Journal of Visualized Experiments

Analysis of the interactions between endobiotics and human gut microbiota using in vitro batch fermentation systems

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59725R2
Full Title:	Analysis of the interactions between endobiotics and human gut microbiota using in vitro batch fermentation systems
Keywords:	endobiotics, Bifidobacteria exopolysaccharides, human gut microbiota, in vitro, batch fermentation, short-chain fatty acid
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	YongZhou, Hunan province, China

Dear Editor,

We would like to submit the enclosed manuscript entitled “Analyze the interactions between xenobiotics to human gut microbiota using in vitro batch fermentation”, which we wish to be considered for publication in “JOVE”.

The human body is host to a complex microbial ecosystem, consisting of approximately 10^{13} microbial cells. Human second genomes of gut microbiota encoded lots of metabolic related genes that expanded host metabolic capabilities. On the other sides, xenobiotics have the potential to alter the gut microbiome community structure and their functions. A study on the interactions between xenobiotics to human gut microbiota become a popular research subject. In this manuscript, we described a protocol to investigate the interactions between bifidobacterial (Bif) EPS and human gut microbiota in vitro by using thin-layer chromatography, 16S rDNA high-throughput sequencing, and gas chromatography. This protocol we described here can also be modified to investigate the interactions between other xenobiotics and gut microbiota.

All authors have read and approved this version of the article, and due care has been taken to ensure the integrity of the work.

Correspondence should be addressed to yinyeshi@126.com.

Thank you very much for your attention to our manuscript.

Sincerely yours,

Dear Dr Steindel:

Thank you and the reviewers for helping us to improve our manuscript (JoVE59725, "Analyze the interactions between xenobiotics to human gut microbiota using in vitro batch fermentation"). All changes have been maded in the revised manuscript. Hopefully, the revised manuscript will meet with your approval.

Thank you so much for your attention to our manuscript.

Sincerely,

Yeshi Yin, PhD

TITLE:

Analysis of Interactions between Endobiotics and Human Gut Microbiota Using In Vitro Bath Fermentation Systems

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KEYWORDS:

endobiotics, *Bifidobacteria exopolysaccharides*, human gut microbiota, in vitro, batch fermentation, short-chain fatty acid

SUMMARY:

Described here is a protocol to investigate the interactions between endobiotics and human gut microbiota using in vitro batch fermentation systems.

ABSTRACT:

Human intestinal microorganisms have recently become an important target of research in promoting human health and preventing diseases. Consequently, investigations of interactions between endobiotics (e.g., drugs and prebiotics) and gut microbiota have become an important research topic. However, in vivo experiments with human volunteers are not ideal for such studies due to bioethics and economic constraints. As a result, animal models have been used to evaluate these interactions in vivo. Nevertheless, animal model studies are still limited by bioethics considerations, in addition to differing compositions and diversities of microbiota in animals vs. humans. An alternative research strategy is the use of batch fermentation experiments that allow evaluation of the interactions between endobiotics and gut microbiota in vitro. To evaluate this strategy, bifidobacterial (Bif) exopolysaccharides (EPS) were used as a

representative xenobiotic. Then, the interactions between Bif EPS and human gut microbiota were investigated using several methods such as thin-layer chromatography (TLC), bacterial community compositional analysis with 16S rRNA gene high-throughput sequencing, and gas chromatography of short-chain fatty acids (SCFAs). Presented here is a protocol to investigate the interactions between endobiotics and human gut microbiota using in vitro batch fermentation systems. Importantly, this protocol can also be modified to investigate general interactions between other endobiotics and gut microbiota.

INTRODUCTION:

Gut microbiota play an important role in the functioning of human intestines and in host health. Consequently, gut microbiota have recently become an important target for disease prevention and therapy[1]. Moreover, gut bacteria interact with host intestinal cells and regulate fundamental host processes, including metabolic activities, nutrient availabilities, immune system modulation, and even brain function and decision-making[2,3]. Endobiotics have considerable potential to influence the bacterial composition and diversity of gut microbiota. Thus, interactions between endobiotics and human gut microbiota have attracted increasing research attention[4-9].

It is difficult to evaluate interactions between endobiotics and human gut microbiota in vivo due to bioethics and economic constraints. For example, experiments investigating the interactions between endobiotics and human gut microbiota cannot be performed without permission of the Food and Drug Administration, and recruitment of volunteers is expensive. Consequently, animal models are often used for such investigations. However, the use of animal models is limited due to different microbiota compositions and diversity in animal- vs. human-associated communities. An alternative in vitro method to explore the interactions between endobiotics and human gut microbiota is through the use of batch culture experiments.

Exopolysaccharides (EPSs) are prebiotics that significantly contribute to the maintenance of human health[10]. Distinct EPSs that consist of different monosaccharide compositions and structures can exhibit distinct functions. Previous analyses have determined the composition of Bif EPSs, which are the representative xenobiotic targeted in the current study[11]. However, host-associated metabolic effects have not been considered with regard to EPS composition and diversity.

The protocol described here uses the fecal microbiota from 12 volunteers to ferment Bif EPSs. Thin-layer chromatography (TLC), 16S rRNA gene high-throughput sequencing, and gas chromatography (GC) are then used in combination to investigate the interactions between EPSs and human gut microbiota. Distinct advantages of this protocol compared to in vivo experiments are its low cost and avoidance of interfering effects from the host's metabolism. Furthermore, the described protocol can be used in other studies that investigate interactions between endobiotics and human gut microbiota.

PROTOCOL:

This protocol follows the guidelines of the ethics committee of Hunan University of Science and Engineering (Hunan, China), and the Zhejiang Gongshang University (Zhejiang, China).

1. **Preparation of bacteria**

1.1. Preparation of bifidobacterium medium broth

1.1.1. Combine the following components in 950 mL of distilled water: meat extract, 5 g/L; yeast extract, 5 g/L; casein peptone, 10 g/L; soytone, 5 g/L; glucose, 10 g/L; K_2HPO_4, 2.04 g/L; $MgSO_4 \cdot 7H_2O$, 0.22 g/L; $MnSO_4.H_20$, 0.05 g/L; NaCl, 5 g/L; Tween 80, 1 mL; salt solution, 40 mL ($CaCl_2.2H_2O$, 0.25 g/L; KH_2PO_4, 1 g/L; $NaHCO_3$, 10 g/L; NaCl, 2 g/L); and resazurin, 0.4 mL (2.5 mg/L). Adjust the pH to 6.8 with 2 M NaOH.

1.1.2. Autoclave at 121 °C for 15 min and allow the broth to cool to room temperature (RT) under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2). Add filter-sterilized cysteine-HCl (0.5 g/L) and mupirocin (5 mg/L) to the medium.

1.2. Add an aliquot (50 µL) of frozen *Bifidobacterium longum* to a culture tube with 5 mL of bifidobacterium medium broth under anaerobic conditions, then culture in an anaerobic incubator for 24 h at 37 °C.

2. **Preparation of bifidobacterial EPSs**

2.1. Preparation of PYG agar medium

2.1.1. Combine the following: peptone, 20 g/L; yeast extract, 10 g/L; glucose, 5 g/L; NaCl, 0.08 g/L; $CaCl_2$, 0.008 g/L; $MgSO_4 \cdot 7H_2O$, 0.008 g/L; K_2HPO_4, 0.04 g/L; KH_2PO_4, 0.04 g/L; $NaHCO_3$, 0.4 g/L; agar, 12 g/L. Adjust the pH to 7.2 using 10 M NaOH.

2.1.2. Autoclave the media at 121 °C for 15 min and cool to ~50 °C. Then, per 1 L of medium, add 0.5 mL of filter-sterilized vitamin K_1 solution (1 g of vitamin K_1 dissolved in 99 mL of 99% ethanol), 5 mL of haemin solution (0.5 g of haemin dissolved in 1 mL of 1 mol/L NaOH, then brought up to 100 mL with distilled water), and 0.5 g of cysteine-HCl.

2.1.3. Before pouring the PYG plates, add filter-sterilized 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal, 0.06 g/L), $LiCl \cdot 3H_2O$ (5.7 g/L) and mupirocin (5 mg/L) to the medium.

NOTE: X-Gal and $LiCl.3H_2O$ allow the identification of *B. longum* colonies on plates via coloration changes.

2.2. Inoculate 20 µL of *B. longum* strains (step 1.2) to PYG plates and place in an anaerobic incubator at 37 °C for 72 h.

2.3. Collect mucoid bacterial colonies from the PYG plates using a weighing scoop, then completely resuspend in 10 mL of phosphate-buffered saline (PBS) using a vortex oscillator.

NOTE: The bacterial and EPS mixtures should be resuspended completely by vortexing or pipetting up and down repeatedly until the fibers are completely dissolved in PBS.

2.4. Centrifuge the suspension at 6,000 x *g* for 5 min.

2.5. Carefully transfer the supernatants to a new centrifuge tube and mix completely with three volumes of cold 99% ethanol by repeated inversion and blending.

2.6. Centrifuge the mixture at 6,000 x *g* for 5 min and completely remove the supernatants.

2.7. Remove the precipitates from the centrifuge tubes by scraping and drying the EPS extracts overnight using a speed vacuum.

3. Preparation of fermentation medium

3.1. Preparation of basic culture medium VI

3.1.1. Combine the following: peptone, 3 g/L; tryptone, 3 g/L; yeast extract, 4.5 g/L; mucin, 0.5 g/L; bile salts No. 3, 0.4 g/L; NaCl, 4.5 g/L; KCl, 2.5 g/L; $MgCl_2 \cdot 6H_2O$, 4.5 g/L; 1 mL Tween 80; $CaCl_2.6H_2O$, 0.2 g/L; KH_2PO_4, 0.4 g/L; $MgSO_4 \cdot 7H_2O$, 3.0 g/L; $MnCl_2 \cdot 4H_2O$, 0.32 g/L; $FeSO_4 \cdot 7H2O$, 0.1 g/L; $CoSO_4 \cdot 7H_2O$, 0.18 g/L; $CaCl_2 \cdot 2H_2O$, 0.1 g/L; $ZnSO_4 \cdot 7H_2O$, 0.18 g/L; $CuSO_4 \cdot 5H_2O$, 0.01 g/L; and $NiCl_2 \cdot 6H_2O$, 0.092 g/L. Adjust the pH to 6.5 with 1 M HCl.

3.1.2. Prepare haemin and cysteine as done in section 2.1 and add after autoclaving and cooling.

3.2. Prepare culture media that contains different carbon sources with a VI base media. Prior to autoclaving, add 8 g/L of Bif EPS fibers to medium VI, comprising group VI_Bif. In addition, add 8 g/L starch to medium VI to represent group VI_Starch. Finally, medium VI without addition of a carbon source is used as the control (group VI).

NOTE: Bif EPS and starch are first dissolved in hot water using a magnetic agitator, then mixed with prepared VI medium.

3.3. Autoclave all media at 121 °C for 15 min and allow to cool to RT.

3.4. Transfer a subsample (5 mL) of each medium to culture tubes in an anaerobic incubator, and store the remaining media at 4 °C.

4. Human fecal sample preparation

4.1. Collect fresh fecal samples immediately following fresh defecation from healthy adult human volunteers using feces containers, and subsequently use for slurry preparation.

NOTE: Prior to sample collection, all the volunteers should be screened to ensure no receiving of antibiotics, probiotics, or prebiotic treatments for at least 3 months prior to donating samples. In addition, all donors must provide informed, written consent.

4.2. Transfer a fresh fecal sample (1 g) to 10 mL of 0.1 M anaerobic PBS (pH 7.0) into glass beakers, then use glass rods to prepare a 10% (w/v) slurry.

4.3. Use a 0.4 mM sieve to filter the fecal slurry. Then, use a subsample of the filtered slurry to inoculate batch culture fermentation experiments, and store the remainder at -80 °C for further analyses.

NOTE: Steps 4.2–4.3 are conducted in an anaerobic chamber.

5. In vitro batch fermentation

5.1. Add filtered fecal slurry (500 µL) to the fermentation medium prepared in step 3.2 within an anaerobic chamber, then incubate at 37 °C.

5.2. Collect 2 mL of fermented samples at 24 h and 48 h in the anaerobic chamber and then centrifuge outside of the chamber at 6,000 x *g* for 3 min.

5.3. Carefully transfer the supernatants to a new centrifuge tube that will be used for polysaccharide degradation analysis and short-chain fatty acids (SCFAs) measurements.

5.4. Store the centrifugation pellets at -80 °C and subsequently use for bacterial genomic DNA extraction.

6. **EPS degradation by human fecal microbiota**

6.1. Load 0.2 µL of fermented supernatants onto pre-coated silica gel-60 TLC aluminum plates, then dry using a hair drier.

6.2. Develop the plates in 20 mL of a formic acid/n-butanol/water (6:4:1, v:v:v) solution and dry using a hair drier.

6.3. Soak the plates in the orcinol reagent to dye, then dry using a hair drier.

6.3.1 Prepare orcinol reagents by dissolving 900 mg of Lichenol in 25 mL of distilled water then adding 375 mL of ethanol. Subsequently, concentrated sulfuric acid should be slowly added and

the solution thoroughly mixed.

6.4. Heat plates at 120 °C for 3 min in a baking oven and evaluate degradation of EPS by measuring TLC bands.

7. Effects of EPS on human intestinal microbiota

7.1. Freeze-thaw the original fecal samples prepared in step 4.3 and fermented samples prepared in step 5.4.

7.2. Extract bacterial genomic DNA (gDNA) from all the samples using a stool bacterial genomic DNA extraction kit following the manufacturer's instructions.

7.3. Determine DNA concentrations, integrities, and size distributions using a micro-spectrophotometer and agar gel electrophoresis.

7.4. Conduct PCR of bacterial 16S rRNA genes from the extracted gDNA using the following previously described forward and reverse primers[12]:

-forward primer (barcoded primer 338F): ACTCCTACGGGAGGCAGCA
-reverse primer (806R): GGACTACHVGGGTWTCTAAT

Use the following thermal cycler conditions:
1) 94 °C for 5 min.
2) 94 °C for 30 s.
3) 55 °C for 30 s.
4) 72 °C for 1 min.
5) Repeat 2–4 for 35 cycles
6) 72 °C for 5 min.
7) 4 °C hold until removal from thermal cycler.

7.5. Conduct high-throughput sequencing of PCR products at a DNA sequencing company using ultra-high throughput microbial community analysis.

7.6. Obtain clean, high quality sequences using the Quantitative Insights into Microbial Ecology (QIIME) sequence analysis pipeline[13].

7.7. Define operational taxonomic units (OTUs) for 16S rRNA gene sequences with greater than 97% nucleotide similarity using bioinformatics tools such as the Mothur software suite[14].

7.8. Choose a representative sequence from each OTU and use the RDP classifier along with the SILVA taxonomic database to classify representative sequences[15].

7.9. Calculate Good's coverage, alpha diversity metrics (including Simpson and Shannon index),

and richness (observed number of OTUs) using bioinformatics tools[16].

8. **Effects of EPS on SCFA production by human intestinal microbiota**

8.1. Add fermented supernatants (1 mL) prepared in step 5.3 to 2 mL centrifuge tubes.

8.2. Add 0.2 mL of 25% (w/v) metaphosphoric acid to each of the samples and thoroughly mix the solutions by vortexing.

8.3. Centrifuge the mixtures at 13,000 *x* g for 20 min and transfer the supernatants to fresh tubes.

8.4. Concomitantly, prepare solutions of 120 mM acetic, propionic, butyric, isobutyric, valeric and isovaleric acids. Then, add 1 mL of each prepared acid to 1.2 mL of 25% (w/v) metaphosphoric acid and use as the standard cocktails.

8.5. Filter the samples using a 0.22 µM membrane.

8.6. Detect SCFA concentrations using high performance gas chromatography according to previously described protocols[11,17].

NOTE: An InertCap FFAP column (0.25 mM x 30 m x 0.25 µM) is used for gas chromatography (GC). SCFA concentrations are then quantified based on peak areas using the single-point internal standard method in the GC Solution software package.

REPRESENTATIVE RESULTS:

The production of mucoid EPS could be observed in *B. longum* cultures on PYG plates after anaerobic incubation for 72 h (**Figure 1A**). Centrifugation of culture scrapes, followed by ethanol precipitation and drying, resulted in the collection of cellulose-like EPS (**Figure 1B**). Dried EPS and soluble starch were then used as carbon sources for fermentation cultures. TLC was used for oligosaccharide separation and purity analysis due to its low cost and rapid results turnaround[18]. Although the degradation rate of starch by human fecal microbiota was faster than that of Bif EPS **(Figure 2**), Bif EPS degradation was clearly observed for some EPS-inoculated samples.

Community compositional analysis via 16S rRNA gene high-throughput sequencing and principal coordinate analysis (PCoA) was then performed to investigate the effects of Bif EPS on human gut microbiota. Samples from the VI_Bif and VI_Starch groups clustered separately from each other in the PCoA analysis (**Figure 3A**), indicating that EPS and starch availability differentially shape human fecal bacterial communities. Linear discriminant analysis effect size (LEfSe) was further used to distinguish the specific bacterial taxa that differed between the VI_Bif and VI_Starch treatments. The genera *Collinsella*, *Coprococcus*, *Parabacteroides*, and *Rhodopseudomonas* were significantly more abundant in the VI_Bif samples than in the

VI_Starch samples (**Figure 3B**). Furthermore, GC measurements were made for several SCFAs to evaluate their production following the addition of different carbon sources. SCFAs that were measured from fermentation cultures included acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids. Following fermentation for 24 h and 48 h, five of the six aforementioned SCFA concentrations were similar among treatments and not statistically different between the VI_Bif, VI_Starch, and VI groups. However, propionic acid concentrations were significantly higher in the VI_Bif group than in the VI_Starch group (**Figure 4**).

FIGURE LEGENDS:

Figure 1: EPS produced by *B. longum*. Frozen *B. longum* was restored in Bifidobacterium medium broth and then streaked onto PYG plates, followed by anaerobic incubation at 37 °C for 72 h (**A**). The EPS produced by bacterial cultures were scraped from plate cultures, precipitated using ethanol, and dried overnight using a speed vacuum (**B**).

Figure 2: TLC analysis of *in vitro* EPS and starch degradation by human gut microbiota. TLC analysis was conducted on 0.2 µL samples collected at 24 h and 48 h from each fermentation culture grown under anaerobic conditions. VI, VI_Starch, and VI_Bif indicate VI media, VI media + starch supplement, and VI media + EPS supplement, respectively. The numbers 1–12 indicate fecal bacterial samples from the 12 volunteers that were used to inoculate the fermentation experiments. The control group represents treatment without additional carbon supplements. This figure is modified from Yin et al.[11].

Figure 3: Effects of Bif EPS availability on human gut microbiota communities. (**A**) PCoA plot of gut microbiota community compositional dissimilarities based on the unweighted UniFrac metric. (**B**) LEfSE analysis of bacterial taxa that were differentially abundant among treatment groups. A cutoff of $p < 0.05$ was used to assess the statistical significance of bacterial taxonomic differences among groups. Ori indicates the gut microbiota of the volunteer fecal samples. VI_Bif and VI_Starch indicate the gut microbiota from fermentation samples using VI media with EPS and starch as carbon substrates, respectively. VI represents the control group with gut microbiota inoculated fermentations in VI media without supplementation of other carbohydrates. This figure is modified from Yin et al.[11]

Figure 4: Effects of EPS availability on SCFA production after 24 h and 48 h of fermentation. Acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids were detected using gas chromatography. VI_Bif, VI_Starch, and VI indicate the samples that were collected after cultivation using VI media + EPS, VI media + starch, and VI media, respectively. All samples were measured in triplicate. The figures were generated using GraphPad Prism Version 5.01. Panels represent organic acid concentrations within each fermented sample for A, acetic acid; B, propionic acid; C, isobutyric acid; D, butyric acid; E, isovaleric acid; and F, valeric acid. This figure is modified from Yin et al.[11]

DISCUSSION:

Significant progress has been made towards understanding human gut microbiota composition and activities over the last decade. As a consequence of these studies, the holobiont concept has emerged, which represents the interactions between hosts and associated microbial communities, such as in between humans and their gut microbiota[19,20]. Furthermore, humans are even now regarded as superorganisms[21], wherein the gut microbiota have been recognized as one of the functional organs in humans[22,23]. The human body hosts a complex microbial ecosystem, consisting of approximately 10^{13} microbial cells[24]. Moreover, the genomes of gut microbiota are considered auxiliary genomes from humans that encode numerous metabolic-related genes that expand the host's metabolic capabilities[4]. However, xenobiotics, including therapeutic drugs and diet-derived bioactive compounds, can potentially alter the gut microbiome community structure and associated functions[5]. Increasing numbers of studies have indicated that interactions between gut microbiota and xenobiotics play important roles in mediating chemical toxicity and causing, or otherwise exacerbating, human diseases[6,7]. Thus, investigations of the interactions between xenobiotics and the human gut microbiota have recently garnered significant research attention.

Mouse models have been the most widely used methods to investigate interactions between microbiota and hosts. However, differences in composition and activities between the gut microbiota of humans and mice[25] may result in inadequate modeling of human interactions through studies of mice. Nevertheless, bioethics considerations require minimal use of mice. An alternative to the above in vivo models is batch fermentation, which can be used to simulate human gut microbiota in vitro[26]. Consequently, fermentation experiments have been used to investigate the interactions between xenobiotics and human gut microbiota. For example, Yin et al.[17] have used batch fermentation experiments to investigate the interactions between polysaccharides and human gut microbiota. The results from this study indicate that some polysaccharides can be metabolized by the human gut microbiota, and that polysaccharides modulate the human bacterial community and metabolites that they produce in vitro, including SCFAs. However, some methodological considerations are critical for use of this protocol. For example, fecal samples should be collected as soon as possible, and an anaerobic chamber should be used to ensure the growth of obligate anaerobes. The latter consideration is particularly critical, because oxygen exposure can lead to the death of some gut bacterial populations and thus, alteration of the bacterial community. In addition, xenobiotics are metabolized in the upper digestive system. Consequently, modeling the interactions between xenobiotics and the lower digestive system microbiota is an important consideration for in vivo modeling.

Batch fermentation experiments have clear advantages over in vivo human and animal studies because they are more economically feasible and convenient. Moreover, they can be used to investigate interindividual variation of human gut microbiota responses to xenobiotic exposure. Moreover, batch fermentation can be easily applied to manipulate microbiota communities and evaluate their associated metabolic functions. However, batch fermentation systems suffer from the limitation of static state dynamics. Future investigations could implement bioreactor chemostats that allow the dynamic modulation of pH, temperature, and peristalsis, while maintaining a steady supply of nutrients and the continuous removal of waste. Such activities

would allow experiments to better mimic in vivo intestinal tracts and provide new insights to supplement those from batch fermentation experiments. An additional limitation of batch fermentation experiments is that they remove all microbiome-host tissue interactions. This could be a particularly important consideration, as some xenobiotics (e.g., methamphetamine) can be co-metabolized by human cells and gut microbiota[27]. Moreover, recent studies have indicated that gut metagenome (GM) can indirectly regulate xenobiotic metabolism via regulating host gene expression regulation[8].

Although developments of batch fermentation systems are still needed, these systems can be widely used for high-throughput and rapid screening of interactions between xenobiotics and human gut microbiota. Elucidating the mechanisms underlying xenobiotic resistance and metabolism in active human gut microbiomes will provide important insights into unexplained patient-to-patient variation in drug efficacy and toxicity[8,9]. Furthermore, a more detailed understanding of how diets and specific food components alter microbial metabolisms and consequently effect host health is the first step towards realizing the goal of personalized medicine via microbiota modulation.

ACKNOWLEDGMENTS:

This study was funded by the National Nature Science Foundation of China (No. 31741109), the Hunan Natural Science Foundation (No. 2018JJ3200), and the construct program of applied characteristic discipline in Hunan University of Science and Engineering. We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

DISCLOSURES:

The authors declare that they have no conflicts of interest. The figures were cited in Yin et al.[11].

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induced conditioned place preference. *Frontiers in Microbiology.* **8** (1), 1620 (2017).

A

B

Figure 2

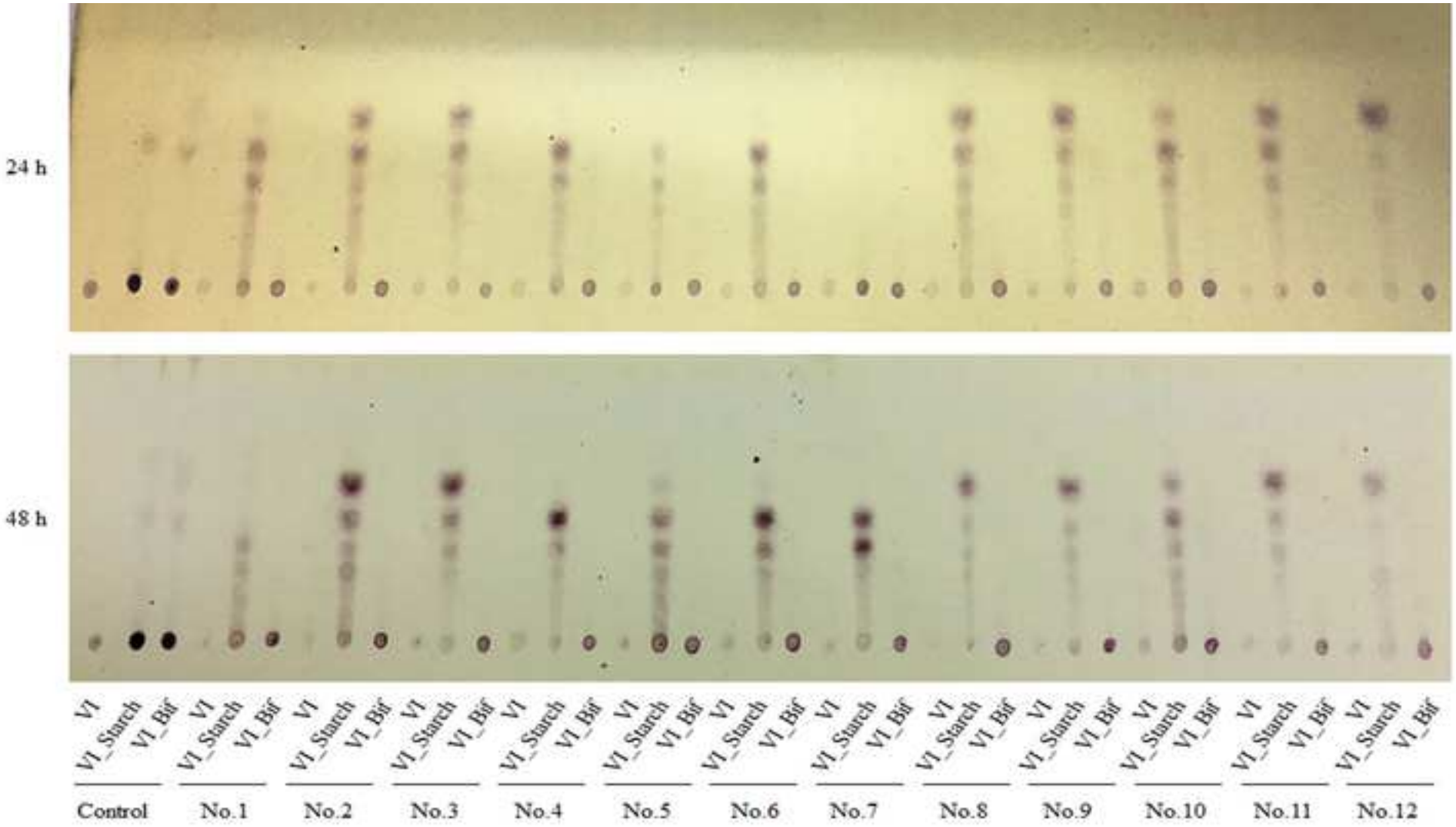

Figure3

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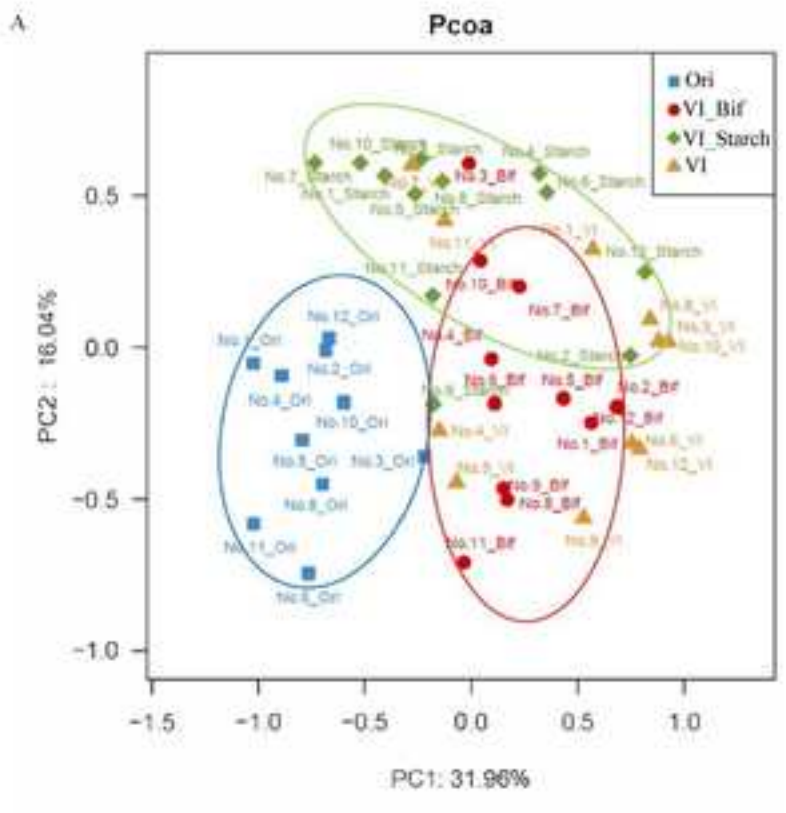

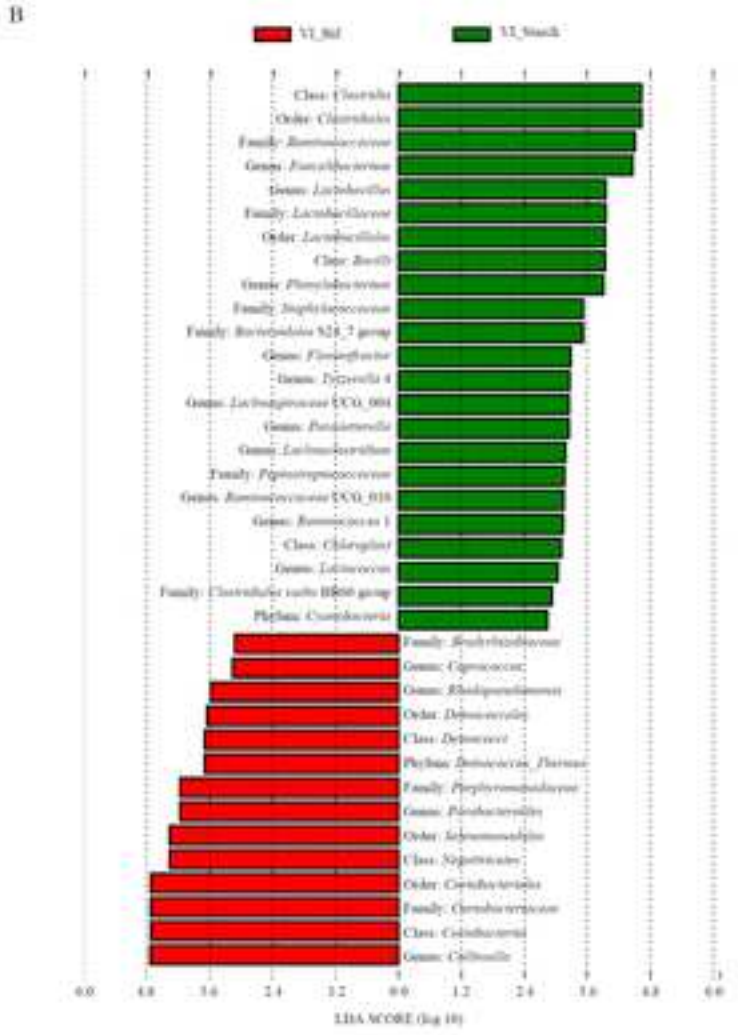

Figure 4

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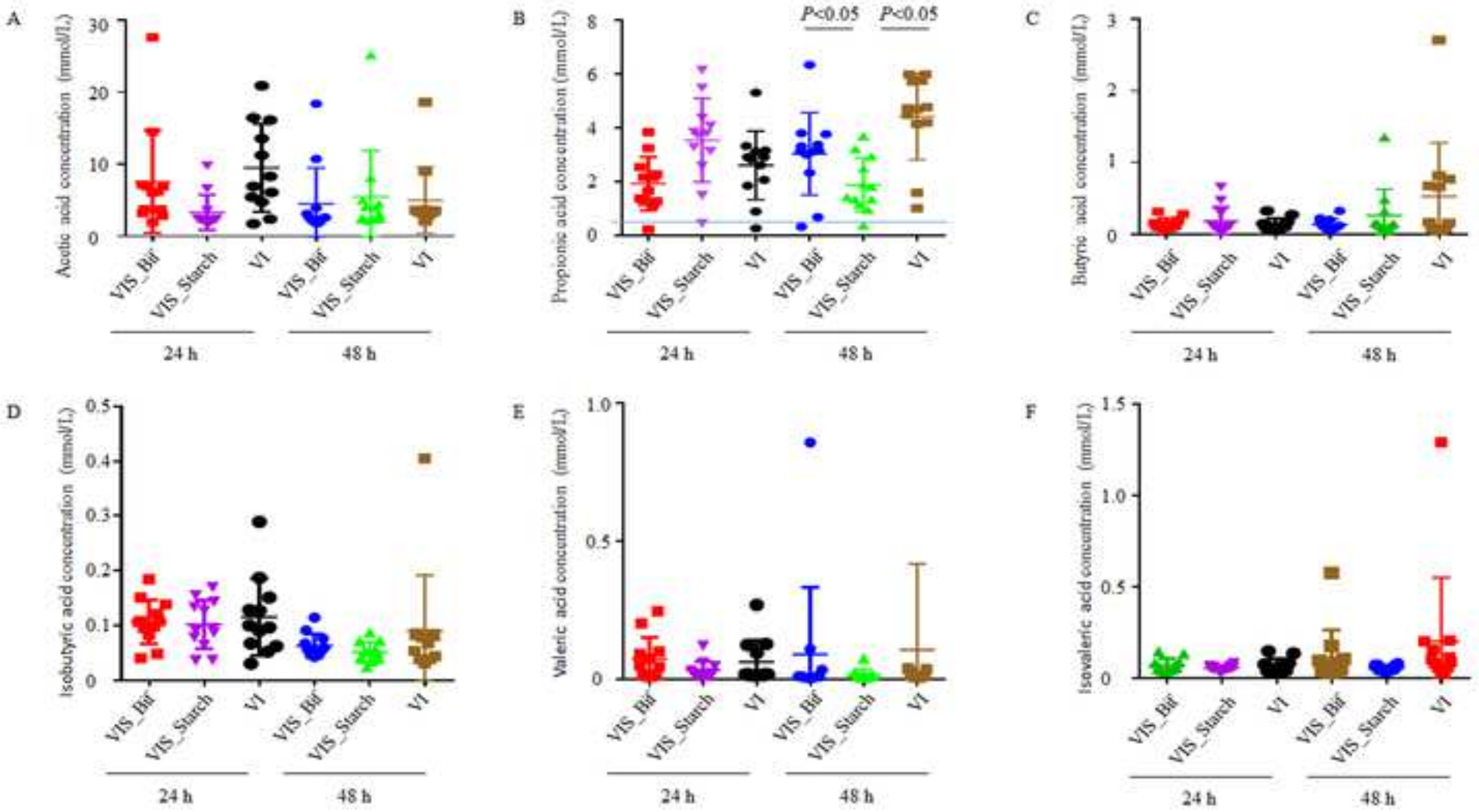

Table of Materials

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Name of Material/Equipment	Company	Catalog Number
0.22 µm membrane filters	Millipore	SLGP033RB
0.4-mm Sieve	Thermo Fischer	308080-99-1
5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X	Solarbio	X1010
Acetic	Sigma-Aldrich	71251
Agar	Solarbio	YZ-1012214
Anaerobic chamber	Electrotek	AW 400SG
Autoclave	SANYO	MLS-3750
Bacto soytone	Sigma-Aldrich	70178
Baking oven	Shanghai Yiheng Scientific Instruments Co., Ltd	DHG-9240A
Beef Extract	Solarbio	G8270
Bifidobacterium longum Reuter	ATCC	ATCC® 51870™
Bile Salts	Solarbio	YZ-1071304
Butyric	Sigma-Aldrich	19215
$CaCl_2$	Solarbio	C7250
Capillary column	SHIMADZU-GL	InertCap FFAP (0.25 mm × 30 n
Casein Peptone	Sigma-Aldrich	39396
Centrifuge	Thermo Scientific	Sorvall ST 8
$CoSO_4.7H_2O$	Solarbio	C7490
$CuSO_4.5H_2O$	Solarbio	203165
Cysteine-HCl	Solarbio	L1550
Ethanol	Sigma-Aldrich	E7023
$FeSO_4.7H2O$	Solarbio	YZ-111614
Formic Acid	Sigma-Aldrich	399388
Gas chromatography	Shimadzu Corporation	GC-2010 Plus
Glass beaker	Fisher Scientific	FB10050
Glucose	Solarbio	G8760
Haemin	Solarbio	H8130
HCl	Sigma-Aldrich	30721
Isobutyric	Sigma-Aldrich	46935-U
Isovaleric Acids	Sigma-Aldrich	129542

K_2HPO_4	Solarbio	D9880
KCl	Solarbio	P9921
KH_2PO_4	Solarbio	P7392
$LiCl.3H_2O$	Solarbio	C8380
Meat Extract	Sigma-Aldrich-Aldrich	70164
Metaphosphoric Acid	Sigma-Aldrich	B7350
$MgCl_2.6H_2O$	Solarbio	M8160
$MgSO_4.7H_2O$	Solarbio	M8300
MISEQ	Illumina	MiSeq 300PE system
$MnSO_4.H_20$	Sigma-Aldrich	M8179
Mupirocin	Solarbio	YZ-1448901
NaCl	Solarbio	YZ-100376
$NaHCO_3$	Sigma-Aldrich	792519
NanoDrop ND-2000	NanoDrop Technologies	ND-2000
NaOH	Sigma-Aldrich	30620
n-butanol	ChemSpider	71-36-3
$NiCl_2$	Solarbio	746460
Orcinol	Sigma-Aldrich	447420
Propionic	Sigma-Aldrich	94425
QIAamp DNA Stool Mini Kit	QIAGEN	51504
Ready-to-use PBS powder	Sangon Biotech (Shanghai) Co., Ltd.	A610100-0001
Resazurin	Solarbio	R8150
Speed Vacuum Concentrator	LABCONCO	CentriVap
Starch	Solarbio	YZ-140602
Sulfuric Acid	Sigma-Aldrich	150692
T100 PCR	BIO-RAD	1861096
TLC aluminium sheets	MerckMillipore	116835
Trypticase Peptone	Sigma-Aldrich	Z699209
Tryptone	Sigma-Aldrich	T7293
Tween 80	Solarbio	T8360

Valeric	Sigma-Aldrich	75054
Vitamin K_1	Sigma-Aldrich	V3501
Vortex oscillator	Scientific Industries	Vortex.Genie2
Yeast Extract	Sigma-Aldrich	Y1625
$ZnSO_4.7H_2O$	Sigma-Aldrich	Z0251

Comments/Description
Use to filter samples
Use to prepare human fecal samples
Use to prepare color plate
Standard sample for SCFA
The component of medium
Bacteria culture and fermentation
Use to autoclave
The component of medium
Use to heat and bake
The component of medium
Bacteria
The component of medium
Standard sample for SCFA
Salt solution of medium
Used to SCFA detection
The component of medium
Use for centrifugation
The component of medium
The component of medium
The component of medium
Use to prepare vitamin K_1
The component of medium
Used to TLC
Used to SCFA detection
Used for slurry preparation
The component of medium
The component of medium
Basic solution used to adjust the pH of the buffers
Standard sample for SCFA
Standard sample for SCFA

Salt solution of medium
The component of medium
Salt solution of medium
Use to prepare color plate
The component of medium
Standard sample for SCFA
The component of medium
Salt solution of medium
DNA sequencing
Salt solution of medium
Antibiotic
Salt solution of medium
Salt solution of medium
Determine DNA concentrations
Basic solution used to adjust the pH of the buffers
Used to TLC
The component of medium
Used to prepare orcinol reagents
Standard sample for SCFA
Extract bacterial genomic DNA

Used to prepare the lipid suspension
Anaerobic Equipment
Use to prepare EPSs
Use to the carbon source
Used to prepare orcinol reagents
PCR amplification
Used to TLC
The component of medium
The component of medium
Salt solution of medium

Standard sample for SCFA

The component of medium

Use to vortexing

The component of medium

The component of medium

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Editorial comments:

1. Title: It's unclear what 'ingredient xenobiotics' means; please clarify.
"Xenobiotics" has been changed to "endobiotics" in the revised manuscript based on the reviewer's comments.

2. Summary: There still is no explicit 10-50 word 'Summary' section in the manuscript outlining the protocol.
The 'summary' section has been corrected in the revised manuscript.

3. Our format requires the imperative tense for the protocol (which it was before; I'm not sure why it was changed); please rewrite.
The revised manuscript has been updated to use the imperative tense in the protocol.

4. 1.1, 2.1, 3.1: You mention adjusting pH after preparation of these media; is this done after autoclaving?
Media pH was adjusted before autoclaving, and this discrepancy has been corrected in the revised manuscript.

5. 6: This is unclear-are TLC plates developed twice (once in formic acid/n-butanol/water, once in orcinol)?
The TLC plates were developed twice, first for developing, and a second time for staining.

6. 7.1-7.4: The filmed steps are somewhat lacking in detail. I'm not sure they necessarily require them (they are fairly standard), but on the other hand I'm not sure if we can film them as is.
The steps for DNA extraction and PCR are standard for the commercial kit used in the protocol and are not necessary to film.

7. Figure 3: Please combine panel A and B into a single panel.
Figure 3 has been modified to combine panels A and B.

8. Why are there 2 ALAs attached? There should only be one.
This discrepancy has been corrected in the submission.

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Requestor type	publisher
Publisher	Not listed below
Format	electronic
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Number of figures/tables /illustrations	4
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Circulation/distribution	<501
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Lead author	Yeshi Yin
Title of targeted journal	Journal of visualized experiments
Publisher	Not listed below
Publisher imprint	Boston, Mass. : MYJoVE Corporation
Expected publication date	Apr 2019
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