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Safety assessment of the commensal strain *Bacteroides xylanisolvens* DSM 23964

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ABSTRACT

We recently isolated and characterized the new strain *Bacteroides xylanisolvens* DSM 23964 and presented it as potential candidate for the first natural probiotic strain of the genus *Bacteroides*. In order to evaluate the safety of this strain for use in food, the following standard toxicity assays were conducted with this strain in both viable and pasteurized form: *in vitro* bacterial reverse mutation assay, *in vitro* chromosomal aberration assay, and 90 day subchronic repeated oral toxicity studies in mice. No mutagenic, clastogenic, or toxic effects were detected even at extremely high doses. In addition, no clinical, hematological, ophthalmological, or histopathological abnormality could be observed after necropsy at any of the doses tested. Hence, the NOAEL could be estimated to be greater than 2.3×10^{11} CFUs, and 2.3×10^{14} for pasteurized bacteria calculated as equivalent for an average 70 kg human being. In addition, the absence of any *in vivo* pathogenic properties of viable *B. xylanisolvens* DSM 23964 cells was confirmed by means of an intraperitoneal abscess formation model in mice which also demonstrated that the bacteria are easily eradicated by the host's immune system. The obtained results support the assumed safety of *B. xylanisolvens* DSM 23964 for use in food.

1. Introduction

Probiotics are live microorganisms, which, when administered in adequate numbers, confer a health benefit on the host (FAO/WHO, 2001; Guarner et al., 2005). Therefore, microbes used as probiotics may theoretically originate from any genera and species. However, besides conferring a health benefit to the host, a probiotic strain must first of all be shown to be safe at intended levels of use in food. Although probiotic strains belonging to species commonly found in fermented food are generally recognized as safe, strains belonging to nontraditional species may evoke greater concern about potentially adverse effects (Franz et al., 2003; Lund and Edlund, 2001), and therefore should be subject to an appropriate series of studies to demonstrate safety. As a consequence, research in the probiotic field has so far concentrated on traditional bacterial species (Sanders et al., 2010). This focus, however, may deter the identification and development of other probiotic strains with novel potentially valuable properties.

The genus *Bacteroides* may provide a source of interesting probiotic strains. First, this genus is the second most abundant in the human gut microbiota, surpassing *Lactobacillus* and *Bifidobacterium* by a factor of 10,000 (Hayashi et al., 2002, 2007). Furthermore, it has been reported to possess many metabolic and immunomodulatory activities that may be beneficial for the human body (Troy and Kasper, 2011; Dasgupta and Kasper, 2010; Comstock, 2009). However, despite this diversity and great potential, this genus did not attract much attention within the probiotic research community. In a recent study, we isolated a new *Bacteroides* strain (DSM 23964) from feces of healthy human individuals which belongs to the non-pathogenic species *B. xylanisolvens* (Ulsemer et al., 2011, in press). We demonstrated that it is (i) resistant towards the action of enzymes of the gastric and intestinal juice, (ii) sensitive to antibiotics, (iii) unlikely to transfer or acquire antibiotic resistance, and (iv) free from any of the virulence activities described for the genus *Bacteroides*. These results suggested the strain *B. xylanisolvens* DSM 23964 to be free of any virulence factors that would preclude it from being considered for further investigations, and eventually leading to the development of the first natural probiotic strain of the genus *Bacteroides*. However, for a complete safety assessment, further *in vitro* and *in vivo* analyses are still required.

Accumulating evidence suggests that, depending on the molecular mechanisms involved, killed probiotic strains may confer the

Abbreviations: BW, body weight; CFU, colony-forming unit; Crl, Charles River Laboratories; DMSO, dimethyl sulfoxide; EC, European Commission; FAO, Food and Agriculture Organization; FECI, Freiburg Ethics Commission International; GCP, Good Clinical Practice; GLP, Good Laboratory Practice; ICH, International Conference on Harmonization; i.p., intraperitoneal; NOAEL, no observed adverse effect level; NMRI, Naval Medical Research Institute; OD, optical density; OECD, Organization for Economic Cooperation and Development; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; UKEMS, United Kingdom Environmental Mutagen Society; WHO, World Health Organization.

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same health benefit as viable strains (Izumo et al., 2011; Kataria et al., 2009; Adams, 2010). Furthermore, the development of new functional ingredients based on an inactivated probiotic strain would (i) greatly facilitate its production and storage, (ii) expand its application possibilities, (iii) allow more correct estimation of dosage and shelf life data, and (iv), perhaps most importantly, avoid concerns about possible unwanted side effects that may be accompanied with the chronic intake of any living probiotic strain (Kataria et al., 2009; Fuller, 1989; Adams, 2010). Therefore, provided that the health benefit conferred by the strain *B. xylanisolvens* DSM 23964 is conserved after heat inactivation, it may be highly advantageous to commercialize it in inactivated form. Hence, and because some studies have revealed adverse effects resulting from the ingestion of heat killed probiotics (Kirjavainen et al., 2003), it is prudent that the safety of both the viable and the heat-inactivated bacteria be demonstrated.

This study presents the results of *in vitro* and *in vivo* toxicological and pathological analyses of the strain *B. xylanisolvens* DSM 23964 in both its viable and heat-inactivated (pasteurized) form.

2. Materials and methods

2.1. Bacterial strains

2.1.1. Test items

B. xylanisolvens DSM 23964 was cultured in Wilkins–Chalgren broth medium under anaerobic conditions, harvested by centrifugation, and lyophilized. The lyophilized *B. xylanisolvens* DSM 23964 material was used for the genotoxicity and oral toxicity studies. It contained 4×10^9 CFU/g (referred to as "viable bacteria"). The lyophilized preparation of pasteurized *B. xylanisolvens* DSM 23964 contained 1.7×10^{12} cells/g (referred to as "pasteurized bacteria"). Viability, purity and bacterial concentration of lyophilized bacterial strains were analyzed under GLP conditions by BIOTECON GmbH (Potsdam, Germany). For the *in vivo* pathogenicity study (abscess formation), fresh overnight cultures of *Bacteroides fragilis* RMA 6971, a clinical isolate obtained from the collection of the Institute for Medical Microbiology at the University of Leipzig, Germany, and of *B. xylanisolvens* DSM 23964 were harvested, washed, resuspended in PBS (5.28 g Na_2HPO_4, 1.44 g KH_2PO_4, 90 g NaCl per liter, pH 7.4), and mixed with 50% (w/w) autoclaved rat feces and 10% (w/v) barium sulfate to final concentrations of 5.0×10^6, 1.5×10^8, and 1.0×10^9 CFUs/ml for *B. fragilis* RMA 6971 and *B. xylanisolvens* DSM 23964.

2.2. In vitro mutagenicity study (Ames test)

This study was conducted at the Laboratory of Pharmacology (Hamburg, Germany) under GLP standards and according to the OECD guideline and the EC directive 2000/32/EC.

2.2.1. Tester strains

The following *Salmonella typhimurium* strains were obtained from Dr. Bruce N. Ames: TA 98 and TA 1537, which primarily respond to frameshift mutagens, and TA 100, TA 102 and TA 1535 which respond to base-pair substitution mutagens. In addition to the mutation in the histidine operon, these strains contain several other mutations that greatly increase their ability to detect mutagens.

2.2.2. Metabolic activation system

Post-mitochondrial fraction (S9 fraction) from rats treated with Aroclor 1254 (Analabs, North Haven, CT, USA) was prepared according to Maron and Ames (1983). Briefly; S9 was prepared from the livers of 20–30 rats. The pooled fraction was tested for its protein content according to Lowry et al. (1951) and for its P-450 content according to Mazel (1971). The values were 38.2 mg/mL protein and 1.97 nmol cytochrome P-450/mg protein, respectively. The S9 fraction was stored in liquid nitrogen. The 5% S9 mix was freshly prepared on the day of the test as follows: 5.0 mL rat liver fraction S9 (Aroclor 1254-induced), 2.0 mL 0.4 M $MgCl_2$ + 1.65 M KCl solution (sterile stock solution), 141.0 mg glucose-6-phosphate, 306.5 mg NADP, 50.0 mL 0.2 M phosphate buffer, pH 7.4 (sterile stock solution), and sterile *aqua ad injectabilia* ad 100 ml. Afterwards, the S9 mix was filter-sterilized by using a 0.45 μm filter and then kept on ice.

2.2.3. General methods

The lyophilized bacteria (viable and pasteurized cultures of *B. xylanisolvens* DSM 23964) were suspended in sterile 0.8% aqueous hydroxypropyl-methylcellulose solution (Fargon, Germany) under anaerobic conditions shortly before the start of the test. The sterile 0.8% hydroxypropylmethylcellulose solution (referred to as "vehicle") was degassed in an anaerobic jar (Oxoid, Wesel, Germany) with an anaerobic bag (Oxoid) for 24 h before use. Pasteurized bacteria were suspended to a final concentration of 118 mg/ml. Viable bacteria were suspended at concentrations of 57.0, 17.0, 5.8, 1.7, and 0.56 mg/ml. The vehicle alone served as negative control. Two independent experiments were carried out each with and without S9 activation; each experiment consisted of three plates per concentration and strain. Metabolic activation was performed with a liver post-mitochondrial fraction (S9 fraction) from Aroclor 1254-treated rats. The positive controls used in this study were: (i) without S9 activation: sodium azide (Sigma, Taufkirchen, Germany) in H_2O was added for strains TA100 and TA1535 (10 mg/plate), 2-nitrofluorene (Riedel de Häen, Seelze, Germany) in DMSO (Merck, Darmstadt, Germany) was applied for strain TA98 (10 μg/plate), 9-aminoacridine (Merck) in ethanol was employed for strain TA1537 (100 μg/plate), and methyl methane sulfonate (Merck) in DMSO was added for strain TA102 (1300 μg/plate); (ii) with S9 activation: 2-amino-anthracene (Oxoid) for strains TA98, TA102 and TA 1537 (2 μg/plate), and cyclophosphamide (Sigma) in *aqua ad injectabilia* (Delta) for strains TA100 and TA1535 (1500 mg/plate).

2.2.4. Plate incorporation test

Sterile top agar containing 0.6% agar and 0.5% NaCl was molten on the day of the test. Ten ml of a sterile solution of 0.5 mM L-histidine HCl and 0.5 mM biotin were added to 100 ml of molten agar. Two milliliter of this top agar were distributed into culture tubes held at 45 °C in a heating block. *Salmonella* cell suspension (0.1 ml, containing approximately 10^8 viable cells in the late exponential or early stationary phase), 0.5 ml of test item suspension (or 0.5 ml solvent, or 0.1 ml positive control), and 0.5 ml of S9 mix were added to the culture tubes. In the assay without metabolic activation, the S9 mix was substituted with 0.5 ml sterile PBS. The test components were mixed and then poured onto a Vogel–Bonner medium E minimal glucose agar plate. The plates were inverted and incubated for 48–72 h at 37 °C. The revertant colonies on the test plates and on the control plates were counted with a colony counter, and the presence of the background lawn on all plates was confirmed. A lawn that was thin compared with the lawn on the negative control plate was considered as evidence of bacterial toxicity.

2.2.5. Pre-incubation test

The test item and test suspension with the *Salmonella* tester strain (containing approximately 10^8 viable cells in the late exponential or early stationary phase) and sterile PBS or the metabolic activation system were pre-incubated for 20 min at 37 °C prior to mixing with the overlay agar and pouring it onto the surface of a