

酸枣仁水提物对焦虑大鼠症状的改善 及其对肠道菌群的影响

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摘要: 选用慢性温和不可预知应激大鼠模型, 探究酸枣仁水提物缓解焦虑症状的潜在作用机制。以行为学(糖水偏好率、旷场得分、暗处所待时间)及肠道菌群为参考指标, 研究酸枣仁水提物对焦虑模型大鼠的行为、肠道菌群多样性、结构及组成的影响。结果显示, 与模型组(CUMS)组相比, 酸枣仁(SZS)组显著改善了慢性压力诱导的焦虑行为(糖水消偏好率及旷场得分显著增加, 在暗箱中所处的时间显著减少); SZS组肠道菌群的alpha多样性显著升高($p < 0.05$), 且菌群结构显著偏离CUMS组; 进一步探究发现, 相比于CUMS组, SZS组中 *Lactobacillus* (CUMS: 29.42%; SZS: 8.08%; $p=0.003$) 和 *Ruminococcaceae_UCG-014* (CUMS: 3.30%; SZS: 2.14%; $p=0.04$) 的丰度显著降低, 而 *Bacteroides* (CUMS: 2.23%; SZS: 5.15%; $p=0.001$), *norank_o_Mollicutes_RF9* (CUMS: 1.01%; SZS: 2.43%; $p=0.019$), *Ruminiclostridium_9* (CUMS: 0.42%; SZS: 0.62%; $p=0.034$), *Parabacteroides* (CUMS: 0.35%; SZS: 0.60%; $p=0.011$), *Butyrivimonas* (CUMS: 0.01%; SZS: 0.03%; $p=0.018$) 及 *Acetatifactor* (CUMS: 0.01%; SZS: 0.03%; $p=0.001$) 的丰度显著升高。结果表明, 酸枣仁可改善CUMS大鼠焦虑相关症状, 且影响了CUMS大鼠的肠道菌群结构, 为明确其潜在作用机制提供了理论依据。

关键词: 焦虑; 肠道菌群; 酸枣仁

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Improvement in Symptoms of Anxiety in Rats by Aqueous Extract of *Semen Ziziphi Spinosae* and Its Effect on the Intestinal Flora

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Abstract: The model of rats suffering from chronic, mild and unpredictable stress was used to explore the potential mechanism underlying the anxiety symptoms-alleviating effect of an aqueous extract of *Semen Ziziphi Spinosae*. Using reference indices associated with behaviors (sucrose preference rate, open field score, and time spent in the dark zone) and the intestinal flora, the effects of the aqueous extract of *Semen Ziziphi Spinosae* on the behaviors, and intestinal flora diversity, structure and composition in anxious model rats were studied. The results showed that compared with the model group (CUMS) group, chronic administration of *Semen Ziziphi Spinosae* significantly ameliorated chronic stress-induced anxiety-like behaviors in rats (sugar water preference rate and open field score increased significantly, while the time spent in the dark box significantly decreased). The alpha diversity of the intestinal flora for the SZS group significantly increased ($p < 0.05$), with the flora structure of the SZS group significantly different from the CUMS group. Further investigation revealed that the SZS group had significantly lower abundance of *Lactobacillus* (CUMS: 29.42%; SZS: 8.08%; $p=0.003$) and *Ruminococcaceae_UCG-014* (CUMS: 3.30%; SZS: 2.14%; $p=0.04$), but higher abundance of *Bacteroides* (CUMS: 2.23%; SZS: 5.15%; $p=0.001$), *norank_o_Mollicutes_RF9* (CUMS: 1.01%; SZS: 2.43%; $p=0.019$), *Ruminiclostridium_9* (CUMS: 0.42%; SZS: 0.62%; $p=0.034$), *Parabacteroides* (CUMS: 0.35%; SZS: 0.60%; $p=0.011$), *Butyrivimonas* (CUMS: 0.01%; SZS: 0.03%; $p=0.018$) and *Acetatifactor* (CUMS: 0.01%; SZS: 0.03%; $p=0.001$), compared with the CUMS group. These results indicated that *Semen Ziziphi Spinosae* could alleviate the anxiety-related symptoms in CUMS rats and affect their intestinal

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flora structure, which provides a theoretical basis for the potential working mechanism.

Key words: anxious; gut microbiota; *Semen Ziziphi spinosae*

2010年全球疾病负担研究报告显示:在过去20年,精神、神经和药物滥用的致残率达到了38%,其中精神和行为性障碍贡献了近1/4^[1]。且神经精神性疾病的发病机制较为复杂且受多种因素影响,如基因、环境、饮食等^[2-4],因此其治愈率偏低。近些年,大量的研究证明肠道微生态失衡与多种疾病的发生存在着密切联系,如糖尿病、肠道性疾病、神经精神性疾病等^[5-8],包括焦虑症、抑郁症等^[9-13]。Nishino等^[9]探究共生微生物对无菌小鼠行为影响的结果显示,无菌小鼠相比于有菌小鼠焦虑程度显著较高,暴露在有菌环境24 h后焦虑程度显著降低,且脑部去甲肾上腺素、多巴胺、5羟色胺的表达量也显著升高;Burokas等^[14]的研究结果显示,补充寡聚果糖和寡聚半乳糖的复合物能够显著缓解慢性压力诱导的焦虑行为及相关生理指标,且显著提高了结肠菌群中双歧杆菌属和乳酸菌属的丰度;Bravo^[15]的研究显示,补充*Lactobacillus rhamnosus*的抑郁小鼠大脑中皮质酮含量显著降低,且其焦虑、抑郁样行为也显著被改善,探究发现伽马氨基丁酸受体GABA_{B1b}和GABA_{Aα2}在各脑区(前额皮质、杏仁核、海马区)的表达量发生显著改变。综上可知,肠道菌群可能在神经精神性疾病的发生中起着重要作用,同时肠道菌群可能成为治疗和预防神经精神性疾病的靶标,且特定微生物可能是治疗焦虑等疾病的助剂。

酸枣仁是一种具有催眠及镇静作用的典型中草药,几千年来常被临床医生用来治疗失眠和焦虑障碍疾病^[16]。其水提取物中包含多种活性物质,如皂苷、环肽、生物碱、黄酮、脂肪酸类等,这些次生代谢产物可以通过调节突触后膜伽马氨基丁酸能活性及5-羟色胺能系统,进而缓解失眠和焦虑障碍等疾病^[17-19]。另外,有研究表明该草药的水提取物还可通过调节扁桃体中央核区的促皮质激素释放因子和去甲肾上腺素激活信号发挥其抗焦虑功效^[16]。尽管大量的前临床及临床试验已经证明酸枣仁具有抗焦虑功效,但都是基于神经保护对其进行研究。而大量研究发现,皂苷、黄酮等物质发挥作用可能是通过调节肠道微生物发挥其缓解结肠炎和气虚的功效^[20,21]。因此,该试验旨在探究酸枣仁对肠道菌群的调节作用,进而基于肠道菌群探究其潜在抗焦虑机制。

1 材料与方法

1.1 实验动物

30只SPF级Sprague-Dawley(SD)雄性大鼠,180±20 g,由广东省医学实验动物中心提供,许可证号:SCXK(粤)2013-002;适应性饲养于华南农业大学实验动物中心SPF级屏障环境内7 d后备用,饲养环境:12 h昼/夜,温度25±1℃,湿度55%~65%。

1.2 材料与试剂

酸枣仁水提取物。提取方法:酸枣仁加10倍量水煮沸后,煮沸提取2 h,过滤后将药材继续加8倍水煮沸后,煮沸提取1 h,过滤后将2次滤液合并一起浓缩至目标浓度(1 g/mL,每1 mL浓缩液含1 g酸枣仁)。

蔗糖,天津市大茂化学试剂厂;QIAmp DNA Stool Mini Kit,德国Qiagen公司;Qiagen Gel Extraction Kit纯化试剂盒,德国Qiagen公司。

1.3 仪器与设备

高速离心机,美国Thermo公司;Gene Amp PCR system 2700,美国Applied Biosystem公司;核酸电泳仪,美国BIO-RAD公司;Gel Doc EQ凝胶成像系统,美国BIO-RAD公司;NanoDrop 2000,美国Thermo公司;HiSeq 2000 DNA测序仪,美国Illumina公司。

1.4 试验方法

1.4.1 动物分组及处理

适应性饲养期间进行1%的糖水偏好训练,1只/笼。随后将其随机分成两组:一组为健康组(n=6),另一组CUMS焦虑模型组(n=24)。建模8周后,根据行为学测试结果将构建成功的焦虑模型大鼠随机分成两组:模型组(n=5)、酸枣仁组(n=5),分别灌胃无菌水及酸枣仁水提取物。随后,进行为期6周的酸枣仁补充试验,经前期研究确定最终灌胃量为180 mg/(kg·d)。灌胃前,使用无菌水将浓缩液稀释成适当浓度(45 mg/mL)。酸枣仁干预期间,继续给予各焦虑模型大鼠应激刺激,为避免模型组大鼠带来干扰,健康组则饲养在相邻房间内,环境安静舒适,食物和水可自由获取。经过6周的酸枣仁干预后,进行行为学测试初步检验干预效果。最后,末次补充酸枣仁24 h后收集各组大鼠粪便样本,保存于-80℃冰箱,用于肠道菌群分析。

1.4.2 CUMS 焦虑大鼠模型构建

参考Gawali和Wu等研究^[22,23],采用慢性不可预知性应激刺激结合孤养的方式构建焦虑模型。建模期间,

每日随机选取1种刺激,连续两天内刺激方式不可重复。

1.4.3 CUMS 焦虑模型评价标准

各组大鼠体重每周称量一次,用于记录大鼠体重变化趋势。

参考文献^[14,24,25]进行行为学测试(糖水偏好实验、旷场实验及明暗穿梭实验),分别采用大鼠的糖水消耗率、旷场得分及暗处所处时间等行为指标衡量大鼠是否焦虑。

1.4.4 肠道菌群 16S rRNA V4-V5 区高通量测序

首先,对粪便前处理,采用粪便基因组提取试剂盒提取粪便细菌基因组;其次,对粪便细菌16S rRNA的V4-V5区进行扩增;然后,然后将样品委托给上海美吉生物医药科技有限公司进行建库、测序;最后,基于有效测序数据进行OTUs (Operational Taxonomic Units (聚类 and 物种分类分析。对OTUs聚类结果进行alpha多样性 (Shannon/Simpson指数)、beta多样性 (PCoA和层级聚类树分析 (Hierarchical clustering tree analysis)分析。为进一步挖掘各组间肠道微生物差异,选用单尾单因素方差分析 (one-way analysis of variance, ANOVA) 对各组物种进行差异统计检验。

1.5 结果统计

选用SPSS 22.0统计软件对行为学实验结果和肠道差异物种进行统计分析(T检验及ANOVA), p 值小于0.05,认为具有统计学差异。

2 结果与讨论

2.1 酸枣仁对焦虑模型大鼠情绪的影响

表1 酸枣仁补充前、后各组行为学结果

Table 1 The result of behaviors test before and after SZS supplementation (X±S)

时间	行为学实验	分组		
		HC (n=6)	CUMS (n=5)	SZS (n=5)
酸枣仁 补充前	糖水偏好实验	78.15±5.22	65.18±13.89**	/
	旷场实验	91.75±19.26	46.18±24.46***	/
	明暗穿梭实验	124.33±35.09	187.95±38.27***	/
酸枣仁 补充后	糖水偏好实验	84.58±6.61	54.27±26.21*	76.69±1.34##
	旷场实验	64.50±32.60	19.08±19.10*	62.65±1.85#
	明暗穿梭实验	135.42±40.95	205.78±32.95*	152.64±2.13#

注: *: 与 HC 组相比, $p<0.05$; #: 与 CUMS 组相比 $p<0.05$; HC: 正常对照; CUMS: 慢性不可预知性应激刺激

2.2 酸枣仁补充对肠道菌群多样性的影响

Shannon 和 Simpson 指数表征着肠道物种的多样

2.1.1 酸枣仁干预下焦虑模型大鼠体重的变化趋势

各组大鼠的体重变化如图1所示,相比于 CUMS 组,在第9周之前, SZS 组大鼠的体重较低;在第9周之后, SZS 组大鼠的体重增长速度明显加快,但体重仍低于 HC 组。体重变化结果表明,酸枣仁能够促进焦虑模型大鼠体重增长。

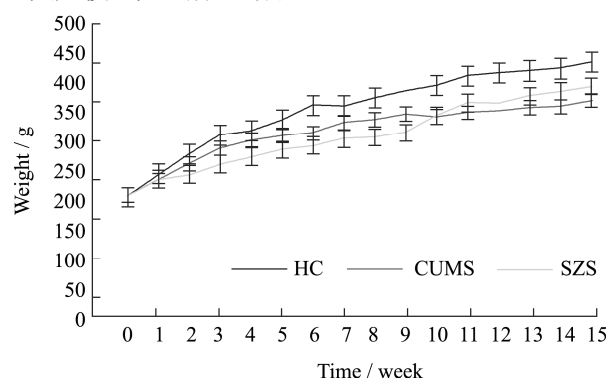


图1 酸枣仁补充前后大鼠体重变化

Fig.1 Changes in body weight of rats before and after SZS supplementation

注: HC 代表健康组; CUMS 代表模型组; SZS 代表酸枣仁添加组。

2.1.2 酸枣仁补充后焦虑大鼠行为学的变化

糖水偏好实验、旷场实验和明暗穿梭实验分别反映了大鼠的快感缺失程度、活跃程度和探索未知的能力。如表1所示,酸枣仁补充前,与 HC 组相比, CUMS 组的糖水偏好率、旷场得分及在暗处所处时间均存在极显著差异 ($p<0.01$)。经过6周的酸枣仁补充后,相比于 CUMS 组, SZS 组的糖水消耗率及旷场得分均显著升高 ($p<0.05$), 且大鼠在暗处所处时间显著缩短 ($p<0.05$)。综上可推测,酸枣仁显著改善了 CUMS 诱导的焦虑症状^[18]。

性。由图2可知,三组间肠道物种多样性存在显著差异(Shannon (CUMS: 4.04; SZS: 4.53, $p=0.04$); Simpson (CUMS: 0.07; SZS: 0.03, $p=0.04$)). 与 HC 组相比,

CUMS 组的物种多样性较低, 但经 6 周的酸枣仁治疗后菌群多样性显著升高。与 Francesco^[26]等的研究结果一致, 表明健康组微生物的多样性和丰富度都较高。肠道微生物多样性的结果显示, 酸枣仁水提取物可增加肠道物种的多样性。

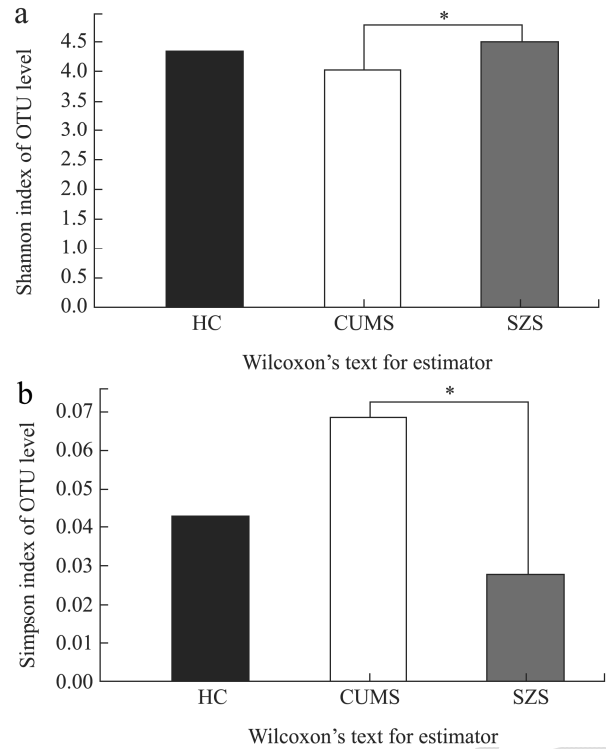


图 2 各组粪便样品物种多样性

Fig.2 Species diversity of three groups

2.3 酸枣仁对肠道菌群结构的调节作用

菌群结构分析结果显示 (图 3), 3 组间肠道菌群结构差异较大, 说明慢性不可预知性压力显著破坏了粪便菌群结构, 而酸枣仁显著影响了粪便菌群结构。从 PCoA 结果图中可以看出, CUMS 组和 HC 组菌群结构差异较大; 经酸枣仁治疗后, SZS 组菌群结构偏离 CUMS 组。另外, 层级聚类树分析 (Hierarchical clustering tree analysis) 结果进一步确定酸枣仁可显著调节菌群结构。由以上结果可知, 酸枣仁显著调节了焦虑模型大鼠菌群结构。

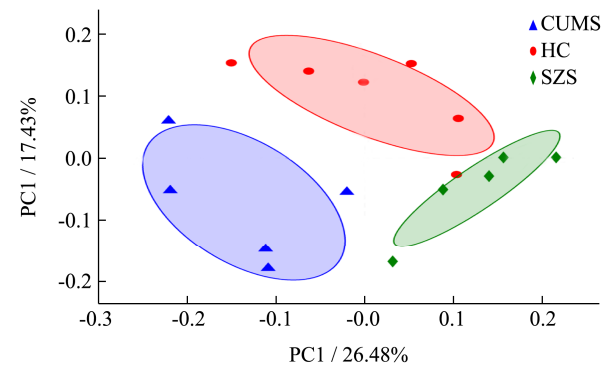


图 3 基于 OTU 水平的 beta 多样性分析

Fig.3 Analysis of beta diversity based on the OTU level

2.4 酸枣仁对肠道群落组成的影响

2.4.1 酸枣仁干预后肠道优势物种的变化

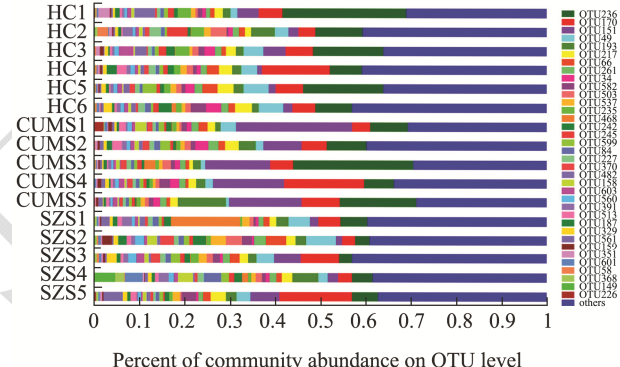


图 4 各组大鼠肠道菌群 OTU 水平相对丰度

Fig.4 Relative abundance of dominant OTUs in microbial communities from different groups

如图 4 所示, 各组大鼠肠道中的优势物种主要属于: 乳酸菌属 (*Lactobacillus*: OTU236 和 OTU151)、*Romboutsia* (OTU170)、未分类拟杆菌目 S24-7 菌科 (norank_f_Bacteroidales_S24-7_group: OTU49、OTU482、OTU217、OTU242、OTU193、OTU582、OTU261、OTU560、OTU329、OTU513)、胃瘤菌科 UCG-013 属 (*Ruminococcaceae*_UCG-013: OTU603)、毛螺旋菌科 UCG-006 属 (*Lachnospiraceae*_UCG-006: OTU235、OTU503)、*Lachnoclostridium* (OTU245)、普雷沃氏菌科 UCG-001 属 (*Prevotellaceae*_UCG-001: OTU66)、*Adlercreutzia* (OTU351) *Anaerosporebacter* (OTU227)、未分类胃瘤菌科 (norank_f_Ruminococcaceae: OTU84)、瘤胃菌属-1 (*Ruminococcus*_1: OTU34、OTU159)、拟杆菌属 (*Bacteroides*: OTU537)、未分类的毛螺旋菌科 (*unclassified_f_Lachnospiraceae*: OTU599、OTU58)、毛螺旋菌科 NK4A136 群属 (*Lachnospiraceae*_NK4A136_group: OTU391、OTU370、OTU158、OTU368、OTU601)、

[*Eubacterium*]*_coprostanoligenes_group* (OTU187)、狭义梭菌属 (*Clostridium_sensu_stricto_1*: OTU561)、*Mucispirillum* (OTU149)、普雷沃氏菌科 NK3B31 群属 (*Prevotellaceae_NK3B31_group*: OTU226)、*Allobaculum* (OTU468)。

由图 4 可知, 乳酸菌属在各组肠道微生物组成中占比相对较高, 其中 OTU151 在 CUMS 中偏高, 在 HC 组和 SZS 组中偏低; 未分类拟杆菌目 S24-7 菌科在肠道菌群中占比也较高, 且相比于 HC 组和 SZS 组, OTU49 在 CUMS 组中偏高; 另外可以看出 CUMS 中的其他 (others) 菌群占比相对于 HC 组和 SZS 组较少, 可推测 CUMS 组中物种相对较少, 进一步验证了 CUMS 组 alpha 多样性较低的结果^[26]。

2.4.2 酸枣仁补充后肠道特定微生物丰度的变化

差异物种结果如表 2 所示, 在门水平, 相比于 CUMS 组, SZS 组中厚壁菌门 (*Firmicutes*) 的相对丰度显著降低, 而拟杆菌门 (*Bacteroidetes*) 的水平则显著升高 ($p<0.05$)。经分析发现, 相比于 HC 组, 厚壁菌门和拟杆菌门的比值 (*Firmicutes/Bacteroidetes*) 在 CUMS 组中明显升高 (HC: 2.34 ± 0.54 ; CUMS: 3.35 ± 1.37 , $p=0.07$), 而经酸枣仁治疗后, 其比值显著降低 (CUMS: 3.35 ± 1.37 ; SZS: 1.72 ± 0.34 , $p=0.01$)。

属水平统计显示, 相比于 HC 组, 乳杆菌属 (*Lactobacillus*)、胃瘤菌科 UCG-014 (*Ruminococcaceae_UCG-014*) 丰度在 CUMS 组中升高, 而拟杆菌属 (*Bacteroides*)、未分类柔嫩菌纲 -RF9 (*norank_o_Mollicutes_RF9*)、*Parabacteroides*、*Jeotgalicoccus*、*Butyricimonas*、*Acetatifactor* 的丰度在 CUMS 组中较低, 但仅有 *Jeotgalicoccus*(HC: 0.20%; CUMS: 0.07%, $p=0.001$)、*Butyricimonas*(HC: 0.02%; CUMS: 0.01%, $p=0.04$)、*Acetatifactor*(HC: 0.02%; CUMS: 0.01%, $p=0.02$)的丰度具有显著性差异。相比于 CUMS 组, SZS 组大鼠肠道中乳杆菌属 (*Lactobacillus*, CUMS: 29.42%; SZS: 8.08%, $p=0.003$)、胃瘤菌科 UCG-014 属 (*Ruminococcaceae_UCG-014*, CUMS: 3.30%; SZS: 2.14%, $p=0.041$) 的水平显著降低, 而拟杆菌属 (*Bacteroides*, CUMS: 2.23%; SZS: 5.15%, $p=0.001$)、未分类柔嫩菌纲-RF9 (*norank_o_Mollicutes_RF9*, CUMS: 1.01%; SZS: 2.43%, $p=0.019$)、*Ruminiclostridium_9*(CUMS: 0.42%; SZS: 0.62%, $p=0.034$)、*Parabacteroides* (CUMS: 0.36%; SZS: 0.60%, $p=0.011$)、*Butyricimonas* (CUMS: 0.01%; SZS: 0.03%, $p=0.018$)、*Acetatifactor* (CUMS: 0.01%; SZS: 0.03%, $p=0.001$) 的丰度在 SZS 组中显著升高。

表 2 各组大鼠物种相对丰度比较

Table 2 Comparison of the relative abundance among three groups (X±S, %)

Taxa	HC/%	CUMS/%	SZS/%	p-value	
				HC vs CUMS	CUMS vs SZS
p_ <i>Firmicutes</i>	65.53±3.99	72.94±6.30	59.03±4.04		0.002
p_ <i>Bacteroidetes</i>	29.03±5.03	23.76±6.29	35.31±5.81		0.022
<i>Firmicutes/Bacteroidetes</i>	2.34±0.54	3.35±1.37	1.72±0.34	0.07	0.010
g_ <i>Lactobacillus</i>	17.37±8.88	29.42±9.65	8.08±2.82		0.003
g_ <i>Bacteroides</i>	2.68±0.76	2.23±0.65	5.15±1.17		0.001
g_ <i>Ruminococcaceae_UCG-014</i>	2.51±0.71	3.30±0.70	2.14±0.44		0.041
g_norank_o_ <i>Mollicutes_RF9</i>	1.69±0.73	1.01±0.36	2.43±0.82		0.019
g_ <i>Ruminiclostridium_9</i>	0.4209±0.10	0.42±0.11	0.62±0.11		0.034
g_ <i>Parabacteroides</i>	0.38±0.07	0.36±0.10	0.60±0.14		0.011
g_ <i>Jeotgalicoccus</i>	0.20±0.07	0.07±0.01	0.07±0.03	0.001	
g_ <i>Butyricimonas</i>	0.02±0.01	0.01±0.01	0.03±0.01	0.04	0.018
g_ <i>Acetatifactor</i>	0.02±0.01	0.01±0.01	0.03±0.01	0.02	0.001

注: HC: 正常对照; CUMS: 慢性不可预知性应激刺激。

对比分析发现, 与前人研究结果类似, 厚壁菌门的丰度在疾病组的肠道中显著升高^[26], 且厚壁菌门与拟杆菌门的比值在自闭症患者组中显著升高^[27,28]。尽管多数乳酸杆菌属的成员有益于机体健康, 但也有些研究证明有些菌种是条件致病菌, 与抑郁症的发生有

着密切联系。Strati^[27]和 Tomova^[28]等研究结果均表明乳酸杆菌在神经精神性疾病人群的肠道中丰度显著升高。*Parabacteroides* 的丰度在自闭症谱系障碍性疾病 (Autism spectrum disorder) 患者组中显著降低^[27], 在益生菌补充组中显著升高^[14], 与本文的变化趋势一

致。*Ruminococcaceae*_UCG-014 和 *Ruminiclostridium*_9 均属于瘤胃菌科,其中 *Ruminococcaceae*_UCG-014 具有抗炎性功能^[29],该菌属在 CUMS 组中丰度升高,而经酸枣仁治疗后该菌属降低;*Ruminiclostridium*_9 是一种与降解纤维素相关的菌属^[30],酸枣仁补充组显著增加了该菌属的丰度。*Bacteroides* 隶属于拟杆菌门,有研究表明该菌属的丰度与短链脂肪酸的产生量呈现正相关关系^[31],有益于机体健康。其它相关研究结果表明,与 HC 组相比, *Jeotgalicoccus*、*Butyricimonas*、*Acetatifactor* 的丰度在模型组中都显著降低,经调查发现这些菌属均与短链脂肪酸(乙酸、丙酸和丁酸)的产生相关^[32-35],其丰度增加有益于维持肠道平衡。*norank_o_Mollicutes*_RF9 在焦虑发生过程中发挥的作用是不可知的,但由本试验可知它们丰度的改变对焦虑的发生和治疗有着一定的关系。由以上结果可知,酸枣仁不仅降低了厚壁菌门与拟杆菌门的比值,而且调节特定微生物的丰度,尤其是短链脂肪酸产生菌的丰度。

3 结论

3.1 酸枣仁组中厚壁菌门与拟杆菌门的比值显著降低, *Bacteroides**Ruminiclostridium*_9, *Parabacteroides*, *Jeotgalicoccus*, *Butyricimonas* 和 *Acetatifactor* 菌属的丰度显著升高,且前人研究表明 *Bacteroides*, *Jeotgalicoccus*, *Butyricimonas* 和 *Acetatifactor* 菌属与短链脂肪酸产生密切相关^[31,33-35]。

3.2 综上所述, CUMS 大鼠肠道菌群失调,口服酸枣仁可改善 CUMS 大鼠的焦虑症状,这可能与其对肠道菌群的调节作用有关。后续工作将聚焦于 CUMS 相关的功能菌群,深入揭示酸枣仁改善 CUMS 症状的具体机制。

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