Food and Chemical Toxicology 103 (2017) 203–213

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Assessing the effect of oral exposure to *Paenibacillus alvei*, a potential biocontrol agent, in male, non-pregnant, pregnant animals and the developing rat fetus

Robert L. Sprando [a, *], Thomas Black [a], Nicholas Olejnik [a], Zachary Keltner [a], Vanessa Topping [a], Martine Ferguson [c], Darcy Hanes [d], Eric Brown [b], Jie Zheng [b]

[a] Division of Toxicology, Office of Applied Research and Safety Assessment, Center for Food Safety & Applied Nutrition, U.S. Food & Drug Administration, Laurel, MD 20708, United States
[b] Division of Molecular Biology, Center for Food Safety & Applied Nutrition, U.S. Food & Drug Administration, College Park, MD 20740, United States
[c] Division of Public Health Informatics, Office of Analytics and Outreach, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, Laurel, MD, United States
[d] Division of Virulence Assessment, Office of Applied Research and Safety Assessment, Center for Food Safety & Applied Nutrition, U.S. Food & Drug Administration, Laurel, MD 2008, United States

ARTICLE INFO

Article history:
Received 29 November 2016
Received in revised form
2 March 2017
Accepted 4 March 2017
Available online 10 March 2017

Keywords:
Biological control
Tomato
Salmonella enterica
Paenibacillus alvei
Oral toxicity
Fetus
Sex differences
Teratology

ABSTRACT

Paenibacillus alvei, a naturally occurring soil microorganism, may be used in the control and/or elimination of human/animal pathogens present on/within produce commodities associated with human consumption. The safety of oral exposure to *P. alvei* in male, nulliparous females, the pregnant dam and developing fetus was assessed. Adult male and female rats received a single oral dose (gavage) of *P. alvei* and tissues were collected at post exposure days 0, 3 and 14. To evaluate the effect of the test organism on fetal development, sperm positive female rats received the test organism every 3 days thereafter throughout gestation. As human exposure would be no more than 1×10^3 CFU/ml the following dose levels were evaluated in both study phases: 0 CFU/ml tryptic soy broth (negative control); 1×10^8 CFU/ml; 1×10^4 CFU/ml or 1×10^2 CFU/ml. Neither sex specific dose-related toxic effects (feed or fluid consumption, body weight gain, and histopathology) nor developmental/reproductive effects including the number of implantations, fetal viability, fetal weight, fetal length and effects on ossification centers were observed. The test organism did not cross the placenta and was not found in the amniotic fluid.
Published by Elsevier Ltd.

1. Introduction

Prevention of foodborne illness as a consequence of bacterial contamination is a major concern to the consumer, food industry and regulatory agencies (Estimate the Global Burden of Foodborne Diseases, 2006; WHO, 2006). *Salmonella*, in particular, represents one of the most common and dangerous foodborne bacterial pathogens Surprisingly, one of the major routes of transmission of *Salmonella* in foods is now through ingestion of fresh produce. This threat is accentuated by several recent widespread and lengthy *Salmonella* contamination events associated with produce including *S.* Newport from tomatoes and cucumbers and *S.* Poona from cucumbers grown both domestically and outside of the U.S (CDC, 2015; Britta Leverentz et al., 2006). Thus, it is extremely important that research be conducted to identify strategies which will minimize, eliminate or control salmonellae associated with this popular food sector. While several strategies have been proposed for controlling plant pathogens on plants including the manipulation of the cultivation environment, using disease-resistant cultivars, fungicides/bactericides/cleaning chemicals, and biocontrol agents, the use of biological agents (e.g. genetically modified microorganisms or organisms which are natural enemies of the target pathogen) seems to be the most advantageous (Cook, 1993; Pal and McSpadden Gardener, 2006).

Previously, two naturally-occurring gram-positive bacteria identified as *Paenibacillus alvei (P. alvei)* were isolated from plants native to the Virginia Eastern Shore tomato growing region.

* Corresponding author. Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, Laurel, MD 20708, United States.
E-mail address: Robert.sprando@fda.hhs.gov (R.L. Sprando).

http://dx.doi.org/10.1016/j.fct.2017.03.009
0278-6915/Published by Elsevier Ltd.

Specifically, strain TS-15 was demonstrated to be highly effective in reducing *Salmonella* Newport on blossoms and leaves of whole tomato plants through the high tunnel trials. Follow-up field trials have demonstrated that the application of *P. alvei* TS-15 has no adverse effect on tomato production in the field in terms of fruit quantity and quality (Allard et al., 2014). While the application of *P. alvei* TS-15 seems to be a promising approach as an early-intervention tool against Salmonella contamination at pre-harvest stage, one consideration for the use of this microorganism as a biocontrol agent is that we ensure this is harmless to humans in the event of an exposure event. Therefore, the focus of this study is to assess the effect of oral exposure to this potential bio control agent in male and nulliparous females as well as in the pregnant animals and the developing fetus.

2. Materials and methods

2.1. Animals and animal husbandry

All procedures involving animal care and handling were reviewed and approved by the CFSAN Institutional Animal Care and Use Committee. Male and Female Sprague-Dawley rats were obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA) and were acclimated in our research facility for up to one week. Light in the study room was provided on a 12-h light/dark cycle (light 7:30 a.m. to 7:30 p.m.), temperature was maintained at 68–79 °F and the relative humidity was maintained at 30–70%. Male and Female Sprague-Dawley rats were fed Purina 5002 M (Ralston-Purina, St. Louis, MO, USA). Ultra-pure water was provided to the animals at all times from the Hydro Pico-Systems water purification system. During the course of the study animal body weights, feed-cup weights and water bottle weights were recorded every day, water bottles were changed every 3 days and feed cups were changed every 6 days. Animals from all experimental groups were observed daily for signs of overt toxicity from the test organism. Signs of toxicity included lethargy, anorexia, hunched posture, ruffled coat and loose stool. Animals, if found in a moribund condition and animals showing severe pain and enduring signs of severe distress were euthanized using carbon dioxide. Animals euthanized for humane reasons are considered equivalent to animals that died on test.

2.2. Test organism

P. alvei strain TS-15 was propagated on tryptic soy agar (TSA) at 35 ± 2 °C. The inoculum of *P. alvei* TS-15 was prepared by rapid concentration of bacterial cells with continuous flow centrifugation (CFC) from 3 L overnight (20 h) culture in tryptic soy broth (TSB) with shaking at 160 ± 10 rpm. The centrifugation was performed at 80 rpm and 200 ml/min flow rate. It was then followed by 1–25 dilutions in water to obtain ~10^8 CFU/ml (high dose). Serial 100-fold dilutions were made to get ~10^4 CFU/ml (medium dose) and ~10^2 CFU/ml (low dose). The final inoculum concentrations were confirmed by directing plating in duplicate and counting the CFUs. The dosing solutions were prepared fresh prior to dosing. Doses were administered in a maximal volume of 10 ml/kg body weight. Animals were weighed daily and exposures for individual animals were based on their body weight.

2.3. Experimental design

The study was divided into two phases. In Phase 1, sex associated sensitivity was assessed in male and nulliparous female rats. In Phase 2, the effect of test organism exposure on gestation and fetal development was assessed.

2.3.1. Experimental design: phase 1: sex associated sensitivity

A total of 168 animals were utilized in Phase 1 of this study. Charles River Rats CD IGS VAF/± rats (n = 84 females; n = 84 males), at 8–10 weeks of age were delivered to our research facility. The animals were acclimated for up to 1 week prior to being released to the study. Male and female rats (n = 21) were assigned to one of 4 experimental treatment groups by weight using a stratified random procedure. This procedure reduces the possibility of large weight differences between groups. The test groups include: Group 1 = Control Group (tryptic soy broth; TSB −1:25 dilution dd water); Group 2 = 1×10^8 *P. alvei* CFU/ml TSB; Group 3 = 1×10^4 *P. alvei* CFU/ml TSB; Group 4 = 1×10^2 *P. alvei* CFU/ml TSB. The test organism was administered to animals in each group by gavage once at a volume of 10 ml/kg body weight at the concentrations stated above. The first day of gavage was considered exposure day 0. Animals (n = 7) from each of the treatment groups were euthanized by carbon dioxide asphyxiation and select tissues were collected at 1 day (24 h), 3 days (72 h) and 14 days (336 h) post exposure.

2.3.2. Experimental design: phase 2: teratology

A total of 150 animals were utilized in Phase 2 of this study. Charles River Rats CD IGS VAF/± rats (n = 100 females; n = 50 males), at 8–10 weeks of age were delivered to our research facility. The animals were acclimated up to 1 week prior to being released to the principal investigator. Male rats used as sires were not exposed to the test organism and were euthanized using carbon dioxide or transferred to an approved training protocol. Following acclimatization, the females were mated two females per male. Each morning after cohabitation each female was examined for the presence of sperm in the vaginal lavage or the presence of vaginal plug during the morning after cohabitation. Sperm-positive females were presumed pregnant (GD 0) and were assigned to the control or one of the three treatment groups by stratified random procedure. The test groups include: Group 1 = Control Group (TSB); Group 2 = 1×10^8 *P. alvei* CFU/ml TSB; Group 3 = 1×10^4 *P. alvei* CFU/ml TSB and Group 4 = 1×10^2 *P. alvei* CFU/ml TSB. The presumed pregnant rats were exposed to *P. alvei* by gavage on gestation days (GD) 0, 3, 6, 9, 12, 15 and day 18. On GD 20, female rats were euthanized by carbon dioxide asphyxiation and cesarean sections were performed to determine conventional toxic and/or developmental effects of the compound on the developing fetus. Amniotic fluid was collected to determine if the organism crossed the placenta. Briefly, the uterus was exposed via a mid-line incising in the abdomen and the uterine horns were exposed. A 1 cc syringe fitted with an 18 gauge needle was utilized to collect amniotic fluid from randomly selected fetuses from each uterine horn, if possible. The collected amniotic fluid, devoid of blood, was pooled for each litter and analyzed for the presence of the test organism (see below). Each uterus was subsequently removed, weighed and examined for the presence and position of resorption sites, implantation sites, and dead or live fetuses. Deciduomas were classified as early deaths. Implantation sites with placentas complete with non-viable fetuses of subnormal size or that were in a macerated condition were considered late deaths (MARTA Glossary Committee, 1986). Runt calculations were performed as described in Collins et al. (2001). The uteri of animals that did not appear pregnant were stained with ammonium sulfide to enhance the observation of implantation sites. Each viable fetus was examined individually and records were kept as to its uterine position, sex, weight, externally visible abnormalities and crown rump length. Fetuses were euthanized using dry ice. Half of the viable fetuses were processed for skeletal abnormalities after staining with Alizarin Red S (Dawson, 1926) and the remaining half were processed for soft-tissue abnormalities by serial sections (Wilson and