Irinotecan is a chemotherapeutic agent used to treat a variety of tumors, including colorectal cancer (CRC). In the intestine, it is transformed into SN-38 by gut microbial enzymes, which is responsible for its toxicity during excretion. Our study highlights the impact of Irinotecan on gut microbiota composition and the role of probiotics in limiting Irinotecan-associated diarrhea and suppressing gut bacterial β-glucuronidase enzymes. To investigate the effect of Irinotecan on the gut microbiota composition, we applied 16s rRNA sequencing in three groups of stool samples from healthy individuals, colon cancer, and Irinotecan treated patients (n=5/group). The gut microbiota was disturbed in individuals with colon cancer and after Irinotecan treatment. In the healthy group, Firmicutes were more abundant than Bacteriodetes and that was the opposite in case of colon-cancer or Irinotecan treated groups. Actinobacteria and Verrucomicrobia were markedly present within the healthy group while Cyanobacteria were noted in colon-cancer and the Irinotecan-treated groups. The family Enterobacteriaceae and genus Dialister were more abundant in colon-cancer group compared to other groups. The abundance of Veillonella, Clostridium, Butryicicoccus and Prevotella were increased in Irinotecan-treated groups compared to other groups. Furthermore, three Lactobacillus sp.; L. plantarum, L. acidophilus, L. rhamnosus were applied in a single and mixed form to in-vitro explore the effect of probiotics on the expression of β-glucuronidase gene from E. coli, the most important member of the family Enterobacteriaceae producing β-glucuronidase. Also, these probiotics were introduced in single and mixed forms in groups of mice before the administration of Irinotecan and their protective effects were explored by assessing the level of reactive oxidative species (ROS) as well as studying the concomitant intestinal inflammation and apoptosis. The usage of Lactobacillus sp. mixture significantly relieved Irinotecan-induced diarrhea through the reduction of both β-glucuronidase expression as well as ROS, in addition to guarding gut epithelium against microbial dysbiosis and proliferative crypt injury.