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TITLE:

An In Vitro Batch-Culture Model to Estimate the Effects of Interventional Regimens on Human Fecal Microbiota

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anaerobic, batch culture, colon, fermentation, in vitro, microbiota, microflora, short-chain fatty acids, pH

SUMMARY:

This protocol describes an in vitro batch-culture fermentation system of human fecal microbiota, using inulin (a well-known prebiotic and one of the most widely studied microbiota modulators) to demonstrate the use of this system in estimating effects of specific interventions on fecal microbiota composition and metabolic activities.

ABSTRACT:

The emerging role of the gut microbiome in several human diseases demands a breakthrough of new tools, techniques and technologies. Such improvements are needed to decipher the utilization of microbiome modulators for human health benefits. However, the large-scale

screening and optimization of modulators to validate microbiome modulation and predict related health benefits may be practically difficult due to the need for large number of animals and/or human subjects. To this end, in vitro or ex vivo models can facilitate preliminary screening of microbiome modulators. Herein, it is optimized and demonstrated an ex vivo fecal microbiota culture system that can be used for examining the effects of various interventions of gut microbiome modulators including probiotics, prebiotics and other food ingredients, aside from nutraceuticals and drugs, on the diversity and composition of the human gut microbiota. Inulin, one of the most widely studied prebiotic compounds and microbiome modulators, is used as an example here to examine its effect on the healthy fecal microbiota composition and its metabolic activities, such as fecal pH and the fecal levels of organic acids including lactate and short-chain fatty acids (SCFAs). The protocol may be useful for studies aimed at estimating the effects of different interventions of modulators on fecal microbiota profiles and at predicting their health impacts.

INTRODUCTION:

The human microbiota is a complex community consisting of bacteria, archaea, viruses and eukaryotic microbes[1], that inhabit the human body internally and externally. Recent evidences have established the fundamental role of the gut microbiota and the gut microbiome (the entire collection of microbes and their genes found in the human gastrointestinal tract) in various human diseases including obesity, diabetes, cardiovascular diseases, and cancer[1-3]. Additionally, the microorganisms living in our gut produce a wide spectrum of metabolites which significantly affect our health and can also contribute to the pathophysiology of several diseases as well as a variety of metabolic functions[4,5]. Abnormal changes (perturbations) in the composition and function of this gut microbial population are generally termed as “gut dysbiosis”. Dysbiosis is usually associated with an unhealthy state of the host and hence can be differentiated from the normal (homeostatic) microbial community associated with a healthy control state of the host. Specific patterns of gut microbiome dysbiosis are often found in various different diseases[1-3,6,7].

The fermentation of undigested food, particularly the fermentable carbohydrates/fibers, by the gut microbiota not only yields energy but also produces divergent metabolites including short-chain fatty acids (SCFAs), lactate, formate, carbon dioxide, methane, hydrogen, and ethanol[6]. In addition, the gut microbiota also produces a number of other bioactive substances such as folate, biotin, trimethylamine-*N*-oxide, serotonin, tryptophan, gamma-aminobutyric acid, dopamine, norepinephrine, acetylcholine, histamine, deoxycholic acid, and 4-ethylphenyl sulfate. This occurs primarily through the utilization of intrinsic metabolic fluxes within the host-microbe niche, which contributes in several body processes, metabolic functions and epigenetic changes[1,8-10]. However, the effects of various interventions on such microbial products remain unkown or unclear due to the lack of easy, efficient and reproducible protocols. The human gut microbiota composition is an extremely complex and diverse ecosystem, and hence, many questions about its role in human health and disease pathology still remain unanswered. The effects of many common gut microbiome modulators (e.g., probiotics, prebiotics, antibiotics, fecal transplantation and infections) on the composition and metabolic functions of the intestinal microbiota remain largely elusive. In addition, the examination and validation of these effects in vivo is difficult, particularly because most of the nutrients and metabolites produced by the gut

microbiota are absorbed or disposed of simultaneously and rapidly in the gut; therefore, measuring the production, amount and processing of these metabolites (e.g., SCFAs) in vivo still remains a practical challenge. Indeed, physiological models such as animals and human subjects are critical for determining the role of gut microbiome and its modulation on host health, but these may not be suitable for large-scale screening of different types of microbiome modulators due to ethical, monetary or time constraints. To this end, in vitro and/or ex vivo models, such as culturing of gut microbiota in vitro and then intervening with different microbiota modulators, can offer time- and money-saving opportunities and hence can allow for preliminary or large-scale screening of various components (such as probiotics, prebiotics, and other interventional compounds) to examine/predict their effects on the fecal microbiota diversity, composition and metabolic profiles. Studies using such in vitro and ex vivo systems of the gut microbiome may facilitate further understanding of the host-microbiome interactions that contribute to host health and disease, and could also lead to finding novel therapies that target the microbiome to ameliorate host health and prevent and treat various diseases[1].

Although the in vitro gut microbiota culture systems cannot truly replicate the actual intestinal conditions, several laboratories have endeavored to develop such models, some of which have been found practicable to some extent and have been successfully used for different purposes. One of the recent gut models is the Simulator of the Human Intestinal Microbial Ecosystem, which mimics the entire human gastrointestinal tract, including the stomach, small intestine, and different regions of the colon. However, such technically complex models may not be accessible to other research facilities worldwide. Therefore, there is still a critical need for the development of new alternative models that are relatively simple, affordable and practical for laboratories studying the microbiome modulators and their effects on gut microbiota and host health. Hence, the use of an in vitro (or ex vivo) fecal microbiota culture system would be useful for studying the effects of such interventions[11,12]. Specifically, the effect of different prebiotics on the microbiota fermentation capacity in terms of periodic changes in the gut microbiota diversity and composition, the fecal pH, and the levels of microbial metabolites including SCFAs and lactate can be studied[13]. Herein, using inulin (one of the most widely studied prebiotic components) as an example of the microbiome modulator, a step-by-step protocol of this simple ex vivo microbiota batch-culture system is described to demonstrate its use to estimate the changes in the fecal microbiota and microbial metabolites following intervention with the microbiome modulators.

PROTOCOL:

CAUTION: Consult the appropriate Material Safety Data Sheets and follow the instructions and guidelines for appropriate Biosafety Level 2 (BSL-2) training. Follow all the culturing steps as per the standard biosafety rules and use a BSL-2 cabinet using aseptic conditions. Furthermore, fecal samples from different models and human subjects may have potential risk of spreading microbial borne diseases. Immediately seek medical aid in the occurrence of any injury and infection. In addition, the use of human and animal stool samples should be approved through institutional ethical committees and must compliant with protocols to use samples and subject information.

1. Preparation of culture media

1.1. Preparation of the culture media, prepare nine types of stock solutions

1.1.1. Solution A (1000 mL): Dissolve 5.4 g of sodium chloride (NaCl), 2.7 g of potassium dihydrogen phosphate (KH_2PO_4), 0.16 g of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), 0.12 g of magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), 0.06 g of manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$), 0.06 g of cobaltous chloride hexahydrate ($CoCl_2 \cdot 6H_2O$), and 5.4 g of ammonium sulfate $(NH_4)_2SO_4$, in deionized water to make total volume to 1000 mL.

1.1.2. Trace mineral solution (1000 mL): Dissolve 500 mg of disodium ethylenediamine-tetraacetate dihydrate (Na_2EDTA), 200 mg of ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), 10 mg of zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), 3 mg of manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$), 30 mg of phosphoric acid (H_3PO_4), 20 mg of $CoCl_2 \cdot 6H_2O$, 1 mg of cupric chloride dihydrate ($CuCl_2 \cdot 2H_2O$), 2 mg of nickel(II) chloride hexahydrate ($NiCl_2 \cdot 6H_2O$) and 3 mg of sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$) in deionized water to make total volume to 1000 mL.

NOTE: This solution is light sensitive, therefore ensure to be stored in dark/black or aluminum wrapped tubes/bottles.

1.1.3. Water soluble vitamin solution (1000 mL): Dissolve 100 mg of thiamine hydrochloride (Thiamin-HCl), 100 mg of D-pantothenic acid, 100 mg of Niacin, 100 mg of pyridoxine, 5 mg of P-aminobenzoic acid and 0.25 mg of Vitamin B12 in deionized water to make total volume to 1000 mL.

1.1.4. Folate: biotin solution (1000 mL): Dissolve 10 mg of folic acid, 2 mg of D-biotin and 100 mg of ammonium bicarbonate (NH_4HCO_3) in deionized water to make total volume to 1000 mL.

1.1.5. Riboflavin solution (1000 mL): Dissolve 10 mg of Riboflavin in 5 mM HEPES (1.19 g/L) solution to make total volume to 1000 mL.

1.1.6. Hemin solution (10 mL): Dissolve 5000 mg of Hemin in 10 mM sodium hydroxide (NaOH) (0.4 g/L) solution to make total volume to 10 mL.

1.1.7. Short-chain fatty acid mix (10 mL): Combine 2.5 mL of N-valerate, 2.5 mL of isovalerate, 2.5 mL of isobutyrate and 2.5 mL: DL-α-methylbutyrate.

NOTE: This solution is recommended to use in fume hood to avoid smell and fumes.

1.1.8. Resazurin (1000 mL): Dissolve 1 g of resazurin in deionized water and make total volume to 1000 mL.

1.2. Medium used for in vitro anaerobic fermentation

1.2.1. To prepare this media, mix 330 mL of Solution A, 330 mL of Solution B, 10 mL of Trace mineral solution, 20 mL of Water soluble vitamin solution, 5 mL of Folate:biotin solution, 5 mL of Riboflavin solution; 2.5 mL of Hemin solution, 0.4 mL of Short chain fatty acid mix, 1 mL of Resazurin, 0.5 g of yeast extract, 4 g of sodium carbonate (Na_2CO_3), 0.5 g of Cysteine HCl-H_2O, and 0.5 g of Trypticase, and add 296.1 mL of distilled water.

1.2.2. Check the pH and ensure it is around 7.0, if not, adjust the pH with 1 N HCl or 1 N NaOH. Sterilize by vacuum filtering the media using a bottle filter under the aseptic workstation.

1.2.3. Alternatively, mix all the components (except vitamin and hemin solutions) and autoclave at 121 °C for 20 min and let it cool down to room temperature. Simultaneously, filter-sterilize vitamin and hemin solutions using 0.22 µm membrane filters and add these to the autoclaved and cooled media before dispensing.

2. Preparation of anaerobic chamber and required material

2.1. Keep all the materials, solutions and tools needed for the fermentation experiment inside the anaerobic chamber at least 48 h before the start of the experiment, to ensure that any residual oxygen associated with tools and soluble oxygen in buffers/solutions is removed and all the materials are acclimatized to the set anaerobic conditions.

NOTE: Materials needed inside the anaerobic chamber (48 h earlier of experiment to start): (i) fermentation media; (ii) anaerobic solution (prepared according to section 4.1), (iii) vortexer, (iv) weighing balance, (v) muslin cheese cloths, (vi) scissors, (vii) funnel, (viii) 1.5, 2.0, 15, 50 mL tubes, (ix) pipettor (2, 20, 200 and 1000 µL) and compatible pipet tips, (x) gun pipet and pipets (5 and 10 mL), (xi) paper tissues, (xii) markers, (xiii) tube stands for different tubes, (xiv) waste box, (xv) O_2 indicator and (xvi) 70% ethanol spray bottle (disinfectant).

3. Preparation of tubes and fibers

3.1. Weight 300 mg of inulin and transfer to a 50 mL tube followed by the aseptically addition of 26 mL of fermentation media already prepared and stored in the anaerobic chamber. Prepare one blank tube for each fecal sample type and experimental tube(s) (according to the number of compounds being tested), in triplicate.

3.2. Leave these tubes inside the anaerobic chamber for around 24 h to allow hydration of samples before starting the fermentation experiment. Ensure the tube temperature is 37 °C at the time of inoculation, therefore bring the tubes in the incubator enclosed within the anaerobic chamber.

4. Preparation of inoculum

4.1. Anaerobic dilution solution (at least 48 h before fermentation experiment): Dissolve 5 g of NaCl, 2 g of glucose and 0.3 g of Cysteine-HCl in deionized water and make total volume to 1000 mL. Autoclave and store it inside the anaerobic chamber at least 48 h before use.

4.2. Fecal inoculum preparation (at the day of fermentation experiment)**:** Weigh 5 g of fresh fecal sample in a 50 mL conical tube, add anaerobic dilution solution for a final volume of 50 mL (1:10 w/v) and vortex for 15 min or until completely homogenized. Filter the homogenized mixture through 4 layers of sterile cheesecloth (autoclaved) and use it immediately for inoculation in the tubes containing media.

NOTE: The fecal samples from a group of subjects can be pooled if the experimental objective is to compare the effect of a given compound/ingredient on overall healthy versus diseased fecal microbiota in general.

5. Fermentation and sampling

5.1. Prepare tubes according to the section 4.2 and inoculate blank/control and experimental tubes with 4 mL of diluted and filtered fecal inoculum. Incubate the inoculated tubes at 37 °C inside the anaerobic chamber. Shake the tubes once every hour by inverting gently to re-suspend the fibers and inoculum.

5.2. Collect samples as frequently as needed, for example, hourly to 0, 3, 6, 9 and 24 h during fermentation by taking a 2 mL aliquot sample out into a 2 mL tube from respective fermenting tubes.

5.3. Measure the pH of the aliquots using a laboratory pH meter (by directly inserting the pH electrode in the sample); centrifuge the remaining sample at 14000 x *g* for 10 min at 4 °C. Immediately freeze the supernatant for SCFAs analysis and store the pellet at -80 °C for microbiome analysis.

6. Short chain fatty acids (SCFAs) and lactate quantification

NOTE: The SCFAs and lactate in the supernatant of microbiome culture can be measured exactly according to the methods detailed elsewhere[13-16].

6.1. Briefly, thaw the snap frozen supernatants obtained in section 5 from control and treatment samples on ice and carry out all further processing steps on ice. Filter the supernatant through a 0.45 µm membrane filter and use the cell-free samples to measure the concentrations of SCFAs and lactate using HPLC system with DAD detector at 210 nm, equipped with an HPX-87H column. Use inject volume of 10 µL for each sample and use H_2SO_4 (0.005 N) to elute the column at a flow rate of 0.6 mL/min at 35 °C.

7. Fecal microbiome analysis

NOTE: Perform microbiome analysis following the methods and pipeline detailed elsewhere[7,13,14,17].

7.1. Briefly, extract genomic DNA from approximately 200 mg of fecal slurry pellets by using a fecal DNA extraction kit[13].

7.2. Amplify the hypervariable region of the bacterial 16S rRNA gene by using the barcoded primers as per the method described elsewhere[13], using the primer sequences as described in the Earth Microbiome Project protocol[18].

7.3. Purify the resulting amplicons with magnetic purification beads and quantify by Picogreen or an equivalent method. Pool the purified PCR products in equal molar concentration and sequence on a sequencer[13].

7.4. Process the resulting sequences for de-multiplexing, quality filtering and clustering, taxonomic assignment and rarefaction, and downstream analyses by using the Quantitative Insights into Microbial Ecology (QIIME) software as per the methods described by Caporaso et al[19].

REPRESENTATIVE RESULTS:

The protocol is used to demonstrate the effect of a specific prebiotic (i.e., inulin on the microbiota composition and metabolic activities in terms of changes in the fecal pH and the concentration of lactate and SCFAs in the feces of healthy human subjects over different time-points following treatment with inulin). The fecal pH, the fecal levels of lactate and SCFAs (**Figure 1**), and the microbiota composition (**Figure 2** and **Figure 3**) are measured at 0 (baseline), 9 and 24 h of incubation with or without inulin. The results demonstrate how fecal microbiota composition and its metabolic activities are modulated during in vitro fermentation with or without inulin treatment.

FIGURE AND TABLE LEGENDS:

Figure 1. **Changes in fecal pH (a), lactate (b) and short-chain fatty acids viz. acetate (c), propionate (d) and butyrate (e) in human feces at 0 (baseline), 9 and 24 h of anaerobic fermentation with or without inulin.** Values presented here are Mean ± SEM of triplicate samples. *P<0.05, **P<0.01, ***P<0.001, vs. baseline.

Figure 2. **Changes in the microbiota diversity and composition in human feces at 0 (baseline), 9 and 24 h of anaerobic fermentation with or without inulin**. (**a**) Weighted and (**b**) unweighted Unifrac measures of beta-diversity visualized using Principle Coordinate Analysis (PCoA). (**c**-**f**) Indices of alpha-diversity viz. phylogenetic diversity (PD whole tree (**c**); species richness (Chao1; **d**); observed number of operational taxonomic units (OTUs, **e**); and species evenness (Shannon index, **f**)). Relative abundance of major phyla (**g**) and genera (**h**). Values presented here are Mean ± SEM of triplicate samples. *P<0.05, **P<0.01, vs. baseline.

Figure 3. **Linear discriminant analysis (LDA) effect size (LEfSe) analysis of gut microbiota changes following 9 h and 24 h incubation with (INU) or without (CTL) inulin.** Taxonomic cladogram derived from LEfSe analysis of 16S sequences (relative abundance ≥ 0.5%) representing the differentially abundant taxa between different group of samples. The brightness of each dot is proportional to its effect size (i.e., the taxon abundance). Only taxa passing LDA threshold value of >2.4 are shown here.

DISCUSSION:

The in vitro fecal slurry fermentation model presented here is a simple single-batch model to approximate the effects of different substrates and microbial strains (e.g., prebiotics and probiotics) on the composition of human fecal microbiota as well as its metabolic activities in terms of fecal pH and SCFAs levels. The results presented herein demonstrate that the inoculation of inulin decreases the fecal pH and significantly increases the levels of SCFAs and lactate in inulin-treated fecal specimen as compared to non-treated fecal microbiota culture (**Figure 1**). In addition, the gut microbiota signature also appears to be different between inulin-treated and untreated samples (**Figure 2** and **Figure 3**). These data exemplify how this system can reflect the effects of inulin on fecal microbiome diversity and composition as well as its metabolic activities. In addition, depending on specific experimental objectives and hypotheses, a variety of other factors can also be measured using this system. Furthermore, in addition to 16S rRNA gene sequencing, other analyses such as whole microbial metagenome sequencing (using Whole genome sequencer) or qPCR and culture methods targeted at specific single or multiple genera, species and strains (e.g., bifidobacteria, lactobacilli, *Akkermansia*, *Enterobacteriacaea*, clostridia, etc.) can also be executed. In addition, different chromatographic procedures for the analysis of SCFAs, such as LC-MS, GC, GC-MS, GC-FID, and HPLC can also be exploited based on experimental requirement and availability. Nevertheless, it should be noted that sample preparation for these procedures may vary according to the instruments and conditions required for operation[20].

Although the use of fresh fecal specimen would yield best reproducible results; however, snap frozen fecal sample (as used in the experiment presented herein) can also be used efficaciously as most of the bacteria can be revived from it once resuscitated to body temperature and no more decomposition happens after the freezing[21]. Using frozen sample can be particularly advantageous when it is impossible to obtain fresh fecal samples from specific donor(s) on planned day of the experiment, or from the same donor when an experiment needs to be replicated. It should be noted, however, that the fecal samples should be snap frozen using liquid nitrogen and immediately stored at -80 °C until further use. In addition, to avoid the exposure of the frozen fecal samples to air (oxygen), the samples should be transferred to the anaerobic chamber as soon as possible/immediately after taking out from the freezer and used right away (repeated thawing should be avoided). All subsequent experiments including sample thawing, inoculum preparation and processing should be done inside the anaerobic chamber.

The intestinal organic environment and pH during nutrient fermentation in large bowel are very important particularly in view of the fact that an abnormally reduced pH indicates increased acidity due to the substrate utilization. Hence, more rapid pH reduction can correspond to more

rapid substrate utilization[22]. Although, in this model, the pH has not been controlled, however, inclusion of identical non-treated control is highly recommended to make direct comparisons. The SCFAs produced in the colon as a result of the fermentation of indigestible polysaccharides by the gut microbiota can further influence diverse mechanisms related to the maintenance of the host health. These metabolites contribute in gluconeogenesis and lipid biosynthesis, act as an energy source for colonocytes, and can also have health benefits including immune modulation, controlled/improved gut barrier functions, and neuromodulation[23-26]. In addition, SCFAs are also known to influence several biological pathways including hormones, endocannabinoid system, cell proliferation and death, bone health, mineral absorption, gut motility, intestinal pH, and invert effects on the gut microbiome and microbial metabolic function. Therefore, knowledge of the profile and concentrations of intestinal/ fecal SCFAs can be an important component while evaluating the efficacy of specific microbiota modulators[6]. Of course, besides SCFAs, the gut microbiome also produces many other important metabolites (e.g., ammonium, vitamins, histamine). Hence, the supernatant specimens collected during such in vitro fermentation experiments can also be evaluated for global metabolomics analyses to discover novel gut microbiome-derived metabolites that can be influenced by specific microbiome modulators.

The system described here has many advantages, such as the ease, simplicity, cost effectiveness, and the general adoptability of the experimental set-up. However, there are few limitations as well. For example, the system does not pertain to the interaction of prebiotics (or other interventions used) in the upper digestive system (e.g., saliva, stomach, small intestine) before being exposed to the microbiota of the large intestine. However, such steps can be adopted and incorporated according to specific experimental requirements. Also, an acidic and/or enzymatic hydrolysis process may need to be performed before fermentation if using specific substrates including whole food or digestible food because only the indigestible part of such foods reaches to the colon to be fermented by the lower gut microbiota. In addition, the composition of gut microbiota may shift due to specific culture conditions during fermentation. For example, it was observed that up to 9 h of incubation, microbiota changes are much closer to normal than at 24 h. However, after 24 h of incubation, although pH and SCFAs levels increased substantially, gut microbiota composition showed that *Proteobacteria* counts increased while the microbial diversity reduced. However, it remains unknown how precisely and closely the increased accumulation of microbial metabolites accompanied with the lowered fecal pH is associated with specific microbiota signatures.

In summary, a simple protocol to simulate the ex vivo microbiota ecosystem is described herein. The system enables researchers to test different interventions for modulation of the microbiota diversity, composition and metabolic function that can influence diverse features of the host intestinal and overall health.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

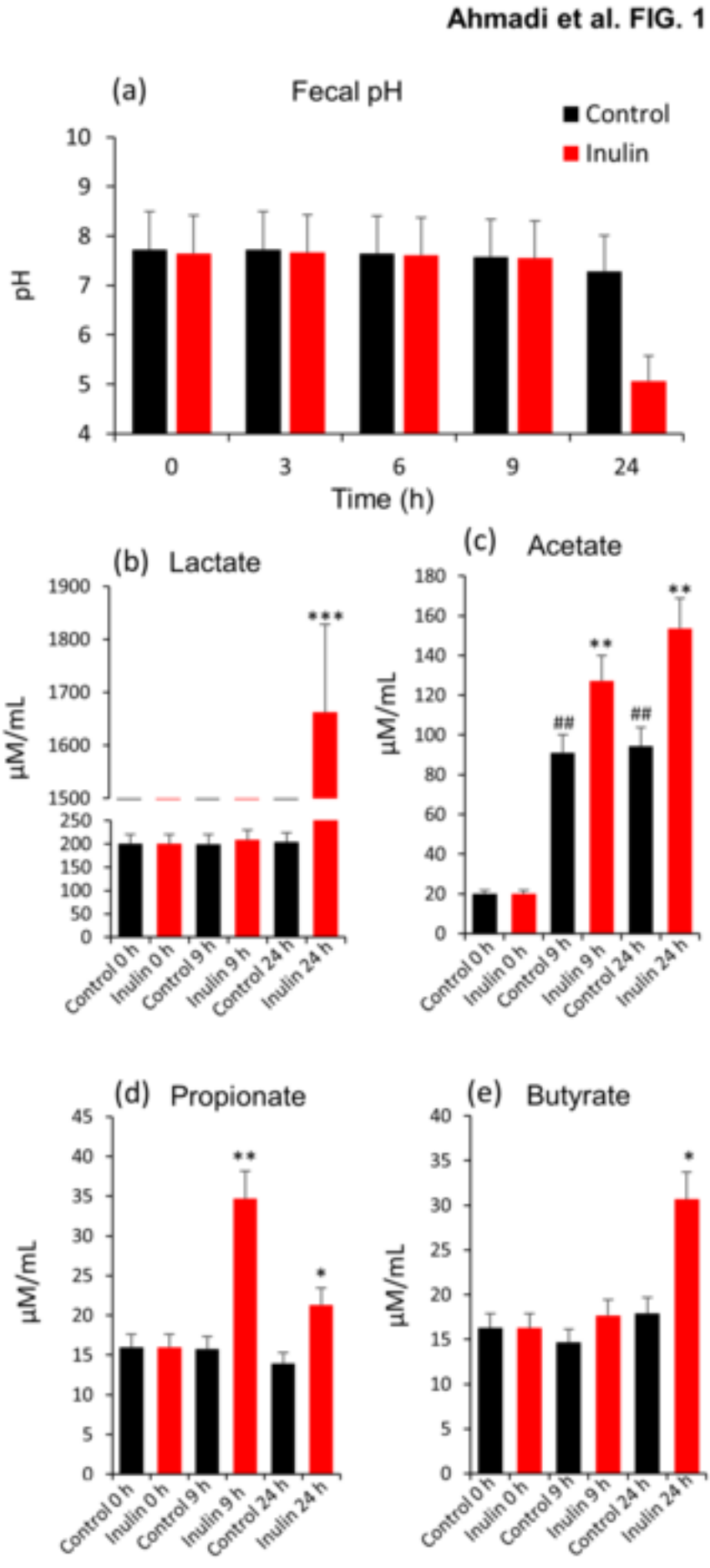


Figure 2

Click here to access/download;Figure;Figure 2-Ahmadi et al.tif

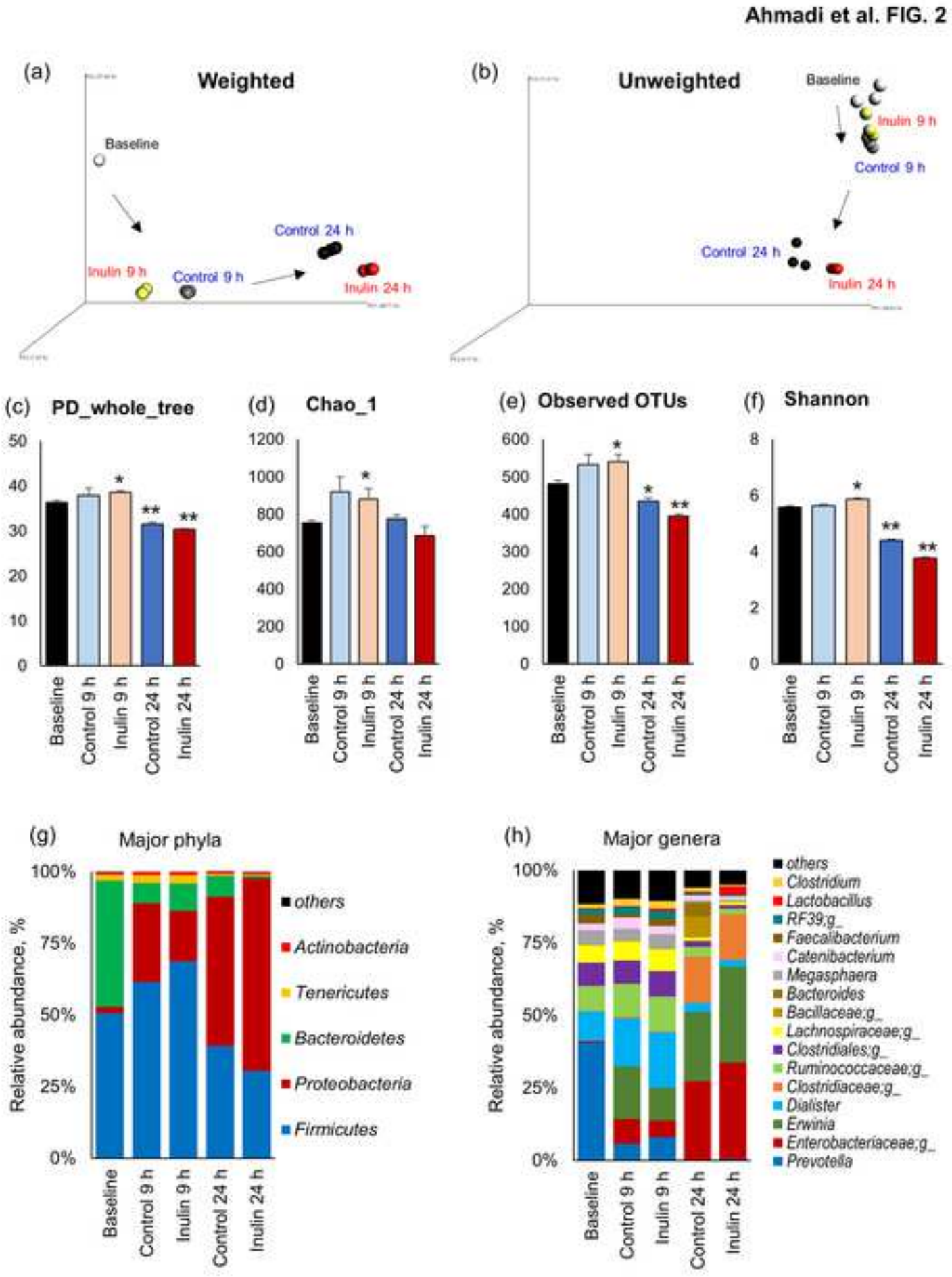
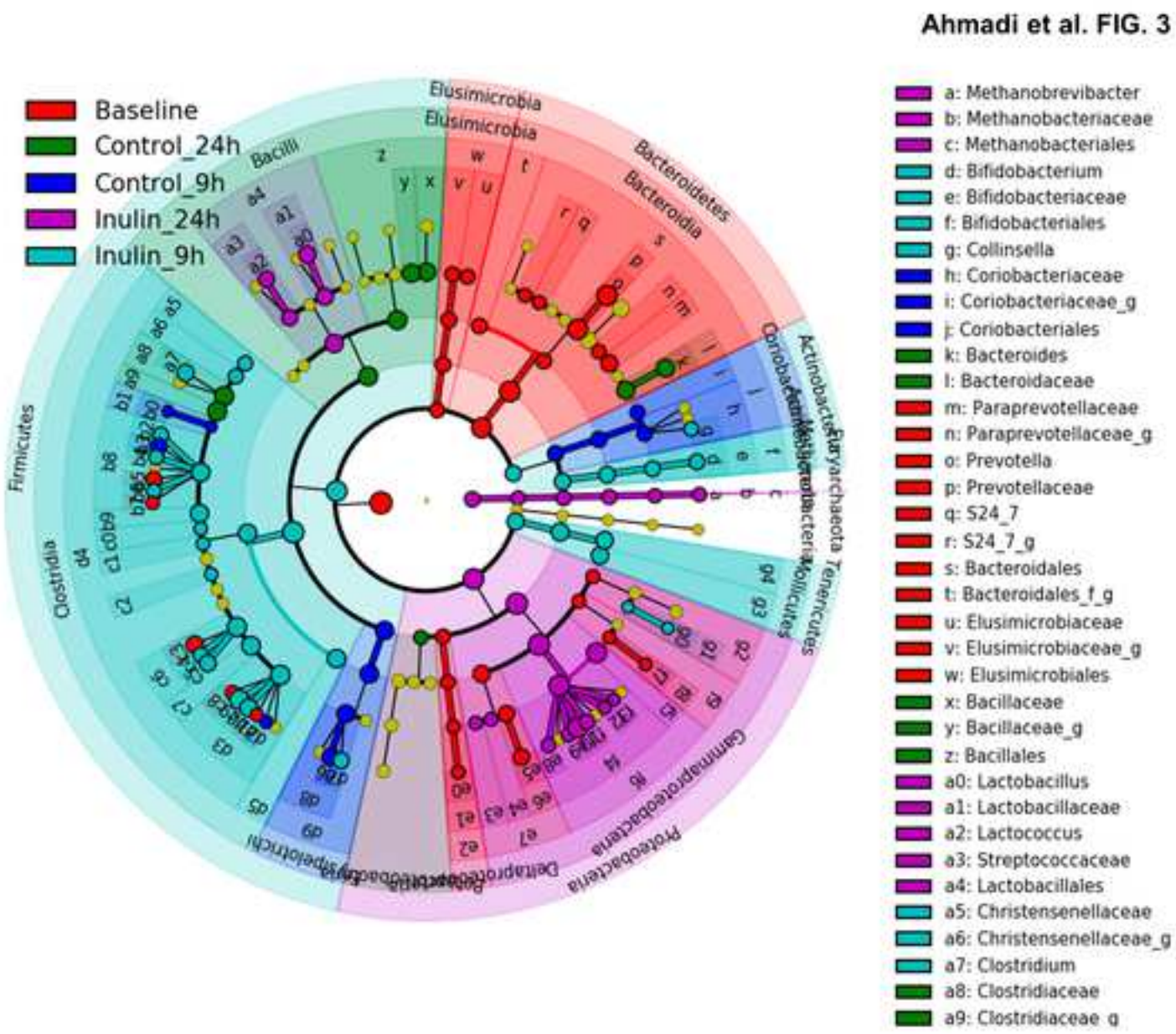


Figure 3

Click here to access/download;Figure;Figure 3-Ahmadi et al.tif



MATERIALS

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Ammonium Bicarbonate (NH_4HCO_3)	Sigma-Aldrich	217255	
Ammonium Sulfate $(NH_4)_2SO_4$	TGI	C2388	Toxic
Calcium Chloride Dihydrate ($CaCl_2 \cdot 2H_2O$)	Sigma-Aldrich	C3306	Irritating
Cobaltous Chloride Hexahydrate ($CoCl_2 \cdot 6H_2O$)	Sigma-Aldrich	255599	
Cupric Chloride Dihydrate ($CuCl_2 \cdot 2H_2O$)	Acros organics	2063450000	Toxic, Irritating
Cysteine-HCl	Sigma-Aldrich	C121800	
D-biotin	Sigma-Aldrich	B4501	
D-Pantothenic acid	Alfa Aesar	A16609	
Disodium Ethylenediaminetetraacetate Dihydrate (Na_2EDT	Biorad	1610729	
DL-α-methylbutyrate	Sigma-Aldrich	W271918	
Ferrous Sulfate Heptahydrate ($FeSO_4 \cdot 7H_2O$)	Sigma-Aldrich	F8263	Toxic
Folic acid	Alfa Aesar	J62937	
Glucose	Sigma-Aldrich	G8270	
Hemin	Sigma-Aldrich	H9039	
Hepes	Alfa Aesar	A14777	
Isobutyrate	Alfa Aesar	L04038	
Isovalerate	Alfa Aesar	A18642	
Magnesium Chloride Hexahydrate ($MgCl_2 \cdot 6H_2O$)	Sigma-Aldrich	M8266	
Manganese Chloride Tetrahydrate ($MnCl_2 \cdot 4H_2O$)	Sigma-Aldrich	221279	
Niacin (Nicotinic acid)	Sigma-Aldrich	N4126	
Nickel(Ii) Chloride Hexahydrate ($NiCl_2 \cdot 6H_2O$)	Alfa Aesar	A14366	Toxic
N-valerate	Sigma-Aldrich	240370	
P-aminobenzoic acid	MP China	102569	Toxic, Irritating
Phosphoric Acid (H_3PO_4)	Sigma-Aldrich	P5811	
Potassium Dihydrogen Phosphate (KH_2PO_4)	Sigma-Aldrich	P5504	
Potassium Hydrogen Phosphate (K_2HPO_4)	Sigma-Aldrich	1551128	
Pyridoxine	Alfa Aesar	A12041	
Resazurin	Sigma-Aldrich	R7017	
Riboflavin	Alfa Aesar	A11764	
Sodium carbonate (Na_2CO_3)	Sigma-Aldrich	1613757	
Sodium chloride (NaCl)	Fisher BioReagents	7647-14-5	
Sodium hydroxide (NaOH)	Fisher Chemicals	S320	
Sodium Molybdate Dihydrate ($Na_2MoO_4 \cdot 2H_2O$)	Acros organics	206375000	
Thiamine Hydrochloride (Thiamin-HCl)	Acros organics	148991000	
Trypticase	BD Biosciences	211921	
Vitamin B12	Sigma-Aldrich	V2876	
Yeast extract	Sigma-Aldrich	70161	
Zinc Sulfate Heptahydrate ($ZnSO_4 \cdot 7H_2O$)	Sigma-Aldrich	Z0251	
0.22 µm membrane filter			
AMPure magnetic purification beads	Agencourt		
Anaerobic chamber with incubatore	Forma anaerobic system, Thermo Scientific, USA		
Bottle filter	Corning		
Cheesecloth			
Illumina MiSeq sequencer	Miseq reagent kit v3		
pH meter			
Qiagen PowerFecal kit	Qiagen		
Quantitative Insights into Microbial Ecology (QIIME) software			
Qubit-3 fluorimeter	InVitrogen		
Vortex	Thermoscientific		
Waters-2695 Alliance HPLC system	Waters Corporation		

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Author(s): Shokouh Ahmadi, Shaohua Wang, Ravinder Nagpal, Rabina Mainali, Sabihe Soleimanian-Zad Dalane Kitzman, Hariom Yadav

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February 12, 2019

To,
The Editor, JoVE

RE: Submission of the revised manuscript (MS#: JoVE59524)

Dear Editor,

On behalf of the all our co-authors, I would like to extend sincere thanks for considering our work and your valuable time and efforts to handle our manuscript entitled, "**An in-vitro batch-culture model to estimate the effects of interventional regimens on human fecal microbiota**". Thanks for to providing your valuable feedback quickly to fix the errors of this manuscript (MS#: JoVE59524). We have addressed all the points raised by editorial office, and the response point-by-point rebuttal is below in this letter and all the changes are included in revised version of the manuscript.

The revised manuscript files are submitted in the manuscript system. We again thank a lot to you, and your whole team of the JoVE for the efforts and consideration of our work to publish in your esteemed journal.

Please feel free to contact me if more information is needed.

Looking forward to hear for your decision soon.

With best regards

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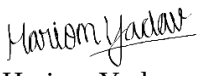

Hariom Yadav

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1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Authors: This has been checked and fixed the errors, if find any.

2. The summary is over the 50 word limit.

Authors: We revised this section and now is 46 words.

3. Please do not abbreviate journal titles for all references.

Authors: References have been adopted from Endnote library using Jove style. However, we have manually fixed this in revised version.

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Authors: There are more than 500 adopter PCR primer sequences, which are not possible to include them here, however we provided the reference, which readers can follow.

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