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# Returning to the Wild: Restoring Natural Levels of Immune Function to Laboratory Animals

**Daniel Ren[1], Dylan Beinart[1], Cindy Pi[1], Susan Poulton[1], Zoie E. Holzknecht[1], Chelsea Swanson[1] and William Parker[1*]**

*[1]Department of Surgery, Duke University Medical Center, Box 2605, Durham, NC 27710, USA.*

***Authors' contributions***

*This work was carried out in collaboration between all authors. Authors WP, ZEH and SP designed the study and wrote the protocol. Author DB performed the statistical analysis and wrote the first draft of the manuscript. Authors DR, CP, SP and ZEH managed the experimental procedures of the study and helped draft the manuscript. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** The loss of biodiversity from the human body, caused by cultural factors in Western society such as widespread use of sewer systems and water treatment facilities, has resulted in a propensity for aberrant immune function, leading to pandemics of allergy, autoimmunity, and other inflammation-related disorders. One approach to combating this problem is through artificial enrichment of the human biota with microbes or other organisms such as helminths. In this study, the extent to which immune function found in wild caught rats could be recapitulated by enriching the biota of laboratory rats was examined.

**Place and Duration of Study:** Department of Surgery, Duke University Medical Center, from June 2009 to present.

**Methodology:** Natural antibody levels against autologous antigen extracts were used as a quantifiable and reproducible marker for immune function, and laboratory rats were immunized to boost their natural antibody levels. Co-housing with wild rodents and colonization with helminths were used as tools for biota enrichment.

**Results:** Three groups of rats with varying levels of biota composition were evaluated; wild rats

**Corresponding author: E-mail: William.Parker@duke.edu;*

(n=8), biota-enriched laboratory rats (n=15) and biota-depleted laboratory rats (n=20). Laboratory rats had approximately 33% of natural IgG and IgM levels compared to wild rodents. Biota enrichment in laboratory animals enhanced levels of natural IgG and IgM to similar extents. Immunization boosted the level to 47%, and co-housing with wild rodents coupled with exposure to helminths boosted the level further to within 72% of levels found in wild rodents.

**Conclusion:** This study indicates that artificial biota enrichment in laboratory animals can significantly enhance immune function that is known to be important for cancer surveillance, wound healing, and a variety of other immune functions.

*Keywords: Biota enrichment; natural antibodies; immune function; rat; wild.*

## 1. INTRODUCTION

The pathogens, parasites, mutualists, and commensal organisms that inhabit the human body are collectively known as the human biota. The agricultural revolution of 10,000 years ago led to a profound increase in population density and concomitant increases in crowd infections. The subsequent industrialisation of human society over the last 200 years greatly reduced the biodiversity of the human biota. Modern sanitation practices have eradicated several disease-causing pathogens that became common after the agricultural revolution, but they have also reduced the population of commensal organisms, which co-evolved with the human immune system over millions of years. There is overwhelming evidence that this “biota depletion” is responsible in part for pandemics of autoimmune and allergic disease in western society [1]. Clinical and laboratory evidence has identified the specific loss of helminths from the human biota as one probable causative factor [2,3]. Current research is focussing on treating autoimmune and allergic diseases by repopulating the biota, a process known as biota-enrichment. Biota-enrichment has already been shown to be effective in treating inflammatory bowel disease [4], multiple sclerosis [5] and experimental models of colitis [6], allergy [7] and type 1 diabetes [8].

The mounting evidence supporting the role of biota depletion in human disease has led to the search for animal models that suitably replicate the changes in immune function associated with biota depletion. Previous studies have found success in comparing the immune systems of wild and laboratory rodents. Devalapalli et al. [9] showed that wild rats have higher levels of ‘natural antibodies’ compared to laboratory rats. Natural antibodies are germ line encoded immunoglobulin molecules produced by cells of innate immune system, in the absence of previous overt exposure [10]. They are important for pathogen clearance as well as cancer immunosurveillance [11]. From an experimental standpoint, natural antibody levels are significant in studies of biota-enrichment because they provide a measurable marker of biota-associated immune activation.

Despite the success of this wild rat model, experiments involving wild animals have inherent flaws stemming from the uncontrolled nature of their environment and the inability to capture and handle them without inducing stress. Additionally, wild animals lack the genetic uniformity of inbred laboratory animals. The aims of the present experiment were (a) to utilize artificial biota-enrichment to mimic the immune stimulation encountered by wild rats, and (b) to evaluate the effectiveness of that enrichment on development of natural immune function. Immune function in wild rats was used as a benchmark for comparison, although it is realized that this benchmark cannot be considered a measure of either “optimal” or maximum immune function for laboratory rodents. Nevertheless, immune function in a cohort of wild caught rodents does serve as an interesting benchmark for comparison with laboratory rodents with various levels of immune stimulation.

Natural antibody levels against autologous antigen extracts were used as a quantifiable and reproducible marker for immune function. The use of markers in banked serum samples is especially useful in assessing immune function in wild animals, allowing for months of sample collection prior to analysis. Previous experiments from our laboratory have found that immunization increases the natural antibody repertoire in laboratory rats [12]. In order to further boost the natural antibody repertoire, a proportion of laboratory rats were immunized, as previously described [12,13], while a separate group of laboratory rats were both immunized and underwent biota-enrichment. The biota-enrichment process included parental exposure

to bedding from wild rats, shared housing with wild rats, and parental colonization with the helminth *Hymenolepis diminuta.* Western blotting was then used to compare the natural antibody repertoire between wild and laboratory rats with and without biota depletion.

## 2. METHODS

### 2.1 Experimental Design

All animal housing and procedures for both laboratory and wild rats were approved by the Duke University Medical Center Institutional Animal Care and Use committee. To probe the effect of biota-enrichment on the immune system of laboratory rats, three groups of rats with varying levels of biota composition were evaluated; wild rats (n=8), biota-enriched laboratory rats (n=15) and biota-depleted laboratory rats (n=20). Additionally, both groups of laboratory rats received an immunization cocktail consisting of peanut extract, fluorescin isothiocyanate labelled keyhole limpet hemocyanin (FITC-KLH) and 2,4 dinitrophenyl conjugated to AminoEthylCarboxyMethyl-FICOLL (DNP-Ficoll, Biosearch Technologies Inc. Novato, CA USA). This mixture of antigens provides broad immunogenic stimulation. The peanut extract promotes an IgE response whilst FITC-KLH and DNP-Ficoll provide T-cell-dependent and T-cell-independent stimulation, respectively. All rats were euthanized by C02 inhalation. Blood was collected from the posterior vena cava and centrifuged, after which sera were stored at -80℃ until use. Once all sera were collected, Western blots were used to quantify the binding of natural antibodies to a set of autologous antigens extracted from 6 different organs. The differences in the natural antibody repertoire between wild rats and laboratory rats with and without biota depletion were taken as representative of biota-associated differences in immune function.

### 2.2 Wild Rats

Live traps were used to catch over 100 wild rats in various urban, suburban, and rural areas in North Carolina. All rats were euthanized immediately, and blood was collected from the posterior vena cava. Subsequently, sera were obtained and stored at -80℃. From this collection of serum, 20 samples from female rats weighing > 250 g were selected for this study. In addition, four female wild rats were captured and selected for housing in a cage placed adjacent to cages housing laboratory rats. (See “Biota enriched laboratory rats”, below.)

### 2.3 Modified Laboratory Animal Housing

To accommodate experiments involving biota enrichment, rats were housed in modified cages consisting of a 40.6 cm high box, with floor dimensions of 61 cm by 35.5 cm. The sides and top of the cages were formed by 1.27 cm by 2.54 cm galvanized steel mesh, instead of the standard, solid plastic. This modification allowed free exchange between the animals and their external environment. A drop-in 7.62 cm deep plastic pan was used as the flooring. Although open air cages were only necessary for animals receiving biota-enrichment, all animals in the experiment (including biota-depleted animals) were housed in these cages to eliminate housing as a potentially confounding variable.

### 2.4 Biota-depleted Laboratory Rats

Male (n=4) and female (n=8) Sprague Dawley rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). These rats were acclimatized in standard (hygienic) animal housing facilities at Duke University for 62 days. Once acclimatized, the rats were bred with the mating process yielding 31 female rats. This experiment exclusively utilized female rats to eliminate gender as a confounding variable. All F1 female rats were weighed at 4 days of age and again at 23 days, immediately prior to weaning. Once weaned, 20 female F1 rats were randomly selected for immunization according to the experimental protocol.

### 2.5 Biota Enriched Laboratory Rats

For the biota-enriched rats, male (n=4) and female (n=8) Sprague Dawley rats were also purchased from Harlan Sprague Dawley but were introduced to a separate animal housing facility that did not contain any other laboratory rodents. This biota enrichment facility was controlled so that housing conditions were identical to those in the standard animal housing. These controls extended to temperature, lighting, cage construction, food and water. F0 rats were housed in this facility for 62 days prior to breeding. In this setting, the F0 rats were exposed to three measures of biota-enrichment as previously described [12,13]. The protocol summary is as follows: Wild rats were captured and introduced to laboratory housing prior to arrival of F0 rats. Upon arrival, F0 rats were housed next to the wild rats, and used bedding

from the wild rat cages was introduced weekly into the cages housing the F0 laboratory rats. Additionally, the F0 rats were exposed to bedding from rats housed under non-standard conditions, which was obtained from a commercial pet supplier. In addition, 56 days prior to breeding, each female F0 rat was fed 3 *Hymenolepis diminuta* cysticercoids in saline.

Of the eight F0 females and four F0 males that entered the biota enrichment facility, 1 male and 2 females were euthanized due to apparent respiratory distress and weight loss. The remaining 3 males and 6 females were bred 62 days after arrival. A further 3 females were not successful breeders. It was later determined that removing the commercial bedding improved the breeding rate. This suggests that undetermined factors related to the commercial bedding, possibly scents from older males, may inhibit the breeding process in laboratory rats.

Out of the 3 females that became pregnant, only 2 females had helminth colonization confirmed by a modified version of the McMaster technique. This confirmation was conducted three weeks after helminth administration, and was repeated 3 times. It is not clear why colonization was not successful in 1 of these female rats, since *Hymenolepis diminuta* readily colonizes laboratory rats and will survive as long as the animal lives [14].

Fifteen F1 females were born to the 3 pregnant F0 females in the biota enrichment facility. These F1 females were exposed to bedding from wild and commercial rats up until weaning at 23 days of age. Once weaned, they remained in the biota enrichment facility and continued to be housed next to wild rats. All 15 of the F1 females in the biota-enrichment facility received immunization according to the experimental protocol.

The F1 laboratory rats were not intentionally colonized with helminths because the life cycle of *Hymenolepis diminuta* requires an intermediate insect host for transmission. This means that *Hymenolepis diminuta* is likely not important to the mammalian biota prior to weaning, before the initiation of foraging. However, the F1 animals may have acquired the helminths naturally after weaning, although this possibility was not assessed.

## 2.6 Immunization Protocol

Rats were immunized with a cocktail of peanut extract, DNP-Ficoll, and RITC-KLH as previously described in detail [12,13]. For this procedure, twenty rats from the standard animal facility and 15 rats from the biota enriched facility each received an immunization protocol consisting of 6 intraperitoneal injections over 14 days. Rats were weighed and given the first injection at 43 days of age, designated day 0. This injection contained 1 mL/kg of the immunization cocktail and 1 mL/kg of Inject Alum (Pierce, Rockford, IL, USA). Using the day 0 weight for dose calculations, the rats were given a 2 mg/kg intraperitoneal injection of peanut extract on days 2, 4, 7 and 9. The immunization protocol concluded on day 14 with a final injection of 1 ml/kg of the immunization cocktail, without the Inject Alum. All laboratory rats were euthanized 28 days after the first injection, at 71 days old.

## 2.7 Immunoblotting

Tissue extracts were prepared as previously described [12]. Once collected, washed and homogenized, organ extracts were loaded onto PVDF membranes for immunoblotting, and membrane strips were prepared as previously described [12]. Sera was randomly selected 8 of the wild rats, 8 of the 20 biota-depleted laboratory rats and 8 of the 15 biota-enriched laboratory rats. Membrane strips were incubated overnight at 4°C with specific rat sera diluted 1/400 in blocking buffer. A control strip was incubated overnight in blocking buffer to account for anti-IgG and anti-IgM conjugate binding directly to organ-derived antigens. Strips were then washed 3 times for 10 minutes each with Tris buffered saline and incubated for 1 hour at room temperature in alkaline-phosphatase conjugated, affinity purified goat antibody diluted at 1/1000 in blocking buffer. As described previously [12], goat antibodies with appropriate specificity were used to detect specific isotypes, and 1-Step™ NBT-BCIP (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate; Thermo Scientific) was used to develop the strips.

## 2.8 Immunoblot Analysis

Immunoblots were scanned and quantified as previously described [12]. Quantity One Software v. 4.6.6 (Bio-Rad Laboratories) was utilized to quantify the amount of natural antibody binding to antigens on each strip. As described previously [12], the image intensity for each blot was manually adjusted, the noise filtered, and natural antibody binding quantified by several measures. The size of a single band was calculated by plotting a curve of the average

intensity of all pixels in each row of the band against the band length. The area under this curve was taken as the size (intensity × $mm^2$) of that band, and the size of the bands on the control strip were subtracted from the corresponding bands on all other strips. The *mean* band size was calculated as the mean of all band sizes in a single strip. The number of bands detected was taken as a measure of the number of antigens recognized by the natural antibody repertoire, and total natural antibody binding for a particular organ extract was taken as the sum intensity of all bands. Both total binding and band size were normalised to the mean of the biota-depleted group. All calculations were performed for IgM and IgG antibody isotypes.

### 2.9 Statistical Analysis

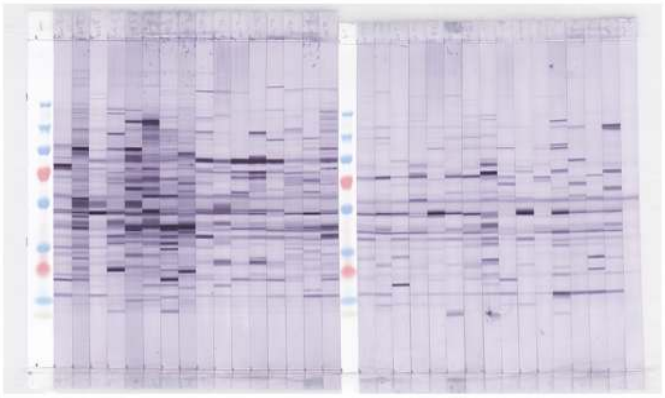
The natural antibody binding characteristics of the sera from wild rats and laboratory rats with and without biota-enrichments were compared with a 1-way ANOVA and Tukey's post hoc tests adjusting for multiple comparisons. All calculations were performed with Graphpad Prism Software (Graphpad Software, La Jolla CA).

## 3. RESULTS

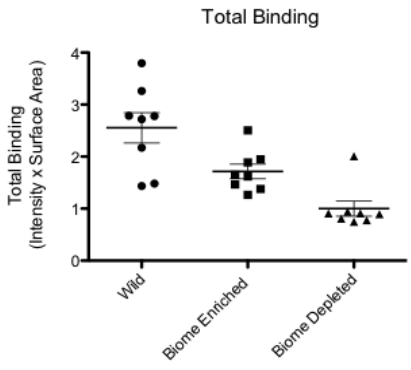
### 3.1 Average Number of Antigens Recognized by Natural Antibodies from Wild Rats and from Laboratory Rats

Western Blots were used to determine the number of antigens recognized by natural antibodies from the sera of wild rats and of laboratory rats with and without biota enrichment (For example, see Fig. 1). The number of antigens recognized was taken as an indicator of the range of the natural antibody repertoire. As described in the Methods, a 1-way ANOVA with Tukey post-hoc testing was used to compare the mean number of bands produced by natural IgM and IgG from each group.

An analysis using ANOVA indicates that, with the exception of kidney antigens, there were



**a)**

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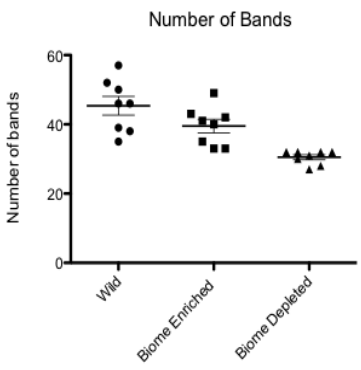


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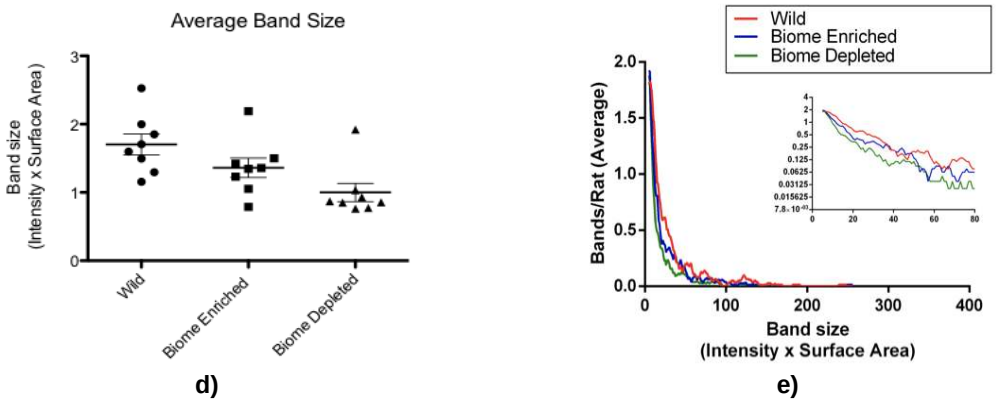
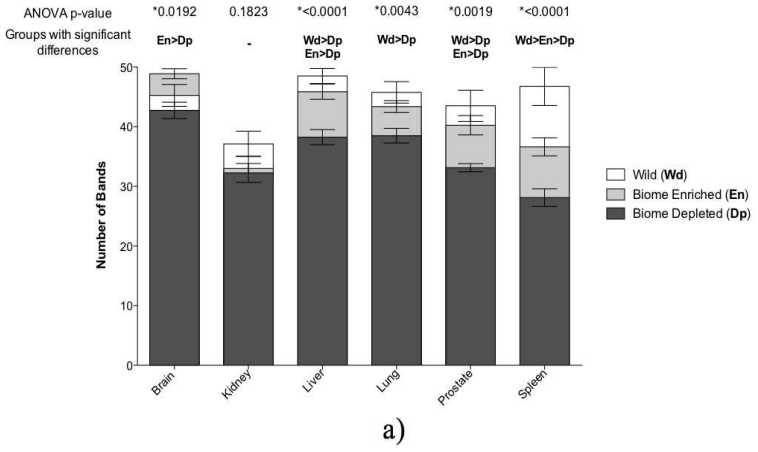



**Fig. 1. Binding of natural IgG from wild rats, biota-enriched rats and biota-depleted laboratory rats to brain-derived antigens as assessed by Western blot**

*The actual blot is shown in panel A. The assay utilized sera from 24 rats, 8 from each category of biota constitution. From left to right: lane 1 is a control, lanes 2-9 used sera from wild rats, lanes 10-17 used sera from biota-enriched laboratory rats, lane 18 is a control, lane 19-26 used sera from biota-depleted laboratory rats, lanes 27-34 used sera from rats not included in this experiment. Quantification of (B) total binding, (C) the number of bands and (D) the average band size produced by natural IgG from wild rats and laboratory rats with and without biota enrichment is also shown. Panel E shows the distribution of band sizes produced by natural IgG from wild rats, biota-enriched laboratory rats and biota-depleted laboratory rats*

significant differences in the number of antigens in all organ extracts recognized by natural IgM from the sera of wild rats and of laboratory rats with and without biota enrichment. The differences were particularly notable with antigens from the liver and the spleen (Fig. 2). The natural IgM from wild rats recognized the largest mean number of antigens in all organ extracts except the brain, whilst the natural IgM from biota depleted rats recognized the smallest mean number of antigens in all organ extracts. On average, the natural IgM from wild rats recognized 8.67% more antigens per organ extract than the IgM from biota-enriched laboratory rats, and 16.8% more antigens per organ extract than the IgM from biota-depleted laboratory rats. However, post-hoc testing determined that the spleen was the only organ extract where there was a significant difference in the number of antigens recognized by natural IgM from wild and biota-enriched rats. In contrast, the difference in the number of antigens recognized by IgM from wild and biota-depleted rats was significant with antigens derived from the liver, lung, prostate and spleen. Comparing the two groups of laboratory mice, natural IgM from biota-enriched rats recognized significantly more antigens than IgM from biota-depleted rats in all organs extracts except the kidney (Fig. 2).

The differences in the range of natural antibody repertoire between the 3 groups were larger with



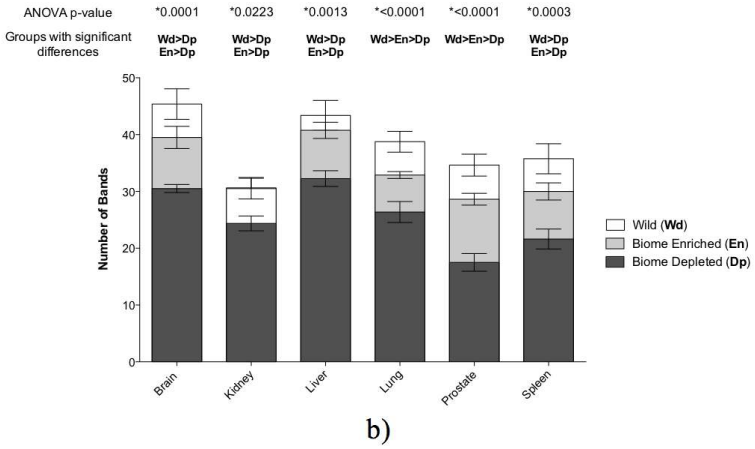



**Fig. 2. Number of antigens recognized by natural IgM (a) and IgG (b) from the sera of wild rats, biota-enriched laboratory rats and biota-depleted rats**

*The figure also contains ANOVA p-values and denotes which groups had significantly different means as assessed by Tukey post-hoc testing*

IgG compared to IgM. An analysis using ANOVA showed that there were significant differences in the number of antigens in all organ extracts recognized by natural IgG from wild rats and from laboratory rats with and without biota enrichment. On average, natural IgG from wild rats recognized 13.2% more antigens per organ than IgG from biota-enriched laboratory rats and 53.1% more antigens per organ than IgG from biota-depleted laboratory rats. Additionally, natural IgG from wild rats recognized significantly more lung and prostate antigens compared to IgG from biota-enriched rats. With every organ extract, the number of antigens recognized by the natural IgG from wild rats and biota-enriched laboratory rats were both significantly larger than the number of antigens recognized by IgG from biota-depleted laboratory rats.

### 3.2 Average Size of Bands Formed by Natural Antibodies from Wild Rats and Laboratory Rats with and without Biota-Enrichment

The size of a single band was calculated as described in the Methods. All values were then normalized to the average band size of the biota depleted laboratory rats.

Whilst the measurement technique does account for band length, pixel intensity is the primary factor that determines band size. Pixel intensity indicates the quantity of antibody bound to the antigen in that band. Therefore, average band size demonstrates the strength of natural antibody binding to antigens extracted from particular organs.

Based on analysis using 1-way ANOVA, the average band sizes produced by natural IgM from the sera of wild rats and from laboratory rats with and without biota depletion were significantly different with antigens extracted from the kidney, liver, prostate and spleen (Fig. 3). The average band sizes produced by IgM from wild rats and from biota-enriched laboratory rats were both significantly greater than the average band sizes produced by IgM from biota-depleted laboratory rats (for antigens from kidney, liver, prostate and spleen). The only significant difference in the average band size produced by IgM from wild rats and biota-enriched laboratory rats was observed when evaluating reactivity toward prostate antigens.

Natural IgM from wild rats produced the largest average band sizes with every organ extract. The average band size per organ extract produced by natural IgM from wild rats was 72.9% greater than the average band size produced by IgM from biota-depleted laboratory rats but only 16% greater than the average band size produced by IgM from biota-enriched laboratory rats.

With the exception of splenic antigens, the average band sizes produced by natural IgG from the sera of wild rats and from laboratory rats with and without biota depletion were significantly different with all organ extracts. The spleen was

also the only organ extract in which the IgG from wild rats did not produce the largest average band size. Similarly, IgG from biota-depleted laboratory rats produced the smallest average band size with antigens from every organ extract except those from the prostate.

Considering binding to all organ extracts, the natural IgG from the sera of wild rats produced average band sizes 40.3% and 57.6% greater than natural IgG from biota enriched laboratory rats and biota depleted laboratory rats respectively. As such, biota enrichment in laboratory rats did not produce as great a difference in the average band size produced by IgG binding compared to the differences seen with IgM binding. In post-hoc testing, no significant differences were observed between the average band sizes produced by IgG from biota-enriched and biota-depleted laboratory rats binding to antigens from any organ.

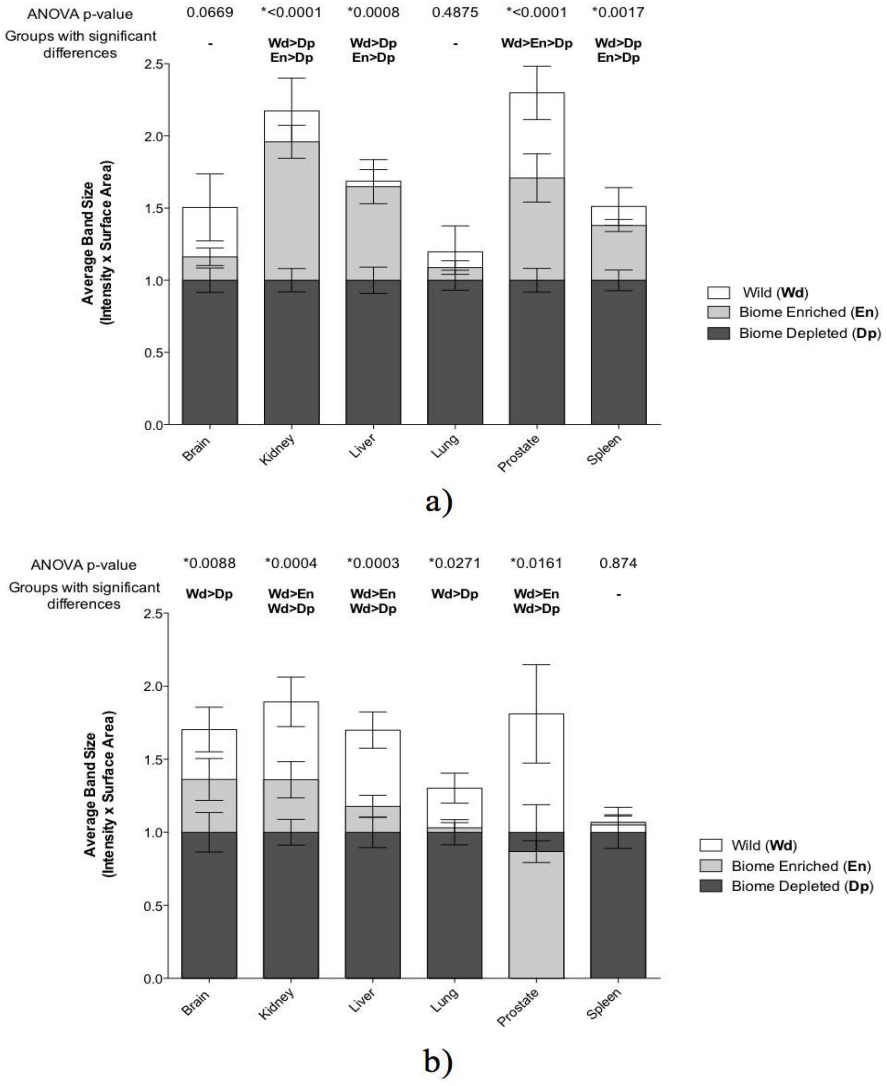



**Fig. 3. Average band size produced by natural IgM (a) and IgG (b) from the sera of wild rats, biota-enriched laboratory rats and biota-depleted laboratory rats binding to antigens extracted from different organs**

*The error bars show standard errors, and the p-values determined by ANOVA and significant differences as assessed by Tukey post-hoc testing are indicated*

### 3.3 Total Binding of Natural Antibodies from Wild Rats and from Laboratory Rats with and without Biota-Enrichment

Total binding refers to the sum of all band sizes produced by the binding of natural antibodies to antigens. Therefore, total binding takes into account the number of bands and the size of each band. This means that total binding demonstrates the range of the natural antibody repertoire as well as the intensity of natural antibody binding. The mean total binding for each group and each organ extract was calculated as described in the Methods. Values have been normalized to the means of the biota-depleted group. Natural antibodies from the sera of wild rats consistently displayed the greatest mean total binding regardless of organ extract or antibody isotype (Fig. 4). On the other hand, natural antibodies from biota-depleted laboratory rats showed the lowest mean total binding in all categories.

The analysis with ANOVA demonstrated that, with the exception of lung antigens, the mean

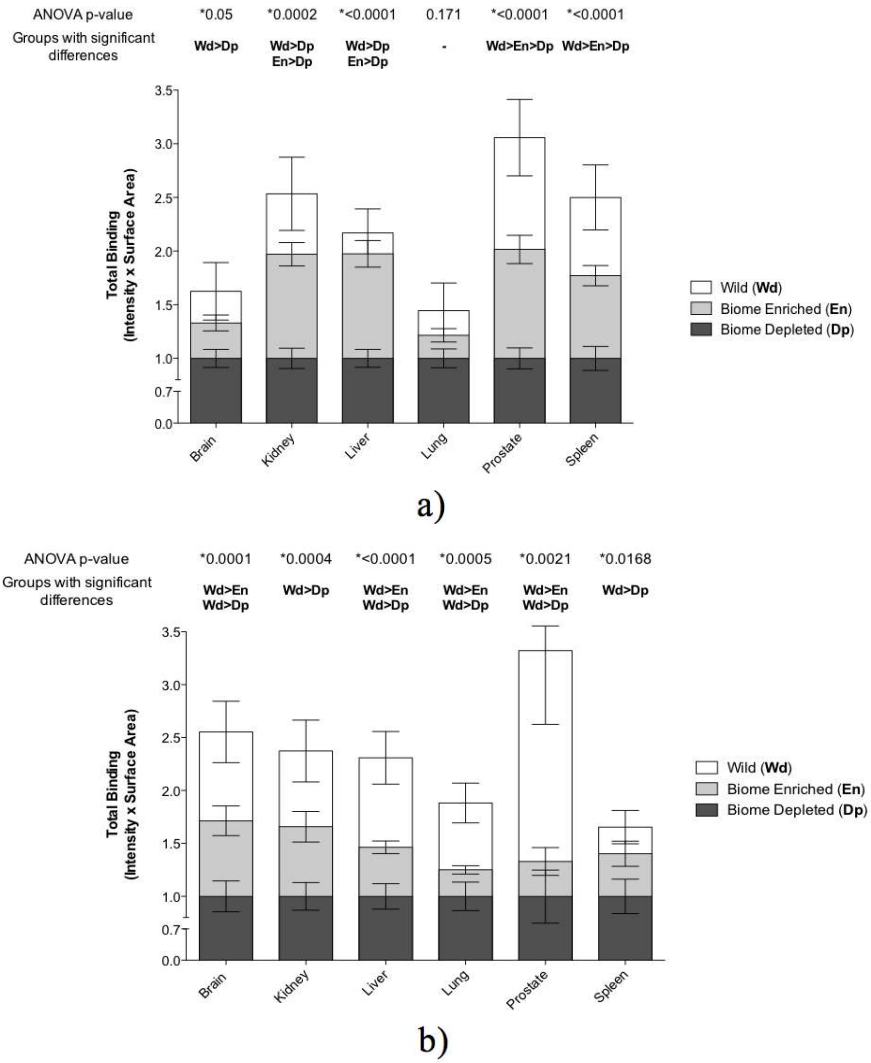



**Fig. 4. Mean total binding produced by natural IgM (a) and IgG (b) from the sera of wild rats, biota-enriched laboratory rats and biota-depleted laboratory rats binding to antigens extracted from different organs**

*The error bars show standard errors, and the p-values determined by ANOVA and significant differences as assessed by Tukey post-hoc testing are indicated*

total binding produced by natural IgM from the sera of wild rats and from laboratory rats with and without biota depletion was significantly different with antigens from all organs tested. On average, the mean total binding produced with each organ extract by natural IgM from wild rats was 122% greater than the mean total binding produced by natural IgM from biota-depleted laboratory rats (Fig. 4). The differences between the total binding produced by IgM from wild rats and biota-depleted laboratory rats were statistically significant with antigens extracted from all organs except the lungs. In comparison, the mean total binding seen with natural IgM from wild rats was on average 28.7% greater with all organ extracts compared to the total binding seen with natural IgM from biota-enriched laboratory rats. The difference between the total binding produced by IgM from wild rats and biota-enriched laboratory rats was only significant when looking at antigens extracted from the prostate and spleen. Meanwhile in laboratory rats, biota enrichment was associated with a significant difference in total IgM binding to antigens from the kidney, liver, prostate and spleen.

Every organ extract displayed a significant difference in the mean total binding produced by natural IgG from the sera of wild rats and laboratory rats with and without biota enrichment. The mean total binding produced in each organ by IgG from wild rats was an average of 135% greater compared to the IgG from biota-depleted laboratory rats. Additionally, the differences between the two groups were significant with antigens extracted from every organ.

Meanwhile, the mean total binding to antigens from each organ produced by IgG from wild rats was an average of 61.3% greater than the mean total binding produced by IgG from biota-enriched laboratory rats. The differences in total binding between these two groups were statistically significant with antigens extracted from the brain, liver, lung and prostate. However, in laboratory rats, biota enrichment was not associated with any significant differences in total IgG binding.

## 4. DISCUSSION

This study examined the effect of biota-enrichment on the natural IgM and IgG antibody repertoire by comparing the natural antibody characteristics of wild rats and laboratory rats with and without biota-enrichment. A previous study examining the effects of biota-enrichment on the natural antibody repertoire found that probiotic administration to chickens enhanced both the production of natural IgG and IgM antibodies [15]. The biota-enrichment process used in this study consisted of parental exposure to bedding from non-controlled environments, parental colonization with the helminth *Hymenolepis diminuta*, shared housing with wild rats and an immunization protocol designed to provide broad antigenic stimulation. Western blots were used to quantitatively assess several measures of the natural antibody repertoire present in the sera of wild rats and laboratory rats with and without biota-enrichment. A 1-way ANOVA was used to compare means from the three groups and indicated that the three groups displayed significant differences in the range of natural antibodies recognizing antigens from various organ extracts, as well as differences in antibody binding affinity and total antibody binding.

On average, the differences between the three groups were larger with IgG antibodies compared to IgM antibodies. Previous experiments from this laboratory established that the immunization protocol alone, without any other biota enrichment conditions, predominantly affects the IgM repertoire of laboratory rats [12]. It was suggested that this trend was due to the short time scale of immunization (14 days), which did not allow for complete antibody isotype switching. Therefore, it seems reasonable that a longer-term protocol, such as the multi-generational biota-enrichment utilized in the current experiment, would affect the IgG repertoire to a greater extent than did a shorter-term protocol.

A key aim of this experiment was to assess how well the process of biota enrichment recreates the immune system of a wild rat in laboratory controlled conditions. This paper compared results obtained using the sera of wild rats and laboratory rats with and without biota enrichment. However, the rats that did not receive biota enrichment *did* undergo immunization. However, previously published results [12] using a group of 7 rats that received neither biota enrichment nor immunization can be incorporated into the analysis, facilitating some comparison of the relative effect of immunization versus biota enrichment in the development of the natural antibody repertoire. The sera from these rats was collected, prepared and analysed under conditions identical to those described in this

report. Therefore, this data can be integrated to quantitatively assess the 'gap' between the immune function of non-immunized laboratory rats and wild rats; and in turn to calculate the extent to which immunization and biota enrichment cumulatively alter the immune function of laboratory rats to resemble that of wild rats.

Fig. 5 displays this data with the natural antibody characteristics of completely untreated laboratory rats, immunized laboratory rats, immunized and biota enriched laboratory rats and wild rats. The data has been normalized to the mean of the wild rats. As can be seen, the laboratory environment contributes to the natural antibody repertoire, particularly in the number of antigens recognized. Immunization alone can reconstitute approximately 20% of the gap in all indices except in the range of the IgM repertoire, where it bridged 43% of the gap. The combination of immunization and biota enrichment bridges the gap in immune function to a greater extent. However, the proportional contribution of immunization and biota enrichment appears to depend on the size of the initial gap. For example, the gap in the range of the natural IgM repertoire between laboratory and wild rats was only 0.37 units to begin with, and combined immunization and biota enrichment was able to bridge 80% of this gap (0.30 units). Alternatively, the gap in IgM antibody binding strength between wild and laboratory rats was 0.49 units. In this case, combined immunization and biota enrichment also raised the mean IgM binding strength by 0.30 units, but this only amounts to 62% of the gap. Therefore, it is possible that immunization and biota enrichment had a greater impact on the range of the antibody repertoire compared to the impact on antibody binding strength. Alternatively, it is possible that, in terms of net gain, the impact was similar on both the range of the natural antibody repertoire and the binding strength. Future research may focus on analysing biota enrichment alone, without any form of immunization.

These results indicate that biota enrichment may be experimentally and clinically useful for its ability to replicate natural (wild) conditions of immune stimulation. There is already evidence that biota enrichment, particularly through helminthic therapy, can effectively treat immune-mediated diseases such as multiple sclerosis and inflammatory bowel disease [16-19]. The impact of biota enrichment on the natural antibody repertoire may account in part for the mechanism underlying observations. Further, if biota enrichment were to positively influence the human natural antibody repertoire, then it could play an important role in pathogen defence and cancer surveillance. Long-term animal studies are required to examine the utility of biota enrichment in pathogen defence and cancer prevention.

Whilst these observations have implications for future research on biota reconstitution, several questions need to be addressed in future studies. This experiment was designed to provide a large variety and quantity of immunostimulation, and therefore does not provide any dose response information. We hypothesize that further stimulation will provide additional increases in immune function, but it is possible that genetic or epigenetic factors in laboratory animals preclude the robust immune function observed in wild animals. Further, the study does not attempt

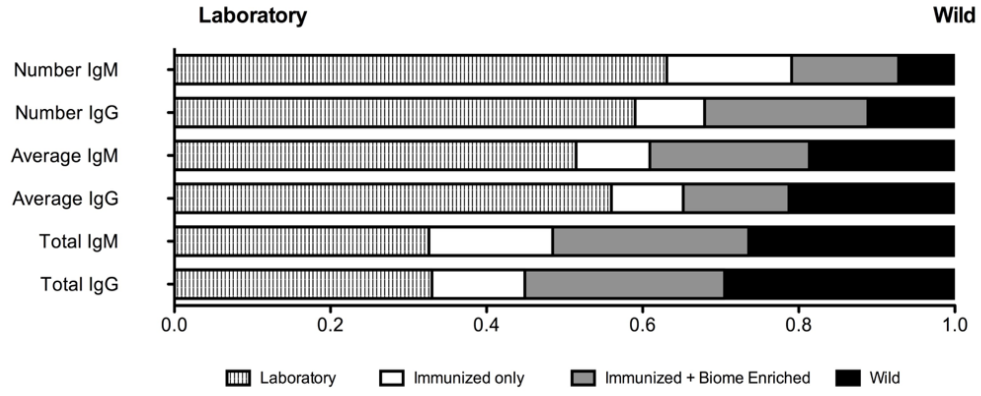



**Fig. 5. Effects of immunization and added biota enrichment on bridging the gap in natural antibody functioning between untreated laboratory rats and wild rats**

to dissect which components of biota enrichment had the most impact on immune function. For example, there may be a difference in the importance of T-cell dependent and T-cell independent antigens in establishing the natural antibody repertoire. In addition, the endpoint measures were limited to the natural antibody repertoire, and additional studies examining the effect of biota enrichment on cellular components of the immune system are worthwhile. Additional work could also be aimed at a more detailed characterization of how biota enrichment protocols actually changed the makeup of the biota, and at artificial introduction of selected species (e.g., roundworms, flukes, protozoans, specific viral components) that were not selectively added in this study. Finally, aside from the biota, there are numerous differences between wild and laboratory rats, including genetic variation and behavioural factors, which may be integral to immune function and are difficult to recapitulate in the laboratory setting. Nevertheless, this study suggests that immunization and biota enrichment can stimulate development of an antibody repertoire that approaches that found in wild animals.

## 5. CONCLUSION

In conclusion, we demonstrate that exposure of laboratory rats to an array of immune stimulation, both artificial (e.g., vaccination) and naturally occurring, effectively stimulates immune function without resulting in substantial morbidity and mortality. These studies highlight the fact that laboratory conditions result in a “baseline” immune system function that is only a fraction, perhaps less than 50%, of the function observed in their natural environment, or their environment of evolutionary adaptedness. These results have implications for public health in Western countries, where populations appear to suffer from decreased immune system regulation and concomitant increases in non-adaptive inflammation and pandemics of inflammatory disease. To the extent that culture-induced biota alteration, and biota depletion in particular, contributes to this milieu of immune dysregulation and disease, these studies support the view that a wide range of immune stimulation might be applied synergistically to prevent and perhaps to treat disease.

## ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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