利用单细胞扩增技术解析母婴间肠道微生物构成及功能

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摘要 微生物在人体中扮演重要角色, 而婴儿早期微生物对日后健康发育至关重要. 微生物可通过产道和母乳这2种主要途径从母亲传递给婴儿. 本研究利用单细胞扩增技术对3对母婴粪便样品(共48份细菌悬液)进行宏基因组测序分析, 旨在探究母婴间肠道微生物构成并对相关功能基因作出分析. 结果显示, 每对母婴间微生物尽管含量不同, 但种类都高度相似(>99%). 三婴儿中, 一个顺产婴儿显示与其母亲有相似的高丰度物种, 如*Roseburia hominis*, *Roseburia intestinalis*, *Eubacterium rectale*, *Eubacterium eligens*等; 另2个剖腹产婴儿显示与皮肤、口腔相关的高丰度物种, 如*Propionibacterium acnes*, *Staphylococcus saprophyticus*/S. *haemolyticus*, *Delftia acidovorans*等. 剖腹产婴儿中拟杆菌少于顺产婴儿, 如*Bacteroides* (*B.*) *fragilis*, *B. helcogenes*, *B. salanitronis*等. 本研究共检测到1283种细菌, 除常见的人体肠道微生物外, 还检测到4个低丰度物种, 分别是*Lactobacillus* (*L.*) *amylovorus*, *L. kefiranofaciens*, *L. sanfranciscensis*和*Bifidobacterium asteroides*. 粪便样品功能宏基因组显示, 婴儿粪便中富有与碳水化合物相关的磷酸转移酶系统(PTS)和 β -葡萄糖苷酶基因. 本研究初次使用单细胞扩增技术对母婴间肠道微生物组成及功能做出探索, 获得了较高测序深度及物种多样性, 发现了之前鲜有报道的低丰度物种, 证实单细胞扩增技术可用于后续不同生态环境微生物领域的研究.

关键词 母婴粪便, 单细胞扩增, 宏基因组, 细菌多样性, 低丰度物种

人体微生物仅占自身重量的1%~3%, 但细胞数超过人体的10倍, 基因数超过人体的100倍^[1,2]. 微生物在人体中扮演重要角色, 可促进机体消化吸收、调节免疫系统、促进婴儿脑及神经发育、维持牙齿健康以及抵抗病原菌入侵等^[3~6]. 人体微生物环境受多种因素影响, 其中婴儿早期微生物状况对日后长期健康发育尤为重要^[7]. 而产妇的年龄、饮食、抗生素使用、孕期病毒感染、分娩方式等均可短期改变婴儿肠道菌群结构并可能对发育产生长期的影响^[8~10].

最早人们认为, 母亲子宫内是无菌环境, 且在羊水破裂时婴儿才首次接触微生物. 这个“子宫无菌”

假说一直持续到多个世纪之后, 并教条地认为子宫内任何细菌的存在都对婴儿相当危险. 直到Jiménez等人^[11]给怀孕小鼠喂食接有遗传标记尿肠球菌(接种)的牛奶, 并在无菌剖腹产后检测子代胎便微生物. 结果显示, 只在喝了接种牛奶小鼠的子代胎便检测到带有遗传标记尿肠球菌, 而在喝了没接种牛奶小鼠的子代胎便中检测不到. 由此为哺乳动物“母婴间微生物传递”提供了有力依据. 除空气、产房、医护人员、母亲皮肤等环境因素外, 婴儿从母亲获得“垂直传递”的微生物途径主要有2种, 即产道和母乳^[9,12~14]. 越来越多的证据表明, 通过母亲垂直传递给婴儿的微生物是婴儿肠道的主要微生物并对后期

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胃肠道成熟及正常发育起关键作用^[15,16]。

新型测序技术的发展加快了多领域、多组学的研究步伐,包括物种的从头测序、疾病的遗传学及进化遗传学等。自然界中只有1%的微生物可培养,因此许多低数量或不可培养的“暗物质”不能通过传统方法实现精准测序。毫无疑问,人体肠道中存在很多这样的“暗物质”。而本研究采用的单细胞扩增方法,其多重置换扩增技术(MDA)与依靠PCR的全基因组扩增方法相比,扩增偏倚可降低3~4个数量级,并可将单个细菌细胞的微量DNA高精度扩增约50亿倍、产生平均长度大于12 kb的扩增子,从而使得基因片段被精确测序^[17-19]。因此本研究采用单细胞扩增技术对3对母婴粪便样品进行深度测序,目的是探究母婴间肠道微生物结构并对相关功能基因作出分析。

1 材料和方法

(i) 样品的制备。本研究招募来自内蒙古医科大学附属医院妇产科的3对母婴(序号分别为19, 26和40, 志愿者信息详见表1)。3名志愿者母亲孕期无服用抗生素情况,3个婴儿均足月生产。在分娩24 h内收集母亲和婴儿共6份粪便样品,采集方法如下:(1) 母亲粪便:将采样纸平铺在马桶上,志愿者用采样勺取15 g左右新鲜样品至50 mL无酶无菌采样管中,加1倍体积的样品保护剂混匀;(2) 胎儿粪便:接产医生用采样勺取15 g左右新鲜样品至50 mL无酶无菌采样管中,加1倍体积的样品保护剂混匀。以上所采粪便样品均用封口膜封口,迅速置于4℃便携冰箱或放满冰袋的泡沫盒中,尽快转移到-80℃超低温冰箱,之后通过干冰运输至测序地点。

样品在冰浴中解冻3~5 min,待融化后进行低速离心去除杂质和真核细胞团块。然后以13000×g离心15 min从上清液中沉淀原核细胞。将沉淀物重新悬浮于含有1% Triton X-100的2 mL磷酸盐缓冲盐水(PBS)中,并在37℃孵育2 h来裂解任何可能残留的真核细胞。随后,通过13000×g离心15 min使细菌沉淀,并将沉淀重悬于500 μL PBS中。最后,再次重复离心步骤以洗涤菌体细胞。

(ii) 梯度稀释和多重置换扩增。为了检测低丰度细菌,将来自每个粪便样品的细菌悬浮液连续稀释以用于随后的扩增反应。用细胞计数室在显微镜下粗略估计每个样品中的细胞数量。每份粪便样品连续稀释直到每个细菌悬浮液中的细胞数大约为

100,每样品取稀释液8份(即8个平行)进行后续扩增反应,编号分别为1, 2, 3...8。使用REPLI-g单细胞试剂盒(Qiagen, Germantown, MD, USA)进行稀释细胞的多重置换扩增(按说明书操作)。

(iii) 文库构建和测序。扩增的DNA片段被随机打断,选取约500 bp长度的片段进行文库的构建。之后使用PerkinElmer LabChip® GX Touch和StepOnePlus™ Real-Time PCR系统对文库质量进行检测。最后使末端配对125 bp的序列在Illumina HiSeq 2500平台进行测序(按说明操作)。每个粪便样品悬浮液产生约5 Gb的数据。

(iv) 序列质量检测和过滤。原始序列中可能含有在文库构建过程中由人工引起的接头污染。因此,为获得高质量、无污染数据,需要执行以下3个步骤:(1) 去除接头污染的Reads;(2) 删除平均得分低于Phred Quality Score值为Q30的序列;(3) 去除含“N”比例≥5%的Reads。之后基于Clean Data进行下游分析。此外,计算基于Q30和GC含量的基础质量。通过比对宿主基因组以除去宿主来源的污染序列。在与细菌(或病毒)基因组参考序列进一步比较之前,丢弃任何宿主来源的序列。利用Burrows-Wheeler aligner (BWA, 0.97a版本)MEM软件来获得更准确的比对结果^[20]。

(v) 序列分类学分配和物种多样性。利用网络软件Metaphlan对属和种水平进行分类^[21];利用R相关包分析α多样性从而比较样本内的物种多样性。

(vi) 序列组装、基因预测及注释。为了获得更全面的物种信息,我们将断裂后的片段组装成基因组(contigs)。通常宏基因组样品中存在很多物种,因此我们整合了SPAdes (版本3.6.2)、内部脚本和宏基因组数据库这些通常用于单一物种分析的方法来提高宏基因组生物信息学基因组组装方法^[22,23]。MetaGeneMark软件用于对组装后序列的基因预测^[24];使用具有90%和95%同一性的CD-HIT去除冗余基因^[25,26];使用相同程序将高质量序列与基因目录比对来确定基因的相对丰度,基于基因相对丰度进行下游差异分析;使用BLAST将高质量序列与几个公共数据库(即NCBI非冗余蛋白质数据库, NR;直系同源数据库, COG;京都基因和基因组百科全书, KEGG)^[27]进行比对;使用Interproscan对域进行搜索^[28]。

(vii) 核苷酸序列登录号。本研究母婴粪便样本测序数据已存入NCBI数据库(登录号: PRJNA482898)。

2 结果与讨论

2.1 测序结果

本研究所采集6份粪便样品(共48份细菌悬液)中共产生2015825666条Raw reads, 每100个细胞悬浮液的平均reads个数为41996368. 过滤不合格序列后, 我们得到1952350984条Clean reads (表S1). 通过计算Shannon指数、Simpson指数、Chao1指数和观察到的物种数量(图1~4), 可以得出大多数粪便悬浮液具有很高的细菌多样性. Shannon-Wiener多样性曲线表明测序深度能满足所有样品测序要求(图1).

2.2 母婴肠道微生物构成

为对样品进行深度测序, 高质量序列被分配到

表 1 母婴详细信息

Table 1 The information of mother and infant

编号	民族	年龄	分娩方式	婴儿体重(kg)	婴儿身高(cm)
19	蒙古族	28	剖腹产	3.5	50
26	汉族	31	剖腹产	3.5	52
40	汉族	27	自然分娩	3.7	53

不同的分类学水平. 本研究共鉴定到702个不同的属, 1283个不同的种, 其中69个种的平均相对含量在1%以上(图5). 正如所期望那样, 每对母婴间微生物尽管含量不同, 但种类都高度相似, 相似度在序号为19, 26和40的母婴间分别为99.06%, 99.53%和100%. 其中顺产婴儿40中高丰度物种与母亲高丰度物种相似, 如*Roseburia hominis*, *Roseburia intestinalis*, *Eubacterium rectale*, *Eubacterium eligens*, *Ruminococcus*

图 1 评价粪便样品微生物多样性的香浓稀疏曲线

Figure 1 Shannon rarefaction curves that estimate the microbial diversity of fecal samples

图2 评价粪便样品微生物多样性的辛普森稀疏曲线

Figure 2 Simpson rarefaction curves that estimate the microbial diversity of fecal samples

torgues, *Coprococcus catus*, *Bacteroides vulgatus*, *Faecalibacterium prausnitzii*等(图6). 而剖腹产婴儿19和26中富有 *Propionibacterium acnes*, *Staphylococcus saprophyticus*/S. *haemolyticus*, *Delftia acidovorans*, *Prevotella melaninogenica*等与皮肤和口腔相关的微生物^[12,29,30]. 通常, *Bacteroides*, *Bifidobacterium*和 *Escherichia/Shigella*是新生儿肠道中最丰富的微生物, 但有研究报道 *Bacteroides*和 *Bifidobacterium*作为婴儿肠道主要微生物的时间要晚于 *Escherichia/Shigella* (3种菌定殖时间分别为产后2.6, 3.6和5.4 d)^[29]. 这也许能解释为什么本研究中顺产婴儿肠道中主要微生物不是 *Bacteroides*或 *Bifidobacterium*, 可能与采样时间为产后24 h之内有关.

本研究中剖腹产婴儿19和26中的拟杆菌均少于顺产婴儿40, 如 *Bacteroides* (*B.*) *fragilis*, *B. helcogenes*, *B. salanitronis*, *B. thetaiotaomicron*, *B. vulgatus*和 *B. xylanisolvens* (图6). 通常, 与顺产婴儿相比, 剖腹产婴儿中有较少的乳杆菌属^[31]. 但本研究中剖腹产婴儿26的 *Lactobacillus reuteri*却显著高于其他2个婴儿($P < 0.01$). 我们推测, 这是由于母亲在怀孕期间增补了罗伊氏乳杆菌 *L. reuteri*或婴儿在剖腹产出生后喝了添有该菌的奶粉. *L. reuteri*是人体内的“土著菌”并主要存在于母乳中, 是目前被研究最广的益生菌之一^[31]. 它可以产生细菌素(广谱的抗菌物质), 调节免疫反应, 缓解婴儿腹绞痛, 降低过敏源敏感性, 有效降低早产儿被念珠菌及真菌感染风险,

图3 评价粪便样品微生物多样性的Chao 1 稀疏曲线
Figure 3 Chao 1 rarefaction curves that estimate the microbial diversity of fecal samples

尤其对由剖腹产引起婴儿肠道菌群的紊乱具快速恢复作用^[31~34]。该菌现被商业化作为婴儿益生菌剂或直接添加到奶粉中^[31]。

以之前报道的母婴肠道微生物为参考^[29,30,35,36]，我们将已知的与之前未曾在母婴肠道报道的微生物分别定义为常见物种和低丰度稀有物种。之前可能因为测序深度不够或DNA含量太少而使得稀有物种不能很好地被检测。本研究利用单细胞扩增技术对每份母婴粪便样品约100个细胞数量的微量DNA大幅扩增后进行宏基因组分析，尤其关注其中的低丰度物种。*L. amylovorus* 最早发现于酸面团中，虽在猪崽中含量丰富，但在人类肠道和粪便中从未被报道^[37,38]。现如今，一些淀粉成分，如马铃薯、玉米、大

米淀粉等被添加到婴儿奶粉中来调节婴儿早期反流频率和胃排空率^[39,40]。而*L. amylovorus*可利用该天然淀粉并通过发酵不同的碳水化合物产D-乳酸和L-乳酸^[41]。*L. kefiranofaciens*是开菲尔中的主要微生物，可调节肠道菌群和免疫系统，增加粪便水分，预防过敏性气道反应，同时具抗过敏、抗菌活性，而这些益生特性的产生归功于其产胞外多糖(EPS)能力^[42~46]。但值得一提的是并不是所有细菌产生的EPS都具益生特性。研究表明，产自*Lactobacillus rhamnosus* RW-9595M的EPS不仅不能被婴儿体内微生物利用、显示任何益生元特性外，相反地通过降低乳杆菌数量来扰乱体内细菌平衡^[44]。*L. sanfranciscensis*存在于酸面团中，因最初分离自旧金山“San Francisco”而取名

图4 评价粪便样品微生物多样性的观测物种稀疏指数

Figure 4 Observed species rarefaction index that estimate the microbial diversity of fecal samples

为“*L. sanfranciscensis*”. 而且此菌种仅存在于酸面团中, 之前仅有一项报道表明分离自果蝇的某菌株和*L. kefiranofaciens*有97%序列同源性^[47]. 它和*L. kefiranofaciens*一样, 都可产生胞外多糖(exopolysaccharide, EPS). 为探究胞外多糖能否改变人肠道菌群并起到“益生元”的作用, 有研究将多个人的粪便和*L. kefiranofaciens*产生的胞外多糖同时混入发酵液发酵, 结果表明由*L. kefiranofaciens*产生的胞外多糖可作为“双歧杆菌增殖因子”促进肠道菌群中双歧杆菌属的增多^[48]. 但该菌和它所产生的胞外多糖在婴儿肠道中的作用仍未见报道.

2.3 宏基因组组装, 基因预测和功能注释

序列组装长度为943759299 bp, 组装的N50值范

围为2494~25750 bp(表S2). 预测的基因数量为100~73294个, 平均长度为513.18~821.9 bp(表S3). 为充分研究粪便样品中细菌编码基因的功能, 使用COG和KEGG数据库对基因进行注释.

COG注释结果显示, 剖腹产婴儿19和26的高丰度基因参与到仅一般功能预测(general function prediction only, R)和功能未知(function unknown, S)这两大COG分类中, 而顺产婴儿40和3位母亲的高丰度基因均参与到复制、重组和修复COG分类(replication, recombination and repair, L)中. 总之, 相比于其他2个婴儿, 顺产婴儿40无论在微生物组成及基因功能上都与成人更为相似.

KEGG注释结果显示, 剖腹产婴儿中富有磷酸转移酶系统(phosphotransferase system, PTS)基因, 顺产

图5 属水平上粪便样品中所鉴定到的细菌相对丰度热图

Figure 5 Heatmap showing the relative abundance of bacteria detected in the fecal samples at genus level

图 6 种水平上粪便样品中所鉴定到的细菌相对丰度热图

Figure 6 Heatmap showing the relative abundance of bacteria detected in the fecal samples at species level

婴儿中富有 β -葡萄糖苷酶(beta-glucosidase)基因(表S4)。初生儿微生物中富含对简单碳水化合物转运的磷酸转移酶系统PTS基因,这与之前的研究相一致,表明对简单碳水化合物的快速内化和转化来维持生长是新生儿肠道微生物的主要特征^[29]。初生儿食物来源主要是母乳,所含糖类为乳糖,而本研究中我们也发现半乳糖激酶(Galactokinase)存在于*L. kefiranofaciens*中。此外,在顺产婴儿中较高丰度编码 β -葡萄糖苷酶[EC: 3.2.1.21]的*bgIX*基因。 β -葡萄糖苷酶是细菌碳水化合物代谢的关键酶,广泛分布于生物体中,在许多生物过程尤其是纤维素的降解中发挥重要作用^[49]。婴儿刚出生时虽还没有摄入固体(植物成分)食物,但 β -葡萄糖苷酶的存在可降解来自母乳的低聚糖和蛋白多糖(如黏蛋白等)来维持肠上皮细胞的功能性稳态^[50]; β -葡萄糖苷酶还可能产自于大肠杆菌或双歧杆菌这些新生儿体内主要微生物中。这些微生物产 β -葡萄糖苷酶目的是通过水解 β -葡萄糖苷糖为葡萄糖来获取能量,从而为日后更好地存活于肠道或宿主间做准备,而这也是*bgIX*同系物在其他物种的主要功能^[49,51]。婴儿奶粉中含有淀粉成分,有些*L. kefiranofaciens*和*L. amylovorus*菌株均可利用淀粉而产生乳酸,本研究中该通路的 β -半乳糖苷酶(EC3.2.1.23)、6-磷酸- β -葡萄糖苷酶(EC3.2.1.86)和苯丙氨酸-tRNA连接酶(EC 6.1.1.20)均被注释。与其他菌株相比,乳酸菌尽管被认为有相对较弱的蛋白水解系统,但仍有足够能力来水解食物中的蛋白质。在*L. kefiranofaciens*中就发现有编码甲酰甘氨酸核苷酸合成酶(phosphoribosylformylglycinamide synthase)的基因。当然,本研究中使用生物信息学方法对母婴粪便微生物注释得到的功能基因仅可作为一种预测手段暗示可能有以上所述特性的存在,其在机体中发挥的确切功能还有待进一步验证。

本研究初次使用单细胞扩增技术对母婴间肠道微生物构成及功能做出探索,获得了较高测序深度

及物种多样性,发现了之前鲜有报道的低丰度物种,证实单细胞扩增技术可用于后续不同生态环境微生物领域的研究。不足之处是样本量太少及没有采集到母乳,因母乳是母婴微生物传递的第二大途径,对婴儿免疫系统发育、抗感染及抗过敏方面起重要作用,因此本研究还不足以全面概述母婴传递机制。此外,基于一些已证实“母婴传递”的研究及在本研究中观察到的母婴间存在高比率共有微生物这个事实,我们姑且认为,母亲是给婴儿传递微生物的一个“储藏所”,但并不能证实这些相同的微生物是从母亲而来还是从其他途径而来。因此,下一步研究我们要加大样本量,使其更具代表性;不仅要证实婴儿肠道中某些微生物确实来自于母亲,还要证实这些微生物可以在婴儿体内转录或定殖,而不仅仅是传递(短暂停留)。另外,现有的任何基于DNA测序而不受限于传统培养的方法,都不能很好地区分活菌和死菌,因此研究中所鉴定到的物种数量和种类都值得我们谨慎考虑。最后,考虑到婴儿肠道微生物是可塑的,可受饮食、益生菌及环境因素等影响,这意味着将来我们可以进一步利用顺产婴儿肠道微生物(或母亲阴道菌群)及益生菌制剂来调节早产儿、剖腹产儿等非自然生产婴儿的肠道菌群,从而为每个婴儿营造一个健康的早期发育微生态环境。

3 结论

本研究首次采用单细胞扩增技术对母婴肠道微生物组成及其功能进行了探索,获得了较高的测序深度及物种多样性。研究结果显示,顺产婴儿肠道与母亲肠道具有相似的高丰度物种,剖腹产婴儿肠道微生物组成与皮肤、口腔微生物密切相关。由于研究之初所获样本数量少,故目前对母婴微生物传递机制的了解仍然十分有限,还需进一步深入研究。

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补充材料

表 S1 序列筛选统计结果

表 S2 序列组装统计结果

表 S3 粪便样品中预测基因统计结果

表 S4 粪便样品中预测基因的 KEGG 注释结果

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Summary for “利用单细胞扩增技术解析母婴间肠道微生物构成及功能”

Analysis of intestinal microbial composition and function of mother-infant pairs by single cell amplification technique

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Microorganism plays an important role in human health, such as promoting digestion and absorption, regulating the host immune system, maintaining dental health, and resistance to pathogens invasion. The microbes of newborn babies are crucial to their health; it may be involved in infant brain development and neurodevelopment. Maternal genotype and diet, antibiotic use, maternal viral infections during pregnancy and mode of delivery are possible factors that can modulate the intestinal microbiome. Our previous studies have found that microbes can be vertically transferred from mother to infant through two main ways, birth canal and the breast milk.

In the natural environment, only 1% of the microbes can be cultured. Thus, many low and non-cultivable taxa cannot be accurately identified by traditional methods. Comparing with PCR-based whole genome amplification methods, the single cell multiple replacement amplification (MDA) technique can reduce amplification bias by 3~4 orders of magnitude, increase the minimal DNA of a single bacterial cell by about 5 billion times, and produce an average length of >12 kb amplification, which allows the gene fragment to be sequenced accurately.

In this experiment, we applied the single-cell amplification technique to analyze 3 pairs of maternal and infant feces (a total of 48 bacterial suspensions) in order to explore the composition of intestinal microorganisms and analyze the related functional genes. The results showed that the fecal microorganisms of each pair of mother and infant were highly similar (>99%) despite in different abundance. Among three infants, one vaginally born infant showed similar high abundance species to its mother, such as *Roseburia hominis*, *Roseburia intestinalis*, *Eubacterium Rectale*, and *Eubacterium eligens*, while the high abundant species of other two C-sections infants were associated with the skin and mouth, such as *Propionibacterium acnes*, *Staphylococcus saprophyticus*, *Haemolyticus*, and *Delftia Acidovorans*. *Bacteroides* (B.), such as *B. fragilis*, *B. helcogenes*, and *B. salanitronis*, were less prevalent in C-section delivered infants compared to the vaginally born infant. A total of 1283 bacterial species were identified in this study. In addition to the previously reported human gut-related species, we detected three low-abundant taxa, namely *Lactobacillus* (L.) *amylovorus*, *L. kefiranoferiens*, *L. sanfranciscensis*, and *Bifidobacterium asteroides*. The functional metagenomes of fecal samples showed that the carbohydrate-related phosphotransferase system (PTS) genes and beta-glucoside genes were enriched in the infant microbiome. In this study, we applied the single-cell amplification technique to explore the composition and function of intestinal microorganisms of the mother and infant, with high sequencing depth and species diversity coverage. We also detected some low abundance species, which were rarely reported before. We believe the present approach is suitable for future analysis of microbiota of different ecological environments.

mother and infant feces, single-cell amplification, metagenomics, bacterial diversity, low-abundant taxa

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