analysis. While phylum-level differences were not statistically significant (Figure 1c, 1d), genus-level differences between genotypes were more easily distinguishable (Figure 2). Whereas the more abundant bacterial genera (such as *Clostridia* and *Helicobacter*) did not differ between transgenic and wild type animals, some of the rarer genera differed markedly. In both cecal lumen and cecal mucosa samples, an unknown genus of the Rikenellaceae family (dark blue bars) was significantly more abundant in wild type animals than either HLA-B27/β2m or β2m animals (p = 0.005 and 0.002, respectively after FDR correction). On the other hand, *Paraprevotella* was higher in transgenic (HLA-B27/β2m or β2m) animals compared to the wild type animals (p = 0.03 in both tissue sites after FDR correction).

In the 3rd cohort of animals, whereas the represented bacterial sequences obtained from the HLA-B27/β2m animals from combined cecal lumen and mucosa samples segregated significantly from the wild type animals (pseudo-F 6.6645, p = 0.001) on principal coordinates analysis, there was a much smaller difference between the HLA-B27/β2m rats and rats that only expressed β2m (pseudo-F 1.4688, p = 0.039) (Figure 3). Table 3 also illustrates that co-housing abrogated some of the differences seen between transgenic animals and wild type animals (cohort 2). This might be expected because rodents are coprophagic. However, in whole cecum preparations from the first cohort of animals, there remained significant differences in the UniFrac distances between the gut microbiota of wild type and HLA-B27/β2m transgenic animals despite co-housing (p = 0.006) (Table 3). HLA-B7, like HLA-B27, is also an MHC I allele, but differs in that it is not associated with arthritis susceptibility and thus serves as an MHC class I control. Significant differences in the gut microbiota were demonstrated in HLA-B7/β2m compared with both wild type controls and HLA-B27/β2m rats (pseudo-F 4.6750, p = 0.001 by permanova for cecal lumen and pseudo-F 5.6001, p = 0.001 by permanova for cecal mucosa). Figure S1 demonstrates the

### Table 2. Rat cohorts used in experiments.

| Cohort     | Housing            | Gender/Genotype | Samples obtained |
|------------|--------------------|-----------------|-----------------|
| 1          | separately housed  | Male/6 WT, 7 (21–3x283–2)F1 | whole cecum     |
| 2          | co-housed          | Male/6 WT, 6 (21–3x283–2)F1 | cecal lumen, cecal mucosa |
| 3          | separately housed by genotype | Male/4 WT, 4 (21–3x283–2)F1, 4 (283–2) | cecal lumen, cecal mucosa |
| Control cohort | Depends on age of cohort | Male/15 WT, 15 (33–3), 15 (120–4) | cecal lumen, cecal mucosa |
|            | Female/11 WT, 13 (33–3), 13 (120–4) |                |                 |

WT: wild type; see Table 1 for genotype designations.

i doi:10.1371/journal.pone.0105684.0002

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| 2      | co-housed          | Male/6 WT, 6 (21–3x283–2)F1 | cecal lumen, cecal mucosa |
| 3      | separately housed by genotype | Male/4 WT, 4 (21–3x283–2)F1, 4 (283–2) | cecal lumen, cecal mucosa |
| Control cohort | Depends on age of cohort | Male/15 WT, 15 (33–3), 15 (120–4) | cecal lumen, cecal mucosa |
|         | Female/11 WT, 13 (33–3), 13 (120–4) | | |
separation of 16s sequences by genotype using principle coordinates analysis and table S2 shows the p values for genotype group comparisons in this control cohort.

Expression of the HLA-B27 transgene is associated with bacterial species-level alterations in the gut microbiota by BRISK

BRISK is a technique that allows for massively parallel deep sequencing of the gut microbiome generated by a Type IIB restriction endonuclease. This provides a representation of the specific microorganisms present in a sample on a species level, as well as the abundance of these organisms. In a typical sample, we were able to amplify about 4 million tag, with ~21% of the DNA identified as from the host animal and representing all chromosomes in the rat genome. Across all samples we identified more than 30,000 tags derived from known bacteria. In total, tags from 909 species were identified in at least one animal. The heat map (Figure 4) shows the relative abundance of specific bacterial...
species from whole cecum preparations of transgenic rats compared to wild type control animals from the first cohort (normalized to amount of host rat DNA), demonstrating an overall difference in the abundance of certain bacterial species. The color code represents the relative abundance of specific bacterial species with each row showing an individual species. Figure 4b quantifies differences between HLA-B27/hb2m transgenic animals and littermate wild type control animals (co-housed animals). Approximately 30 of these showed significant differences in HLA-B27/β2m transgenic animals compared to wild type animals as determined by iterative Monte Carlo analysis of the dataset. Three of the main differences included alterations in the abundance of Faecalibacterium prausnitzii, Bacteroides vulgatus, and Akkermansia muciniphila.

**Bacteroides vulgatus** is more abundant in HLA-B27 transgenic animals than non-co-housed wild type animals

We next attempted to confirm HLA-B27-dependent differences in colonization of these three bacterial species using species-specific real time quantitative PCR. Normalization was performed using 16S rRNA gene PCR. The differences in F. prausnitzii could not be confirmed by qPCR. However, we found a marked reduction in Akkermansia muciniphila and an increase in
Bacteroides vulgatus prevalence in the transgenic HLA-B27/hb2m animals compared to the wild type rats housed alone (Figure 5). However, the differences were reduced (with levels of these bacteria in wild-type mice comparable to transgenic) when wild type animals were co-housed with the transgenic animals, presumably due to coprophagia. Although there were significant differences in Bacteroides vulgatus by BRISK in both co-housed and non-co-housed animals, when confirmatory PCR was performed, a difference was only demonstrated between non-co-housed animals. In cohort 3, Akkermansia muciniphila was not present in co-housed and non-co-housed rat cecal samples (neither cecal mucosa nor cecal lumen), suggesting that this species may have colonized this specific group of wild type animals only.

Discussion

To our knowledge this is the first study that shows that expression of the human transgene HLA-B27 (with hb2m) is associated with alterations in the gut microbiota. The purpose of this study was to establish the difference seen in the gut microbiota due to HLA-B27, but whether or not specific changes are causative of disease, a result of disease, or unrelated to disease has not yet been established and will require further investigations. Germ-free studies in the 33–3 and 21–4 lines strongly suggest that gut microbiota play an important role in the disease state in this animal model [21–23]. The effect of the germ free state on arthritis in the (21–3×283–2) F1 rats which do not develop colitis remains to be studied.

The rat age in our study (in the first 3 cohorts) was specifically chosen to precede arthritis onset so that the genotype effect could be delineated from the disease effect. At 67–70 days, [21–3×283–2] F1 males show only very localized inflammation in the male genital tract, but no gastrointestinal inflammation or joint inflammation [35]. It is possible that HLA-B27 animals have a distinct T cell selection repertoire resulting in bacterial peptide presentation in a way that affects which microbes are allowed to thrive and/or are eliminated. However, studies that cross a CD8−/− background to the Lewis 33–3 rat have shown no effect on
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disease prevalence or severity, thus diminishing the validity of this theory [51]. While there is no evidence that the 21–3×203–21 animals used in this study have gut inflammation both historically and by lack of increased cytokine production, the 33–3 rats do have subclinical inflammation from an early age (8 weeks old). Sartor and colleagues have shown that gut inflammation in the Fischer HLA-B27 33–3 transgenic rats which develop clinically evident inflammatory colitis alters the transcriptional profile of Bacteroides thetaiotaomicron, which is thought potentially to be a causative organism in colitis, and may result in an augmented adaptive immune response to bacterial antigen [52]. Analogously to the human disease, ankylosing spondylitis, in which 50–60% of patients without GI symptoms have microscopic gut inflammation on histological analyses of biopsy specimens from routine colonoscopy, it is possible that transgenic rats have subclinical gut inflammation that causes changes in the gut microbiota, or alternatively, that transgene expression directly changes the members of the gut microbiota which results in alterations in immunity and/or gut permeability.

We have demonstrated by species-specific sequencing and qPCR confirmation that Bacteroides vulgatus is overabundant in the HLA-B27 rat cecum. This organism was found in monoassociation studies in germ-free HLA-B27 transgenic Fischer rats to induce colitis [23,53]. While the Lewis transgenic rats used in the first 3 cohorts of our study do not develop colitis, it is possible that B. vulgatus is pathogenic in the development of arthritis. Testing this hypothesis would require longitudinal correlation of the presence of B. vulgatus with arthritis disease score severity, and/or monoassociation of germ-free rats with B. vulgatus to induce arthritis. It is also possible that the overabundance of B. vulgatus only reflects more global changes in the microbiota and is not directly related to disease.

We also found that Paraprevotella was more abundant in both transgenic animals (HLA-B27/h2m and B2m) by 16s rRNA gene sequencing compared with wild type animals. Prevotella species, which are related to Paraprevotella have been implicated in the pathogenesis of rheumatoid arthritis in humans [54].

While the components of gut microbiota in different groups of animals can differ depending on the specific micro-environment, as shown by the failure to confirm loss of abundance of Akkermansia in our repeat experiments, an overall consistent trend was observed that transgenic animals had different microbiota compared to wild type animals, even in the absence of gut inflammation. Given that individual bacterial species might be inconsistent between groups of transgenic animals, the HLA-B27 disease association might rather be due to broader differences in the metabolic profiles of the bacterial species present, and thus, it is possible that disease and altered immunity may result from the overall milieu of bacterial metabolites interacting with the host rather than an alteration of individual bacterial species.

The transgenic animals that only expressed hB2m without HLA-B27 exhibited microbiota differences that were more similar to HLA-B27-expressing animals than wild type animals despite being separately housed, although there were some microbiota differences unique to HLA-B27. The hB2m transgenic animals remain completely healthy in this cohort, while all of HLA-B27/hB2m rats develop epidymoorchitis and a majority develop arthritis. One potential explanation for these results could be that the changes in microbiota are at least in part due to the expression of hB2m rather than HLA-B27, but these alterations are not pathogenic unless HLA-B27 is also present, causing disease either by effecting additional changes in the microbiome, and/or by contributing its effect(s) on the immune response. Another potential explanation is that human B2m may form a functional complex with rat MHC class I heavy chains, provoking a change in the microbiota that by itself is insufficient to cause disease. There is evidence that overexpression of human HLA-DR*0401 and* 0402, HLA-DR4 subtypes that cause mice to be susceptible or resistant to arthritis, respectively, results in genotype-specific alterations in the gut microbiota [53]. We also show in this study that another MHC I molecule, HLA-B7, can be associated with changes in the gut microbiota differentially from both wild type and HLA-B27 animals. Overall, our results support the concept that changes in MHC expression influence the composition of the gut microbiota. Effects of HLA-B27 that cause disease could turn out to be an unusual specific case within this general principle.

One potential limitation to our study is that random drift, as well as seasonal changes, can contribute to differences detected in the gut microbiome. However, because we compared the microbiota between genotypic groups harvested at the same time, this should not be a large factor in the outcome of our analyses. For instance, within cohort 1, we harvested all genotypes at the same time (as within cohort 2 and 3). Since we have not attempted to pool the three cohorts (which were indeed euthanized at different times) for analyses, random drift should be much less of a factor. It should also be noted that B27+/hB2m+ rats were littermates of B27-/ hB2m+ rats in that particular cohort (cohort 3), so these two groups should have the same founder microbiome and differences seen between these two genotypes would argue against drift being the main factor. In terms of differences due to different founder microbiomes, this is theoretically a problem with the wild type rats even though husbandry was similar for all rats at a particular time. While this does not necessarily mitigate the founder effect, we have at least demonstrated that in co-housed rats some genotypic differences exist.

Sequencing techniques have advanced significantly in the last decade to allow for more rapid and in-depth methods of identifying, in a culture-independent fashion, the members of a complex microbial community such as the gut. It is important to note that there are strengths and limitations to the two methods used here. For instance, while the 16s rRNA gene sequencing method can provide better diversity comparisons between two groups in a cohort using principal coordinates analysis, detailed species-specific information was not readily available with the taxa we used in our analysis. It is sensitive to dramatic changes in gut microbiota such as occur with overt clinical gut inflammation, but may fail to find identify potentially crucial differences on a species level. On the other hand, while the BRISK technique is able to identify species-level differences, its sensitivity is limited by identification of specific tags within the database. While 16S sequences are known for many bacteria, BRISK can generally only identify organisms for which full genome sequences are available. Potentially pathogenic organisms may be among the species that have not been identified or described. Despite these limitations, we have identified substantial microbial biodiversity differences in the gut between wild type and transgenic rats. These findings can potentially direct us to better understand immune-mediated diseases associated with HLA-B27.

Supporting Information
Figure S1 16S rRNA gene sequencing principal coordinates analysis demonstrates significant differences between HLA type in Lewis rats between 2–6 months of age from A, cecal lumen and B, cecal mucosa. B27/B2M: HLA-B27/human β2-microglobulin; B7/B2M: HLA-B7/human β2-microglobulin. Cecal mucosal and luminal contents from rats in various cohorts were collected using sterile swabs and frozen in

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sterile 15 ml centrifuge tubes. Samples were shipped to OHSU in dry ice, where genomic DNA was extracted using Qiagen DNAeasy kit and sequenced using 16S rDNA gene sequencing. Control cohorts were handled as follows: 2 month old rats: Weaned at 21 days after birth and singly housed until 2 months of age and sacrificed. 3–4 month old rats: Weaned at 21 days and cohoused with litter mates (random transgenic and WT) usually 2–3 rats per cage until almost 3 months of age when they were sacrificed. 6 month old rats: Weaned at 21 days and cohoused with litter mates (random transgenic and WT) usually 2–3 rats per cage until almost 3 months of age and then singly housed. Some of these animals were cohoused for mating for a few weeks intermittently.

**Table S1 Primers used for quantitative PCR.**

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**Table S2 Bonferroni corrected p values corresponding to Figure S1 demonstrating differences seen in control cohort Lewis rats including HLA-B7 transgenic rats.**

**Acknowledgments**

We would like to thank the following people for their help and insight in the preparation of the data and statistical analysis: Christina Mete, Dongseok Choi, Stuart Gardiner, Steve Planck, and Daniel McDonald.

**Author Contributions**

Conceived and designed the experiments: PL MA MB JDT RK RVG RAC TGR JTR. Performed the experiments: PL MA MB AYL LA PS SD YP MD CLL TG GA YVBB. Analyzed the data: PL MB MA AYL CLL TG YVBB. Contributed reagents/materials/analysis tools: AYL EDC MD JDT RK CLL YVBB. Contributed to the writing of the manuscript: PL MB MA RK RVG RAC TGR JTR JWD.
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