



复合益生菌制剂缓解 cuprizone 诱导的小鼠神经脱髓鞘

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杨昊, 李琳, 刘青青, 康行, 杨晓丹, 刘海霞, 樊卫平. 复合益生菌制剂缓解 cuprizone 诱导的小鼠神经脱髓鞘. 微生物学报, 2022, 62(3): 774–784.

Yang Hao, Li Lin, Liu Qingqing, Kang Xing, Yang Xiaodan, Liu Haixia, Fan Weiping. Compound probiotics alleviate cuprizone-induced demyelination in mice. *Acta Microbiologica Sinica*, 2022, 62(3): 774–784.

摘要:【目的】探索复合益生菌制剂对双环己酮草酰二脲(cuprizone, CPZ)诱导的小鼠脑内神经脱髓鞘的作用。【方法】将 27 只小鼠随机分为正常组(NC)、模型组(CPZ)和益生菌处理组(probiotics)。NC 组小鼠正常饲养, CPZ 组和 probiotics 组小鼠饲养含 0.2% CPZ 的饲料且每天分别灌饲 0.2 mL 生理盐水及同体积(75 亿菌落形成单位活菌)的复合益生菌制剂, 6 周后处死小鼠。通过快蓝染色(LFB)观察小鼠脑内神经髓鞘的完整性; 苏木素-伊红染色(HE)、阿利新蓝染色及免疫荧光染色分别评估回肠及结肠组织病理学改变、黏蛋白的表达及紧密连接蛋白 ZO-1、occludin 的表达; ELISA 检测脑组织匀浆脂多糖 LPS 水平; Western blotting 检测脑组织 TLR4/NF- κ B 信号通路的蛋白表达。【结果】与 CPZ 组相比, 复合益生菌制剂干预后, 发生在 CPZ 组小鼠大脑胼胝体内的神经脱髓鞘明显减少; 回肠绒毛排列整齐致密, 结肠腺体增多, 炎细胞浸润减少, 黏蛋白及紧密连接蛋白表达均增加; 脑组织 LPS 水平显著降低, TLR4、NF- κ B 及 P-I κ B/I κ B 蛋白表达量下降。【结论】复合益生菌制剂可能通过降低肠壁通透性缓解 CPZ 诱导的小鼠中枢神经系统脱髓鞘。

关键词: 双环己酮草酰二脲; 多发性硬化症(MS); 脱髓鞘; 益生菌; 肠屏障

基金项目: 山西省省筹资金资助回国留学人员科研项目(2020-079); 山西省应用基础研究面上青年基金(201901D211321)
Supported by the Scientific Research Funding Project for Returned Overseas Students in Shanxi Province (2020-079) and by the Yourth Fund of the Applied Basic Research in Shanxi Province (201901D211321)

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Received: 7 July 2021; Revised: 14 September 2021; Published online: 25 November 2021

Compound probiotics alleviate cuprizone-induced demyelination in mice

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Abstract: [Objective] To explore the effects of compound probiotics on demyelination of the mice induced by *bis*-cyclohexanone-oxalyldihydrazone (cuprizone, CPZ). **[Methods]** Twenty-seven mice were randomly assigned into normal (NC) group, model (CPZ) group, and probiotics group. The mice in the NC group were fed with normal diet, and those in the CPZ group and probiotics group with the diet containing 0.2% CPZ. The mice in the CPZ group and probiotics group were fed with 0.2 mL normal saline and 0.2 mL compound probiotics (7.5 billion colony-forming units) per day, respectively. All the mice were sacrificed after 6 weeks. Luxol fast blue (LFB) staining was employed to observe myelin sheath in the cerebrum. The histopathological change and the expression of mucin and tight junction proteins (ZO-1 and occludin) in ileum and colon were detected by hematoxylin-eosin (HE) staining, Alcian blue staining, and immunofluorescence staining, respectively. The content of lipopolysaccharide (LPS) in brain homogenate was detected by ELISA. The expression of proteins involved in the Toll-like receptor 4 (TLR4)/nuclear factor kappa-B (NF- κ B) signaling pathway was detected by Western blotting. **[Results]** Compared with the CPZ group, compound probiotics significantly alleviated the demyelination in corpus callosum. In addition, the mice in the probiotics group showed neat and dense ileum villi, increased colon glands, decreased infiltration of inflammatory cells, and up-regulated expression of mucin and tight junction proteins, declined LPS level, and down-regulated expression of TLR4, NF- κ B, and P-I κ B/I κ B in the brain. **[Conclusion]** Compound probiotics may alleviate CPZ-induced demyelination in mice by reducing intestinal wall permeability.

Keywords: cuprizone; multiple sclerosis (MS); demyelination; probiotics; intestinal barrier

多发性硬化症(multiple sclerosis, MS)是一种中枢神经系统(central nervous system, CNS)的自身免疫性疾病,与脱髓鞘和轴突损伤相关,最终可导致神经变性,表现出许多炎症性自身免疫性疾病的特征,包括血脑屏障(blood-brain barrier, BBB)的破坏^[1]。MS的病因和具体发病机制尚不明确。双环己酮草酰二脲(cuprizone, CPZ)诱导小鼠脱髓鞘是目前公认的毒性脱髓鞘模型之一,常用于模拟人类多发性硬化的病理过程^[2]。

肠道微生物群与多种自身免疫性疾病(autoimmune diseases, AID)有关,包括炎症性肠病、类风湿性关节炎和多发性硬化症等^[3], MS患者通常表现出肠道屏障受损的迹象。健康的肠道微生物群落可维持完整的肠道屏障、抑制病原体的定殖以及调节宿主的生理和免疫反应^[4]。适当补充益生菌有益于肠道微生态平衡,利于宿主健康^[5]。有证据表明,益生菌有助于调节肠道黏液层,进而保护肠上皮细胞,利于肠道内环境的稳定^[6-7]。Gharehkhani Digehsara

等的研究发现, 给 CPZ 诱导的脑内神经脱髓鞘小鼠灌饲干酪乳杆菌(*Lactobacillus casei*) T2 可降低白细胞介素-17 (interleukin-17, IL-17)并缓解脱髓鞘^[8]。在实验性自身免疫性脑脊髓炎(experimental autoimmune encephalomyelitis, EAE)小鼠中, 灌饲来源于乳杆菌属(*Lactobacillus*)、双歧杆菌属(*Bifidobacterium*)和链球菌属(*Streptococcus*)的不同菌株组成的复合益生菌制剂能够逆转已经建立的脱髓鞘症状, 并缓解免疫反应和中枢神经系统的炎症状态^[9]。

BBB 的破坏是 MS 疾病发生和延续的关键, 在 CPZ 诱导的小鼠脑内神经脱髓鞘模型中, BBB 的通透性增加^[10]。Braniste 等的研究结果强调了肠道微生物群是维持 BBB 完整性的潜在调节因子, 主要表现为缺乏正常肠道微生物群的无菌小鼠 BBB 通透性的增加^[11]。因此推测, 通过保护肠道屏障间接保护 BBB 的完整性可能是 MS 一个有前景的治疗策略。本研究采用喂饲 CPZ 法建立小鼠脑内神经脱髓鞘模型并用复合益生菌制剂进行干预, 通过对各组小鼠胼胝体脱髓鞘程度、肠屏障完整性、脑组织脂多糖(lipopolysaccharide, LPS)水平以及 TLR4/NF- κ B 炎症通路蛋白表达水平的检测, 探索改善肠屏障功能缓解小鼠 MS 病变的作用, 为益生菌治疗 MS 提供理论依据。

1 材料与方法

1.1 实验动物

7 周龄雌性 C57BL/6 小鼠, 体重 18–22 g, 购于山西医科大学动物中心, 合格证编号: SCXK(晋)2019–004。小鼠饲养于 12 h 明暗昼夜循环的恒温通风环境, 自由饮食进水, 适应性饲养 1 周后开始实验。

1.2 主要试剂

益生菌冻干粉制剂, 每克含 100 亿菌落形成单位活菌(乳双歧杆菌 HN019、两歧双歧杆菌 Bb06、动物双歧杆菌 BB-12、乳双歧杆菌 Bi07、长双歧杆菌 R175、动物双歧杆菌 B94、鼠李糖乳杆菌 GG、干酪乳杆菌 LC11、瑞士乳杆菌 R52、副干酪乳杆菌 Lpc37、植物乳杆菌 L1012、罗伊氏乳杆菌 HA188、鼠李糖乳杆菌 R11、嗜酸乳杆菌 NCFM、嗜热链球菌 St21)及菊粉、低聚半乳糖和低聚果糖益生元, 由中科宜康(北京)生物科技有限公司惠赠; cuprizone 购自 Sigma-Aldrich 公司; 标准阿利新蓝染色液试剂盒与 Luxol Fast Blue 染色试剂盒购自北京索莱宝科技有限公司; 牛血清白蛋白、苏木素-伊红染色试剂盒及 BCA 蛋白定量试剂盒购自博士德生物; 兔抗鼠 occludin 一抗、兔抗鼠 ZO-1 一抗、兔抗鼠 TLR4 一抗、兔抗鼠 NF- κ B P65 一抗、兔抗鼠 I κ B 一抗及兔抗鼠 p-I κ B 一抗均购自 Abcam 公司; DAPI 染色液与 AlexaFluor555 标记山羊抗兔 IgG 荧光二抗购自生工生物工程(上海)股份有限公司; ELISA 试剂盒购自武汉云克隆科技股份有限公司。

1.3 小鼠分组和模型建立

将 27 只小鼠随机分为正常组(NC)、模型组(CPZ)和益生菌处理组(probiotics), 每组 9 只。图 1A 所示, NC 组小鼠正常饲喂, CPZ 组和 probiotics 组小鼠饲喂含 0.2% CPZ 的饲料, 每天给 CPZ 组小鼠灌胃 0.2 mL 生理盐水, probiotics 组灌胃 0.2 mL 复合益生菌制剂(含 0.75 g, 75 亿菌落形成单位活菌), 每周记录体重, 6 周后处死小鼠, 无菌收集脑、回肠及结肠组织。

1.4 快蓝染色检测胼胝体病理改变

脑组织置于 10%福尔马林中固定 48 h, 脱水、包埋及切片。将大脑组织切片经二甲苯及梯度酒精脱蜡后用快蓝染色液(luxol fast blue,

LFB)室温过夜染色;蒸馏水冲洗;Luxol 分化液分化 5 s;70%乙醇分化 30 s 至灰白质清晰;蒸馏水冲洗;伊红染色,复染 30 s,蒸馏水冲洗;95%、100%乙醇脱水;二甲苯透明;中性树胶封片,镜下观察脑组织脱髓鞘情况。

1.5 苏木素-伊红染色及阿利新蓝染色分别检测肠组织病理改变及黏蛋白表达

结肠、回肠组织置于 10%福尔马林中固定 48 h,脱水、包埋及切片。组织切片脱蜡水化用苏木素-伊红(hematoxylin-eosin, HE)染色,显微镜观察组织病理学变化;切片脱蜡水化,用阿利新蓝酸化工作液浸泡 5 min;阿利新蓝染色液浸染 50 min;流水冲洗;核固红染色液复染 5 min;流水冲洗;常规乙醇脱水;二甲苯透明;中性树胶封片,镜下观察肠组织黏蛋白表达情况,根据 Ho 等的计数方法进行杯状细胞计数^[12],计算回肠绒毛及结肠隐窝产酸性黏蛋白的杯状细胞数量。

1.6 免疫荧光染色检测肠组织紧密连接蛋白的表达

回肠与结肠切片经脱蜡水化,高温修复,过氧化氢阻断内源性过氧化物酶,胎牛血清(BSA)封闭;分别滴加紧密连接蛋白 occludin 和 ZO-1 的一抗(1:200),4 °C 过夜;Alexa Fluor 555 标记山羊抗兔 IgG 荧光二抗(1:150),避光孵育 40 min;滴加 4',6-diamidino-2-phenylindol (DAPI)染色液染核,倒置荧光显微镜观察 ZO-1、occludin 的表达情况。

1.7 ELISA 检测脑匀浆 LPS

称取 100 mg 脑组织置于无菌 EP 管中,加入 0.9 mL 无菌 PBS 超声粉碎;4 °C、12 000×g 离心 20 min,取上清。酶联免疫吸附测定(ELISA)法检测各组小鼠脑匀浆脂多糖(lipopolysaccharide, LPS)含量,检测步骤按照说明书进行。使用酶标仪依序测量各孔在 450 nm

处的吸光度值,并根据标准曲线计算各孔中的样品含量。

1.8 Western blotting 检测脑组织 TLR4/NF-κB 信号通路

提取小鼠脑组织蛋白并定量,各组取等质量蛋白进行 SDS-聚丙烯酰胺凝胶电泳。将蛋白转印至硝酸纤维素膜(nitrocellulose filter membrane, NC 膜)上,5%脱脂牛奶室温封闭 2 h,TLR4、NF-κB p65、IκB 及 P-IκB 一抗 4 °C 孵育过夜,二抗室温孵育 2 h 后,避光加入 ECL 发光液,化学发光显影检测。

1.9 统计学分析

使用 Image-J 软件的面测量参数工具对显微镜图片进行分析,获取半定量数据。利用 GraphPad Prism 8.0.2 进行数据分析,组间比较采用单因素方差分析, $P<0.05$ 认为差异具有统计学意义。

2 结果与分析

2.1 动物实验流程及小鼠体重监测结果

如图 1B 所示,CPZ 饲喂一周后小鼠体重明显降低,持续饲养可逐渐恢复至初始体重,与 NC 组相比,CPZ 可显著抑制小鼠体重增加;probiotics 组小鼠第一周末体重轻微减轻,但直至实验结束体重水平始终高于 CPZ 组,说明复合益生菌制剂处理可改善 CPZ 导致的小鼠体重下降。

2.2 胼胝体病理检测结果

LFB 可染色髓鞘显示髓鞘的完整性。如图 2 所示,脑组织经 LFB 染色,NC 组小鼠大脑胼胝体区着色明显,而 CPZ 组着色显著减少($P<0.0001$),提示 CPZ 组小鼠的髓鞘脱落;probiotics 组着色更接近于 NC 组,显著多于 CPZ 组($P<0.001$),表明复合益生菌制剂可抑制 CPZ 诱导的小鼠胼胝体脱髓鞘。

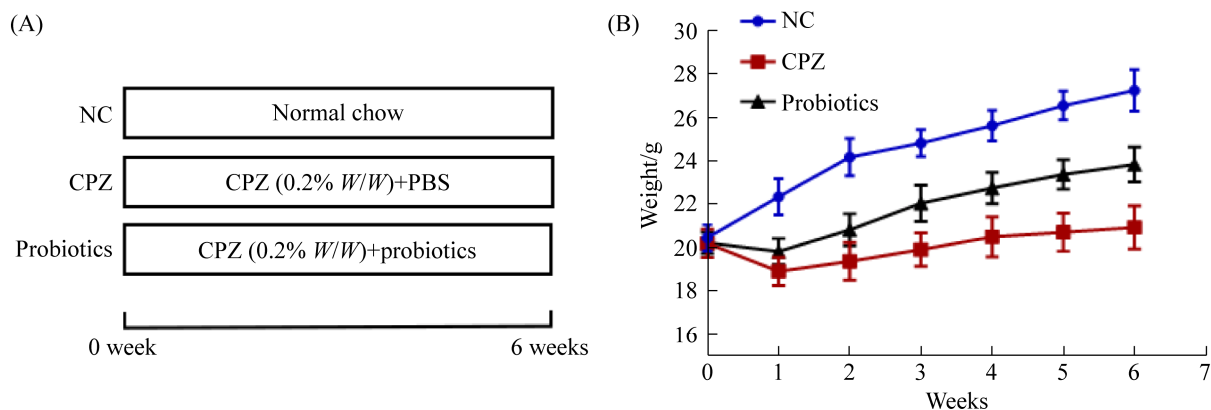


图 1 动物实验流程及各组小鼠体重变化图

Figure 1 Animal experiment process and body weight changes of each group.

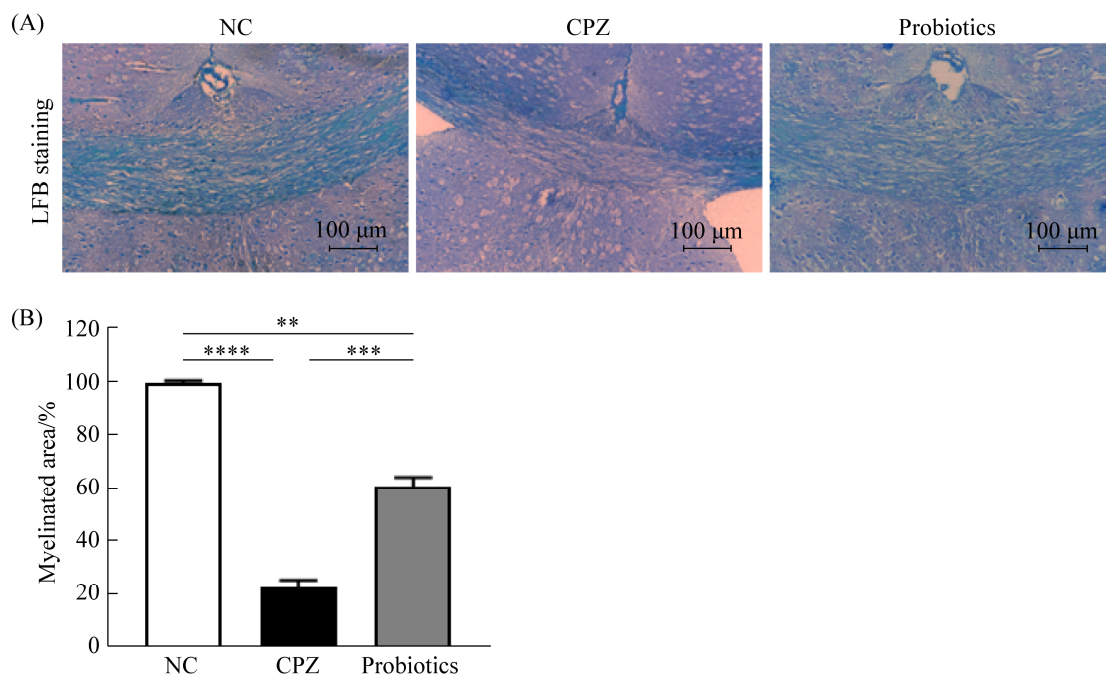


图 2 各组小鼠大脑胼胝体 LFB 染色

Figure 2 LFB staining of the corpus callosum of mice in each group. A: representative LFB staining images of the corpus callosum of mice in each group (200 \times); B: myelinated area of the corpus callosum. NC: normal; CPZ: cuprizone. ****: $P < 0.0001$; ***: $P < 0.001$; **: $P < 0.01$.

2.3 肠组织病理检测结果

HE 染色结果显示, 与 NC 组小鼠相比, CPZ 组小鼠回肠绒毛断裂、稀疏、排列紊乱, 结肠腺体减少, 有明显炎细胞浸润。复合益生菌制剂干

预后的小鼠回肠黏膜结构完整, 绒毛致密且排列整齐, 结肠腺体增多且无炎细胞浸润, 甚至优于正常小鼠, 见图 3。以上结果表明, 复合益生菌制剂可明显抑制 CPZ 诱导的小鼠肠道病理改变。

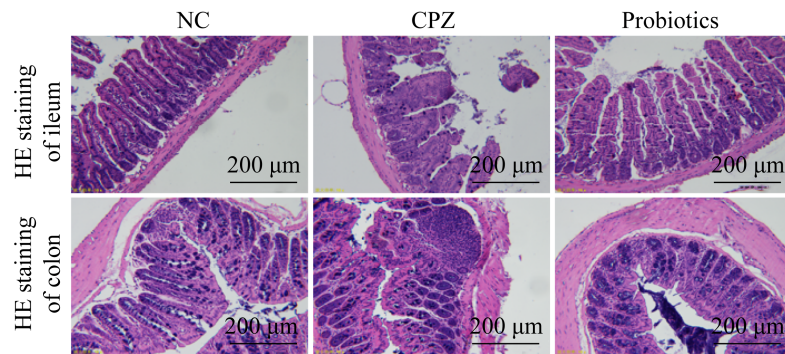


图3 各组小鼠回肠及结肠 HE 染色结果

Figure 3 HE staining of ileum and colon in each group (200×). NC: normal; CPZ: cuprizone.

2.4 肠组织黏蛋白及紧密连接蛋白检测结果

阿利新蓝染色结果显示, 与 NC 组相比, CPZ 组回肠($P<0.01$)及结肠($P<0.05$)的杯状细胞耗竭明显, 黏蛋白分泌显著减少, 复合益生菌

制剂干预后回肠($P<0.01$)及结肠($P<0.001$)杯状细胞数量明显增多, 黏蛋白分泌显著增加, 见图 4。肠屏障功能的维持靠紧密连接蛋白支撑, 回肠与结肠组织 ZO-1 和 occludin 的免疫荧光染色结果显示, 与 NC 组相比, CPZ 组 ZO-1、

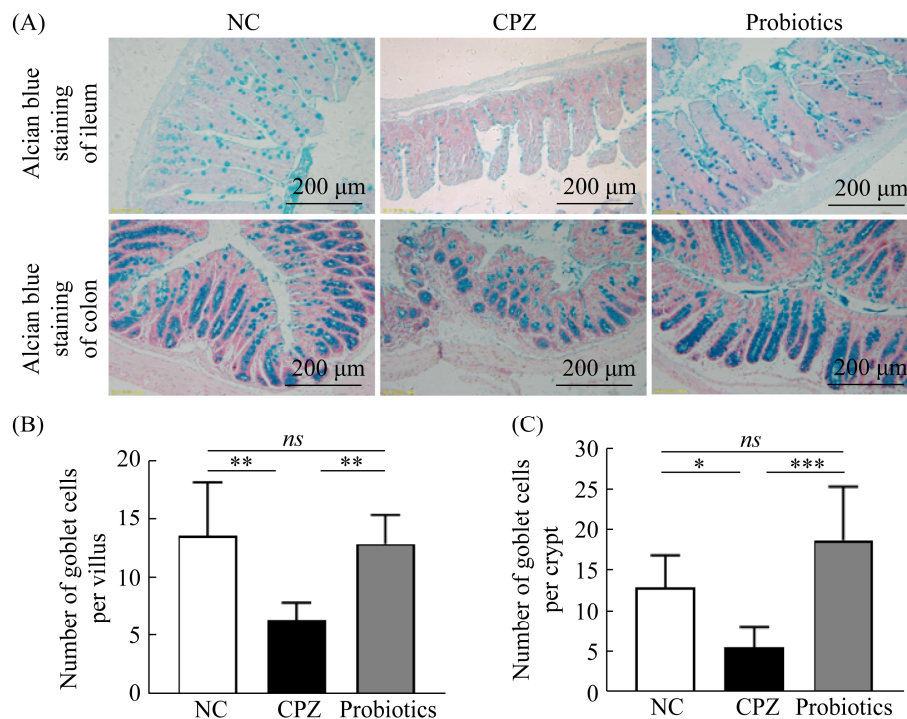


图4 各组小鼠回肠及结肠的阿利新蓝染色结果

Figure 4 Alcian blue staining of ileum and colon of mice in each group. A: representative alcian blue images of the ileum and colon of mice in each group (200×); B: the number of acid-mucin-producing goblet cells per villus; C: the number of acid-mucin-producing goblet cells per crypt. NC: normal; CPZ: cuprizone. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$; ns>0.05.

occludin 蛋白表达较低($P<0.001$), 提示 CPZ 组小鼠的肠屏障被破坏。复合益生菌制剂干预后小鼠的紧密连接蛋白 ZO-1、occludin 表达量明显增加($P<0.01$)。结果参见图 5 和图 6。以上结果说明, 复合益生菌制剂处理可有效保护肠屏障的完整性, 逆转 CPZ 诱导的小鼠肠道通透性的增加。

2.5 脑组织 LPS 及 TLR4/NF- κ B 信号通路检测结果

ELISA 检测脑匀浆 LPS 结果显示, 与 NC 组相比, CPZ 组小鼠脑匀浆的 LPS 显著升高

($P<0.001$), 复合益生菌干预后 LPS 水平明显下降($P<0.01$), 见图 7A。Western blotting 检测脑组织 TLR4/NF- κ B 信号通路蛋白的结果如图 7B、C 所示, 与 NC 组相比, CPZ 组小鼠脑组织中 TLR4、NF- κ B 及 P-I κ B/I κ B 蛋白表达量均升高($P<0.01$ or $P<0.05$); 与 CPZ 组小鼠相比, 复合益生菌干预组小鼠的 TLR4、NF- κ B 及 P-I κ B/I κ B 蛋白表达量均下降($P<0.05$)。以上结果表明, 复合益生菌制剂干预降低了 CPZ 引起的肠源性 LPS 的移位, 且显著抑制了脑组织中 TLR4/NF- κ B 信号通路。

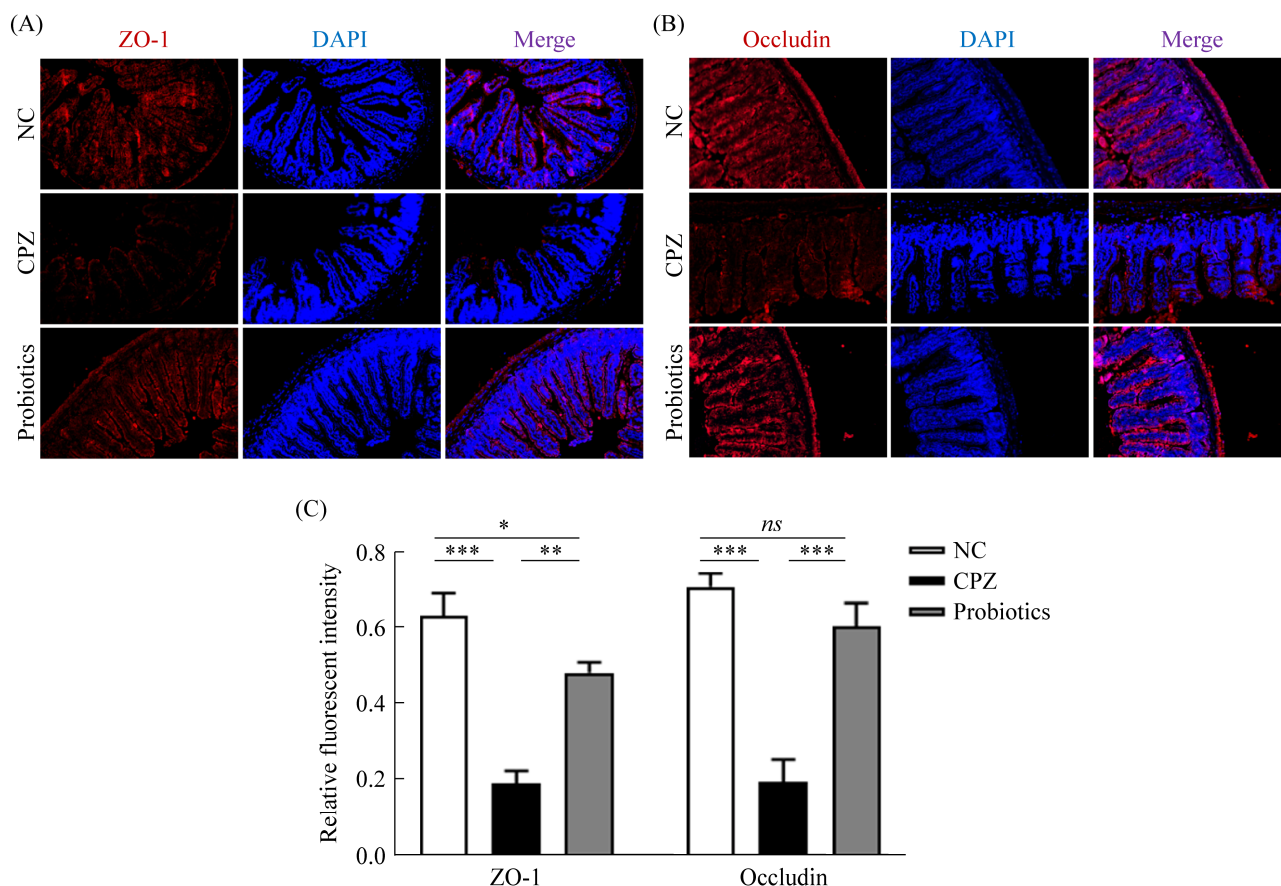


图 5 各组小鼠回肠紧密连接蛋白的免疫荧光检测结果

Figure 5 Immunofluorescence detection of tight junction proteins in ileum of mice in each group. A: representative ileum histology assessed by ZO-1 immunofluorescence staining (200 \times); B: representative ileum histology assessed by occludin immunofluorescence staining; C: quantification of staining intensity of ZO-1 and occludin of ileum in each group. NC: normal; CPZ: cuprizone. ***: $P<0.001$; **: $P<0.01$; *: $P<0.05$; ns>0.05.

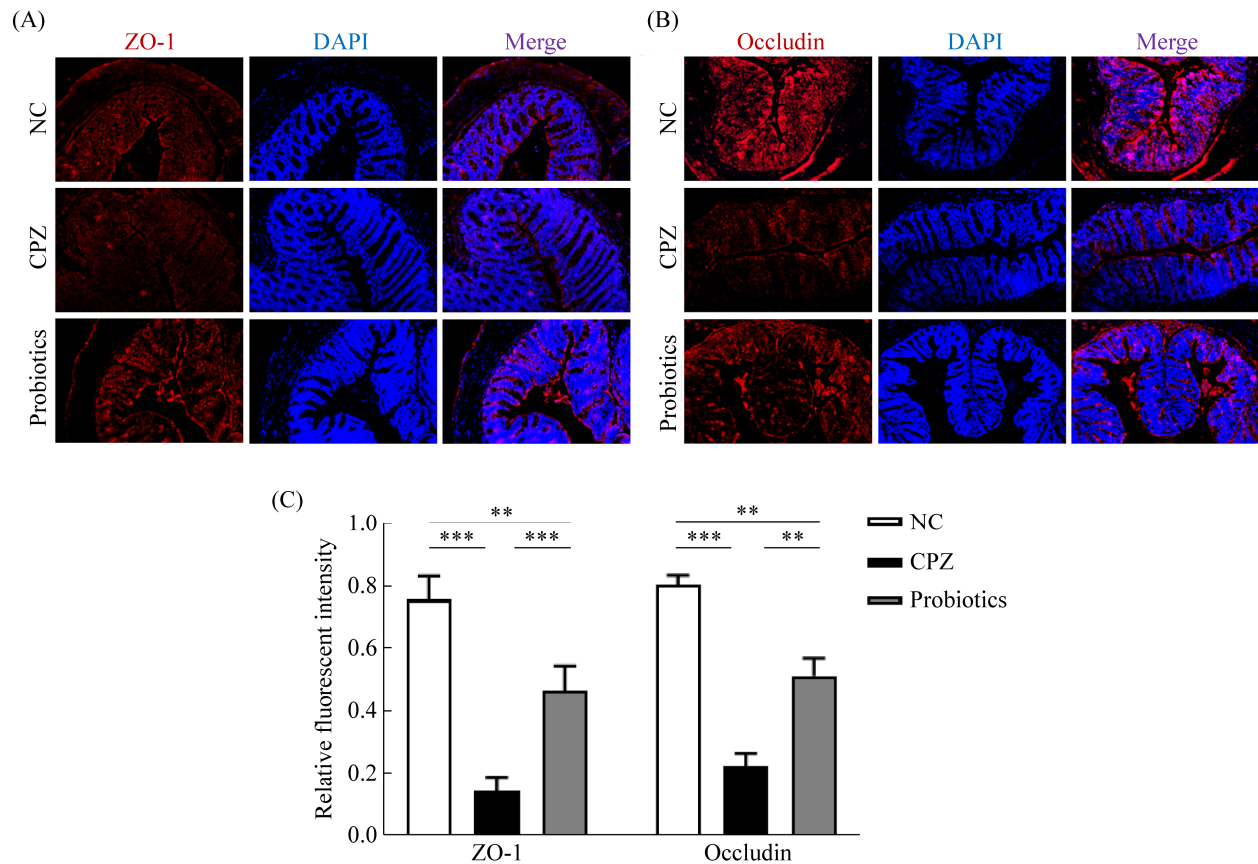


图 6 各组小鼠结肠紧密连接蛋白的免疫荧光检测结果

Figure 6 Immunofluorescence detection of tight junction proteins in colon of mice in each group. A: representative colon histology assessed by ZO-1 immunofluorescence staining (200 \times); B: representative colon histology assessed by occludin immunofluorescence staining; C: quantification of staining intensity of ZO-1 and occludin of colon in each group. NC: normal; CPZ: cuprizone. ***: $P < 0.001$; **: $P < 0.01$.

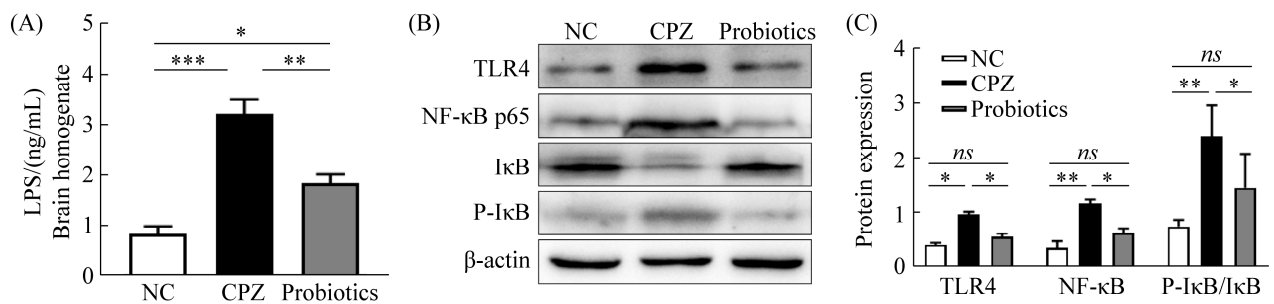


图 7 各组小鼠脑匀浆 LPS 及脑组织 TLR4/NF-κB 信号通路检测结果

Figure 7 LPS levels in brain homogenates and TLR4/NF-κB signaling pathway in brain tissues of mice in each group. A: brain homogenates concentration of LPS in each group; B: TLR4, NF-κB, IκB and P-IκB expression in the brain tissues from each group; C: the relative intensity of TLR4, NF-κB and P-IκB/IκB were quantified. NC: normal; CPZ: cuprizone. ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$; ns: $P > 0.05$.

3 讨论与结论

MS 是一种慢性 CNS 炎症性脱髓鞘疾病,是好发于年轻女性的常见非创伤性致残疾病^[13]。MS 的治疗以免疫抑制剂和免疫调节剂为主,这些疗法虽可部分缓解症状但需要长期用药且伴有较多的毒副作用^[14]。研究显示,MS 患者常表现出肠道潜在有益菌减少,有害菌增多的微生态失调^[15],如产生黏蛋白^[16]及维持肠道屏障完整性的细菌减少^[17]。脑-肠轴是肠道微生物群、肠道屏障与神经系统之间的相互作用,涉及神经、内分泌和免疫系统,可能与炎症性脱髓鞘病理改变有关^[18]。Kouchaki 等的研究表明,罗伊氏乳杆菌(*Lactobacillus reuteri*)可缓解 MS 患者的临床症状^[19]。本研究表明,连续饲喂含 CPZ 的饲料会破坏小鼠的肠屏障,表现在肠道紧密连接蛋白 ZO-1 和 occludin 的表达显著减少,黏蛋白表达降低,而复合益生菌制剂可抑制 CPZ 的作用使回肠及结肠紧密连接蛋白及黏蛋白表达显著增高,肠壁通透性下降,说明复合益生菌制剂缓解 CPZ 诱导的小鼠脱髓鞘病变与其修复肠道屏障完整性有关。

有研究发现,移植了 SPF 小鼠粪便的无菌小鼠 BBB 的通透性降低,这为肠道微生物群和 BBB 完整性之间的因果关系提供了证据^[11]。Al-Ghezi 等发现,EAE 小鼠的脑组织裂解物中 LPS 浓度显著增加,表明肠源性 LPS 通过 BBB 进入了脑组织^[20]。TLR4 是 LPS 的受体,LPS 可通过激活 TLR4/NF- κ B 信号通路诱导炎症反应^[21]。而 LPS 诱导的促炎反应能够激活小胶质细胞,破坏 BBB^[22]。Yang 等发现给衰老加速的小鼠(SAMP8)灌饲由乳酸双歧杆菌(*Bifidobacterium lactis*)、干酪乳杆菌、两歧双歧杆菌(*Bifidobacterium bifidum*)和嗜酸乳杆菌

(*Lactobacillus acidophilus*)组成的复合益生菌制剂(ProBiotic-4),可显著减轻老年 SAMP8 小鼠肠道屏障及 BBB 的破坏,降低血清和脑内 LPS 浓度,抑制 TLR4/NF- κ B 信号通路,使脑内神经炎症得到显著改善^[23]。肠道微生态失调常伴随着肠漏、血清 LPS 升高和 BBB 的破坏^[24-25]。肠壁通透性增加时,细菌成分及其代谢物可能穿过肠屏障、BBB,影响 CNS 的发育和功能,促进脱髓鞘等神经系统病变^[26-27]。

由此推测,本研究复合益生菌制剂可能通过降低肠壁通透性,减少细菌成分如 LPS 的渗漏,进而通过抑制 LPS-TLR4/NF- κ B 炎症通路缓解脑组织的炎症,使 CPZ 诱导的小鼠脱髓鞘病变减轻。

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