Alterations in gut microbiota modulate host physiological functions, including immune responses and they play a role in the pathophysiology of several diseases, including cancer. Radiotherapy (RT), an established curative and palliative cancer treatment, exerts potent immune modulatory effects, inducing tumor-associated antigen (TAA) cross-priming with anti-tumor CD8+ T cell elicitation and abscopal effects. Herein, we tested whether the gut microbiota modulates anti-tumor immune response following RT. Vancomycin, an antibiotic that acts mainly on gram-positive bacteria and is restricted to the gut, potentiated the RT-induced anti-tumor immune response and tumor growth inhibition. This synergy was dependent on cytolytic CD8+ T cells and on IFN-γ. Notably, butyrate, a metabolite produced by the vancomycin-depleted gut bacteria, abrogated the vancomycin effect. In conclusion, gram-positive bacteria depletion by vancomycin enhances the anti-tumor activity of RT, which has important clinical ramifications.