Int J Clin Exp Pathol 2016;9(3):3143-3151
www.ijcep.com /ISSN:1936-2625/IJCEP0018761

Original Article

Effect of simulated microgravity on porphyromonas gingivalis strain ATCC33277 growth and gene expression

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Received October 29, 2015; Accepted December 25, 2015; Epub March 1, 2016; Published March 15, 2016

Abstract: Background: P. gingivalis is the prime aetiological bacteria of initiation and progression of periodontal disease. It is also the risk factor of many system diseases, such as cardiovascular disease, diabetes and pulmonary infection. This study is aiming to demonstrate the effects of stimulated microgravity on p. gingivalis ATCC33277. Methods: We performed the rotary cell culture system (RCCS) to stimulate the microgravity effectively and analyzed the transcriptional adaptation and the changes of growth and genes expression profile by high-throughput RNA sequencing (RNA-Seq). Results: We found the SMG condition can enhance the bacterium growth, and 61 genes were differentially expressed in SMG conditions compared to that of NG condition among all the genes of P. g ATCC33277 by global gene expression analysis. These genes were involved in a wide range of biological processes and biological pathways, including modulation of DNA replication, recombination, and repair; energy metabolism; two-component system; one carbon pool by folate; microbial metabolism in diverse environments. Under the SMG 9 genes were up-regulated. Simultaneously 52 were down-regulated. Conclusion: Our study demonstrated changes in global gene expression under SMG condition. The DEGs were linked to 666 gene ontology (GO) terms. These findings provide a foundation for future studies on genetic and molecular mechanisms associated with oral bacteria changes on SMG condition, and may provide better safeguard for the astronaunts.

Keywords: P. gingivalis ATCC33277, microgravity, growth, gene expression

Introduction

Periodontitis is a difficult-to-eradicate periodontium chronic infection caused by multifactors [1]. It influences people's quality of life at various aspects for its highly prevalence rate and nasty consequence. P. gingivalis is strongly implicated as aetiological agents of periodontal diseases [2]. Besides, P. gingivalis infection is also associated with many serious systemic diseases, such as atherosclerosis [3], pulmonary infection [4], diabetes [5], preterm delivery of low birth-weight infants [6] and Rheumatoid arthritis [7], etc. It is irritating that we can find *P.* gingivalis even in the oral cavities of oral healthy population [8]. Previous research found that many bacteria change their growth and other physiology when exposed to microgravity, also their genes associated with starvation response, acid stress, osmotic stress, oxidative stress, biofilm formation may up or down expressed [9-13]. Up to now, as we known, there is no research on how the P. gingivalis will response to microgravity.

Gravity can produce two effects on an object: motion and/or weight. As long as gravity is present, one or both of these reactions will occur and can be analyzed. If without the significant equal and opposite resistance needed to impart weight, an orbiting object simply experiences a continuous state of gravity-induced free fall (i.e., accelerated motion) around the earth. This state is astronauts suffered during spaceflights commonly referred as microgravity, the condition of weightlessness that causes severe physiological alterations in the human body [14]. When exposed to microgravity, cells experience reduction of gravity resulting in a relative lack of sedimentation, low shear stress and low turbulence [15].

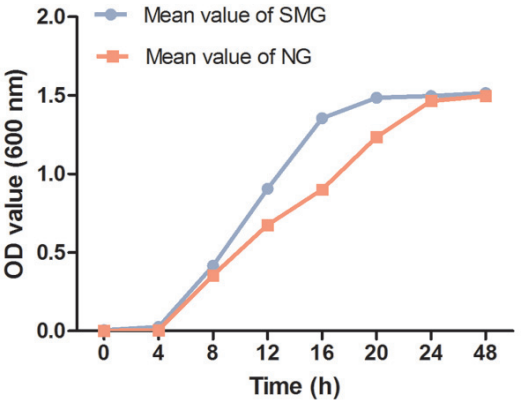

Figure 1. The growth curves of P. gingivalis in SMG group and NG group from beginning to 48 h.

Although the Effects of microgravity on many bacteria had been analyzed, the effect of microgravity on P. gingivalis has not been extensively studied. In this study, we focused on the effects of microgravity on P. Gingivalis ATCC33277.

Methods and materials

Bacterial source and culture condition

Porphyromonas gingivalis strain ATCC33277 was obtained from the culture collection of the stomatological hospital, the Capital University of Medical Sciences. *P.* gingivalis was coated or streaked on BHI-HM blood agar plates incubated at 37°C, 90% N_2, 5% H_2, and 5% CO_2 in a MK3 anaerobic workstation (Don Whitley Scientific, Beijing, China) with a gas composition of 5% CO_2, 5% H_2 and 90% N_2. And then the strain was inoculated in liquid BHI medium incubated at 37°C, 90% N_2, 5% H_2, and 5% CO_2 in a MK3 anaerobic workstation.

Control of bacteria amount

Bacterial cell density was monitored by measuring the absorbance at 600 nm (OD600). To investigate the growth of P. gingivalis, initial cultures were grown at 37°C to an optical density at 600 nm (OD600) of 0.6, and then diluted 1/100 in fresh medium, followed by incubation at 37°C. Bacterial growth was monitored from this point by measuring OD600.

Groups of experiments

For the stimulated microgravity experiment, P. gingivalis were then transferred into a 55 mL high-aspect-ratio vessel (HARV Synthecon) filled with culture medium in the rotary cell culture system (RCCS). We used two RCCS and placed them vertically and horizontally separately in the MK3 anaerobic workstation. The rotational speed of the vessel was set according to the weight, size and density of bacteria to maintain free-fall conditions, and an average microgravity of 10^{-2} ×g at a speed ranging from 20 rpm to 25 rpm to maintain the complexes in a relatively steady position within the vessel. The bacteria cultured in vertically vessels were under the stimulated microgravity condition (SMG), while the bacteria cultured in horizontal vessels were under normal gravity conditions as control group (NG).

Collection of bacterium solution

After 48 h of cultured, the bacteria were harvested by centrifugation (8,000 g) at 4°C for 5 min and pellets were washed with 50 mM Tris-HCl (pH 7.8) at 4°C, and then stored at -80°C until RNA extraction. At least two independent cultures were prepared for each time point.

RNA extraction

Total RNA of *P.* gingivalis was extracted with Trizol reagent (Invitrogen, Grand Island, NY, USA), and digested with DNase I (RNase-free; MBI Fermentas, St. Leon-Roth, Germany) to remove trace DNA. RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

RNA sequencing (RNA-Seq) and differentially expressed genes (DEGs) analysis

The sequencing library was prepared using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following previous studies [16, 17]. Briefly, 3 μg of total RNA from per specimen was obtained to use for mRNA enrichment selection by poly-T oligo-attached magnetic beads. Then divalent cations under elevated temperature in NEBNext First Strand

Table 1. Summary of Illumina RNA-seq data

Sample name	SMG1	SMG2	NG1	NG2
Total reads	11631288	11092378	10083732	11484150
Total mapped	11506830 (98.93%)	10975968 (98.95%)	7391457 (73.3%)	11461207 (99.8%)
Percent mapped	98.93%	98.95%	73.3%	99.8%
Multiple mapped	719910 (6.19%)	713705 (6.43%)	403175 (4.00%)	677062 (5.90%)
Uniquely mapped	10786920 (92.74%)	10262263 (92.52%)	6988282 (69.3%)	10784145 (93.9%)
Read-1	5392301 (46.36%)	5129061 (46.24%)	3495461 (34.66%)	5391525 (46.95%)
Read-2	5394619 (46.38%)	5133202 (46.28%)	3492821 (34.64%)	5392620 (46.96%)
Reads map to '+'	5392944 (46.37%)	5130775 (46.25%)	3492962 (34.64%)	5391964 (46.95%)
Reads map to '-'	5393976 (46.37%)	5131488 (46.26%)	3495320 (34.66%)	5392181 (46.95%)

P.S: Total reads: the statistics numbers of sequences after sequencing data filtered (Clean data); Total mapped: the statistics numbers of sequences positioned on the genome sequence; Multiple mapped: the statistics numbers of sequences with multiple mapped on the reference sequence; Uniquely mapped: the statistics numbers of sequences with uniquely mapped on the reference sequence; Reads map to '+', Reads map to '-': the statistics of plus strand and minus strand in the genome.

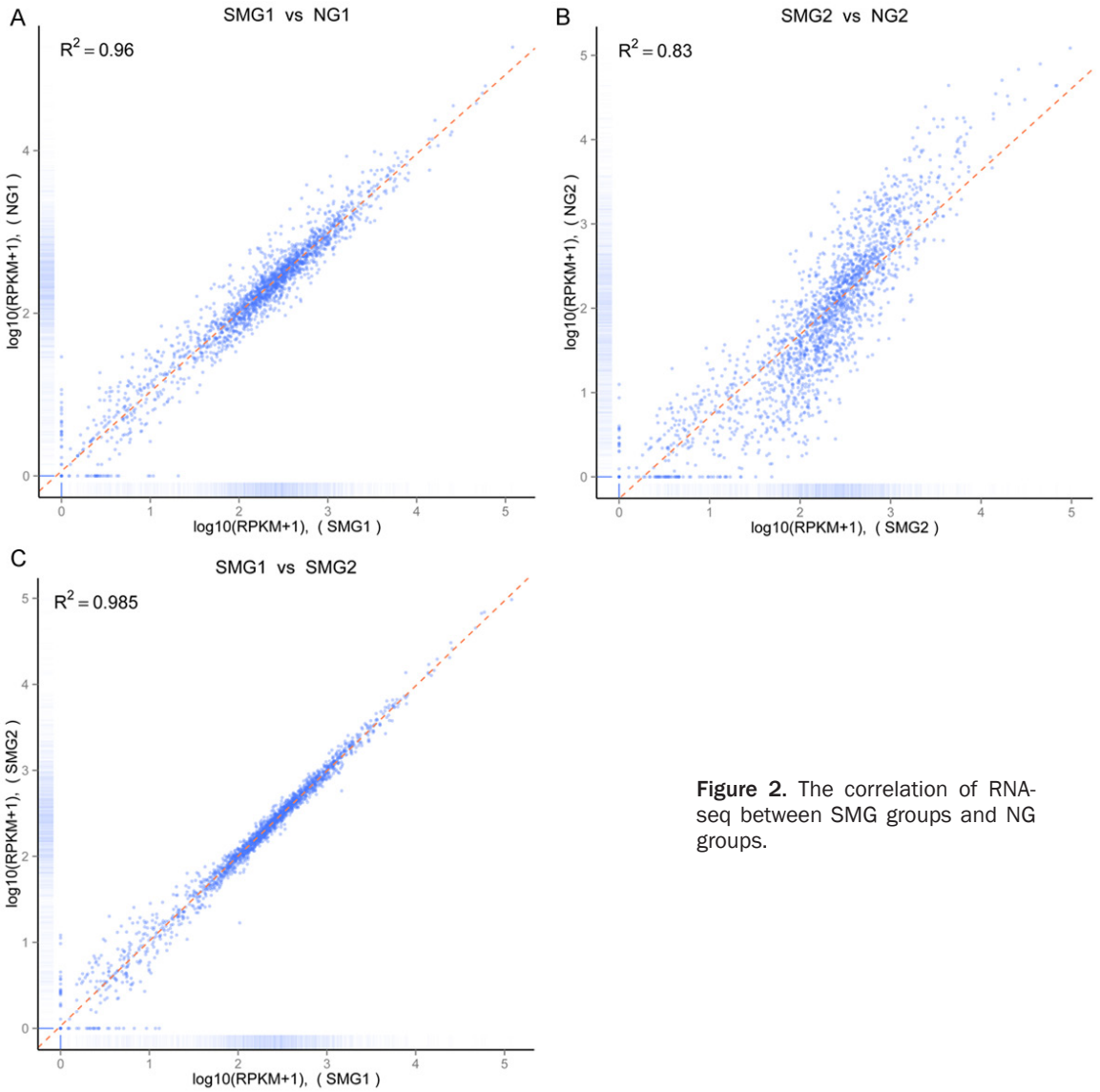

Figure 2. The correlation of RNA-seq between SMG groups and NG groups.

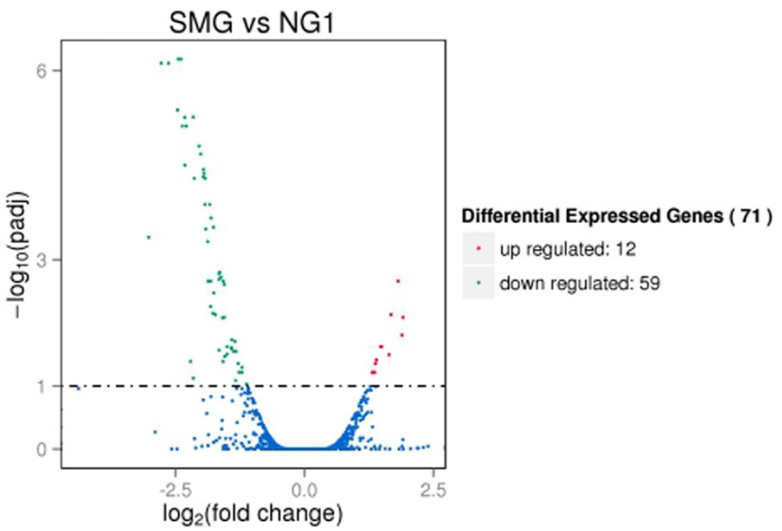

Figure 3. Scatter plot of RPKM distribution in SMG and NG transcripts. The blue dots provide the transcripts with RPKM less than 2-fold changes between SMG and NG.

Synthesis Reaction Buffer (5×) was used to obtain the mRNA fragment. Using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H) the first strand cDNA was synthesized by obtained mRNA as template. We followed that the second strand cDNA was synthesized after adding RNase H and DNA polymerase I. After exonuclease/polymerase activities the remaining overhangs were converted into blunt ends. Dual-strand cDNA with blunt-end was adenylated at 3' end, and the NEBNext Adaptor with hairpin loop structure was ligated to prepare for hybridization. Then the cDNA library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA) to preferentially select 150~200 bp cDNA in length. Before PCR, 3 μl USER Enzyme (NEB, USA) was added into size-selected and adaptor-ligated cDNA at 37°C for 15 min then 5 min at 95°C. PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index Primer. Finally, products of PCR were purified and the quality of cDNA library was assessed on the Agilent Bioanalyzer 2100 system.

Statistical analysis

In this study all experiments were repeated at least three times. The data are presented as the mean ± SD. SPSS software, version 16.0 was used to do the statistical analysis with Student's t-test. $P<0.05$ was accepted as statistically significant. Fisher's exact test and a hypothesis test method were performed to detect the significant differential expression of transcripts based on the Poisson distribution at $P<0.01$. In this article only v2 values of the former method were reported due to more confidence on statistical results. In order to ensure the credibility of expression information, the significant differential expression was only that the comparison with q-value was less than 0.05 and expression fold change was greater than 2 fold in the Cuffdiff output.

Results

Growth of P. gingivalis under SMG condition

To investigate differences in the growth of P. gingivalis under the normal gravity condition (NG) and stimulated microgravity condition (SMG), we measured the growth curves of P. gingivalis in the two groups from beginning to 48 h (**Figure 1**). Although the maximum biomass of P. gingivalis (OD600) was not significantly different, the growth rate of SMG was a little higher than that of NG. It was similar to the expression of streptococcus mutans, which our team described previously. The finding indicated that the bacteria had responded to SMG condition.

Analysis results of RNA-seq

In order to gain the deeper insight on the mechanisms of *P.* gingivalis ATCC33277 response to SMG condition, we performed RNA-seq analysis on transcriptomes from two biological replicates. In **Table 1**, over 99% of all clean reads aligned to coding regions of the P. gingivalis genome. And in **Figure 2**, the overall expression levels in the two biological replicates of each group were highly similar to each other ($R^2>0.8$), illustrating that our RNA-seq data was of suitable quality for transcriptome analysis. In total, 5,880,032 base pairs raw sequence data corresponding 5,815,644 clean reads and 5,613,464 basepairs raw sequence data corresponding 5,546,189 clean reads were gener-

Table 2. The 12 up-regulated P. gingivalis genes after cultured in SMG condition for 48 h

Gene_id	Readcount_SMG	Readcount_NG1	Log2 Fold Change	*P*val	*P*adj
PGN_0065	6666.032048	2142.812946	1.6373	0.00089406	0.032007
PGN_0583	1125.799033	400.1103011	1.4925	0.00057801	0.023864
PGN_0654	1503.639115	539.8313586	1.4779	0.00058176	0.023864
PGN_0970	1301.504144	501.7256156	1.3752	0.0013675	0.044142
PGN_1115	2109.741999	599.5303559	1.8152	3.53E-05	0.0021807
PGN_1617	1519.897298	577.9371016	1.395	0.0011154	0.03853
PGN_1733	12687.3018	3428.246675	1.8878	0.00034244	0.01568
PGN_1818	8018.247529	2133.921606	1.9098	0.00017049	0.0081877
PGN_1819	868.4556856	271.8209664	1.6758	0.00014619	0.0073903

Table 3. The top ten down-regulated P. gingivalis genes after cultured in SMG condition for 48 h

Gene_id	Log2 fold change	Padj	Gene name	Gene description
PGN_0554	-3.0189	0.00044	-	Hypothetical protein
PGN_0287	-2.777	7.70E-07	mfa1	Mfa1 fimbrilin
PGN_1953	-2.6355	7.70E-07	-	TonB-dependent outer membrane receptor
PGN_0180	-2.4624	4.23E-06	fimA	FimA type I fimbrilin
PGN_1905	-2.3934	6.61E-07	-	Hypothetical protein
PGN_0564	-2.3705	7.60E-06	sod	Superoxide dismutase Fe-Mn
PGN_0183	-2.319	5.54E-06	fimC	Minor component FimC
PGN_0604	-2.317	3.16E-05	-	Ferritin
PGN_0291	-2.2922	7.62E-06	-	Hypothetical protein
PGN_2058	-2.2106	0.040827	-	Hypothetical protein

ated from the stimulated microgravity groups (SMG1 and SMG2), respectively. 5,127,995 base-pairs raw sequence data corresponding 5,041,866 clean reads and 5,805,403 base-pairs raw sequence data corresponding 5,742,075 clean reads were generated from the normal gravity groups (NG1 and NG2).

Gene expression of P. gingivalis under SMG condition

Between SMG group and NG group, overall differential gene expression was observed. To further examine this, DEGs were identified using the DEseq package. A total of 71 DEGs were identified, including 12 up-regulated and 59 down-regulated genes (**Figure 3**). Among the 12 up-regulated genes (**Table 2**), 3 have not statistical significance. And also in the 59 down-regulated genes, 7 did not have statistical significance. Thus we identified 61 DEGs, containing 9 up-regulated and 52 down-regulated genes. This suggests that microgravity have effects on genes expression of P. gingivalis ATCC33277.

Among the 9 up-regulated genes, 3 genes (PGN_0583, PGN_0654, PGN_1819) were predicted genes with unknown function coded by hypothetical protein (HPs). Two of the remaining six genes encode HagA (hemagglutinin gene A) which is involved in the adhesion and invasion of P. gingivalis in human gingival epithelial cells. The others genes encode *traG* (conjugate transposon protein); RNA polymerase sigma-70 factor ECF subfamily; Metallophosphoesteras (a hydrolytic enzyme involved metal ions which can hydrolysis important phosphate substances and play extreme roles in the biochemistry reaction).

Among the top 10 down-regulated genes (**Table 3**), six were predicted genes of unknown function coded hypothetical protein (HPs) too. One of the remaining four genes encodes Fimbria (PEN_0180), which are associated with the colonization, the formation of biofilm of *P.* gingivalis. The other genes encode sod (PEN_0564), TonB-dependent outer membrane receptor (PEN_1953) and ferritin (PEN_0604).

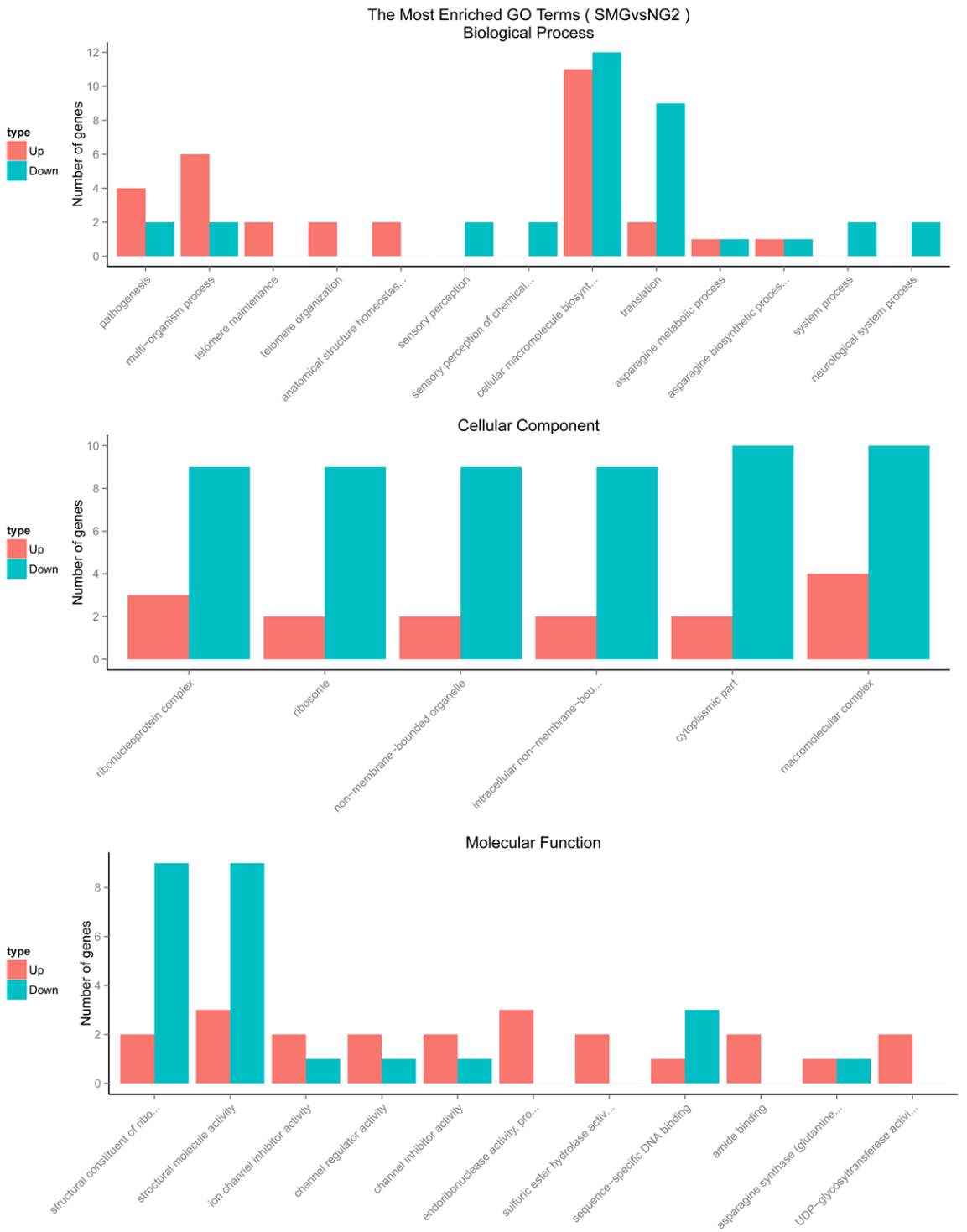

Figure 4. The number distribution of DEGs on biological process, cellular component and molecular function by GO term.

The analysis of GO and KEGG pathway

In the three main categories, biological process, cellular component and molecular function of GO, the different subcategories were at many aspects, including “superoxide metabolic process”, “cellular homeostasis” “spread of virus in multi-cellular”, “cellular homeostasis”,

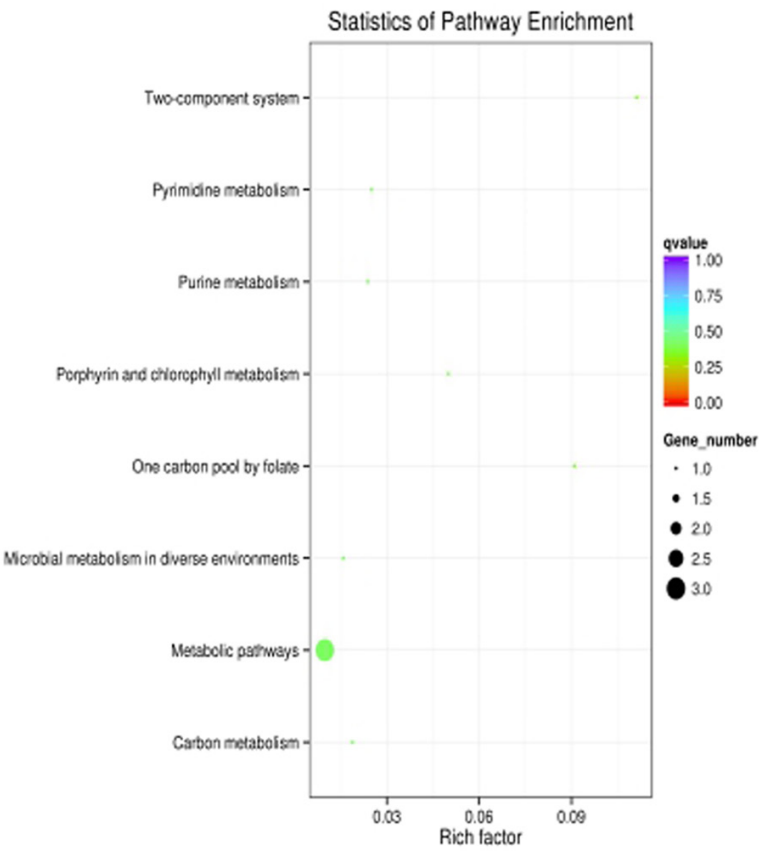

Figure 5. Eight pathways were predicted by KEGG statistics.

"oxidoreductase activity", etc (**Figure 4**). And then the biological functions associated with DEGs were further analyzed in terms of enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and a total of 8 pathways were predicted in **Figure 5**, including "Porphyrin and chlorophyll metabolism", "microbial metabolism in diverse environments", "One carbon pool by folate", and "two-component system", "Pyrimidine metabolism", "Purine metabolism", "Carbon metabolism", and "Metabolic pathways".

Discussion

Microgravity is a condition that the physical gravity of human bodies is reduced, and also the major condition to human in space [18, 19]. It can induce a series of changes in organisms from bacteria to humans. Gravity and sudden changes of gravitational forces may have significant impact on cells, and organ physiology [20]. For example, exposed to microgravity status for a long time, human body may suffer of loss bone, immunosuppression [21], cardiovascular problems [22], changes in metabolism and body temperature activity [23, 24], and so on. The microorganisms in human bodies are also influenced under microgravity status.

Periodontal disease including periodontitis is one of the common and complex diseases in human [25]. It is mainly caused by a variety of bacteria. P. gingivalis is an anaerobic, gram-negative bacterium that is implicated as a prime aetiological agent of initiation and progression of periodontal disease [26, 27]. In spite of the astronauts have perfect healthy physical and mental quality after strict selection at all aspects, the organism and bacteria special response to the special living condition into consideration. In addition few reporters revealed P. gingivalis responsed to microgravity, thus we aimed to develop a microgravity in vitro condition with obligate anaerobic culture system for P. gingivalis ATCC33277.

Many reporters have revealed that microgravity can affect cell growth and gene expression. Nancy et al. found that 4-8% T lymphocyte gene expression differentially in microgravity status [28]. Pardo et al. revealed that under microgravity condition, among 1000 osteoblast genes, 88 genes were obviously down-regulated and 52 genes were up-regulated [29]. And our results in this study were similar with the previous results.

We used RCCS to stimulated microgravity to detect the growth and genes expression of P. gingivalis ATCC33277. The growth rate of P. gingivalis ATCC33277 in SMG group was a little higher than that in NG group. This result indicated that microgravity condition may increase the growth rate of P. gingivalis ATCC33277. Almost all clean reads aligned to coding regions of the P. gingivalis genome, and overall expression levels in SMG and NG group were highly similar by analysis of RNA-seq. By

differentially expressed genes (DEGs) analysis, we identified up-regulated genes and down-regulated genes under SMG condition. In addition to further explore the differentially expressed genes, the analysis of GO and KEGG pathway were also performed. We got the differential genes number enriched on the GO term and the pathways via KEGG enrichment.

The essential key point of this study was the operational time, because some studies showed the bacteria could recuperate their characters if we moved them back from the microgravity to normal gravity condition for some time later. Our study revealed that microgravity can affect the growth and gene expression of P. gingivalis ATCC33277. The results of this study will provide deeper insight into transcriptional regulation of the microgravity on P. Gingivalis.

Acknowledgements

14QN11 and BZZ14J001.

Disclosure of conflict of interest

None.

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