TITLE: Non-recombinant *Bacillus subtilis* endospores as microparticles adjuvant increases humoral immune response and protection conferred by a subunit vaccine against *enterotoxigenic Escherichia coli.*

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ABSTRACT:

Bacillus subtilis microparticles spores have received attention regarding potential biotechnological applications. *B. subtilis* spores have also been shown to behave as particulate vaccine adjuvants, promoting the increase of antibody responses after co-administration with antigens either admixed or adsorbed on the spore surface, but the adjuvants potential as a mucosal vaccine for via the oral inoculation has never been demonstrated. For this reason, this study was evaluated the potential adjuvant properties of *B. subtilis* endospores using the oral vaccine and a lethal neonatal mouse enterotoxigenic Escherichia coli (ETEC) challenge model. Groups of 10 female DBA/2 mice were inoculated via the orogastric .Vaccination regimen consisted of three doses given at 2-week intervals. Sham-vaccinated mice had with the same volumes of PBS administered. CFA/I fimbriae were administered only or combined with LTK63 adjuvant and/or B. subtilis spores. For the challenge model, one week before the last dose, male mice were introduced into the cages of the vaccinated females for a maximum period of 1 week. The final numbers of challenged newborns ranged from 16 to 41 for each tested condition. Serum, fecal sample and milk were collected 2 weeks after the last dose to monitor antibody titers. Statistical analyses were performed using ANOVA with Bonferroni's, Mantel-Cox test and a two-tailed Fisher exact test. Our results demonstrated that vaccination of female DBA/2 mice with CFA/I fimbriae each given with endospores or genetically attenuated LT adjuvant (LTK63) by orogastric delivery, induced significantly increased antigen-specific serum IgG and fecal IgA titers and detectable milk IgA antibody responses. Combined administration using LTK63 and spores as adjuvant showed a synergistic effect on the induced CFA/I-specific systemic and secreted antibody titers. Neonates born to and suckled by dams antenatally vaccinated with each of these regimens showed 13 to 98% survival after a 20× LD50 challenge with H10407(ETEC), compared to 100% mortality in pups from dams vaccinated with the sham vaccine, spores or LTK63 only. Crossover experiments showed that high pup survival rates after ETEC challenge were associated with suckling but not birthing from vaccinated dams, suggesting that vaccine-specific milk antibodies are protective. These findings indicate that *B. subtilis* spores may increase protection against ETEC and contribute to the development of mucosal subunit vaccines.

Keywords: ETEC, vaccine, toxin, bacillus, endospores.

Development Agency: CAPES, FAPESB and Universidade Estadual de Santa Cruz.