Regulatory Toxicology and Pharmacology 62 (2012) 336–346

Contents lists available at SciVerse ScienceDirect

Regulatory Toxicology and Pharmacology

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

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

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ARTICLE INFO

Article history:
Received 31 August 2011
Available online 6 November 2011

Keywords:
Bacteroides xylanisolvens
Bacteroides
Probiotics
GRAS
Safety
Mutagenicity
Clastogenic activity
Toxicity
Abscess formation

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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0273-2300
doi:10.1016/j.yrtph.2011.10.014

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

minimal agar plate. 0.5 ml of the test item suspension, 0.1 ml of *Salmonella* tester strain suspension and 0.5 ml of S9 mix or sterile PBS were mixed with 2 ml of overlay agar. Tubes were aerated during pre-incubation by using a shaker. The remaining steps were the same as described for the plate incorporation method.

For the statistical evaluation of the results a test item was considered to show a positive response, if (i) the number of revertants was significantly increased ($p \leqslant 0.05$, *U*-test according to Colquhoun, 1971) compared with the solvent control (at least 2-fold increase for TA 98, TA 100 and TA 102, and at least 3-fold increase for TA 1535 and TA 1537) in both experiments, and (ii) in addition, a significant ($p \leqslant 0.05$) concentration (log value)-related effect (Colquhoun) was observed. Positive results had to be reproducible, and the histidine independence of the revertants had to be confirmed by streaking random samples on histidine-free agar plates. Cytotoxicity was defined as a reduction in the number of colonies by more than 50% compared with the solvent control and/or a scarce background lawn.

2.3. In vitro assessment of the clastogenic activity (in vitro chromosomal aberration assay)

This test was conducted by the Laboratory of Pharmacology (Hamburg, Germany) under GLP standards and according to OECD guideline 437, EC directive 2000/32/EC, and ICH guideline S2B: (CPMP/ICH/174/95).

2.3.1. Metabolic activation system

A 'S9 mix' (prepared according to 2.2.2.) containing 10% rat liver post-mitochondrial fraction S9 was prepared.

2.3.2. Source of blood and culture conditions

Human peripheral blood was obtained by venipuncture from healthy human donors known to be not on any medication, and collected in heparinized vials. Small inocula of whole blood (0.5 ml) were added to tubes containing 4.5 ml of complete culture medium (500 ml chromosome medium 1A with phytohemagglutinin and 5 ml penicillin/streptomycin [10,000 IU/mL]). The tubes were sealed and incubated for 48 h at 37 °C with occasional shaking to prevent clumping. The cell pellets were resuspended in 4.0 ml treatment medium (500 ml Ham's F-10 and 13.1 ml fetal calf serum) with 0.5 ml S9 mix, or in 4.5 ml treatment medium including the test item at the final concentrations indicated above. The tubes were incubated for 4 h in a shaking water bath at 37 °C. After this period, the tubes were centrifuged, and the cells were washed with 5 ml of treatment medium to remove the test item and S9 mix. After a further centrifugation the cell pellet was resuspended in 5 ml of complete medium, and incubated for a further 20 h.

2.3.3. Preliminary toxicity study

A preliminary toxicity study was conducted to establish the top concentration for the main cytogenetic test. Each treatment was tested in the absence and in the presence of S9 mix. One thousand lymphocytes per culture were examined at a magnification of 400×; the mitotic index was calculated as the percentage of examined lymphocytes which were in mitosis (metaphase). The clastogenic activity of the following doses of test items was analyzed: 5.9 mg pasteurized bacteria/ml vehicle, or 0.028, 0.085, 0.29, 0.85 and 2.8 mg viable bacteria/ml vehicle. Mitomycin C (Sigma) was used as positive control for the study in the absence of metabolic activation, and cyclophosphamide (Sigma) as the positive control for the study in the presence of metabolic activation. The vehicle was used as negative reference item.

2.3.4. Main study

In a second set of experiments a continuous treatment of 24 h without metabolic activation was carried out, and the 4 h treatment with metabolic activation was repeated. Two hours before termination cell division was arrested by the addition of 0.5 ml of a 10 μg/ml solution of the spindle poison colcemid (Fluka, Buchs, Switzerland) to each culture. The tubes were capped and incubated for a further two hours. The cells were harvested by centrifugation at 80–100*g*, and the cell pellets were resuspended in hypotonic potassium chloride solution (0.56%) for 17 min. Following another centrifugation step of 10 min the cells were fixed in freshly prepared methanol/glacial acetic acid fixative (4:1, v/v). For each treatment and culture 100 metaphases were examined. All cultures were run in duplicate using blood from different donors. The observed aberrations were noted and scored according to Savage (1976). In addition, the total number of gaps was recorded in 100 metaphases for each culture. Metaphases which differed from the normal diploid complement (46 cases) were excluded from evaluation. However, test item-related variations of the normal chromosome number were noted (polyploidy/endoreduplication). To examine the toxicity of the test item 1000 cells were scored, and the mitotic index was calculated as percentage of cells in metaphase.

For a statistical evaluation the assessment was carried out by comparing the number of chromosome aberrations of the samples with those of the control, using the recommendations of the UKEMS guideline (Kirkland, 1989). In this study, therefore, only the total numbers of cells with aberrations (except of gap damages) were analyzed. The test item was judged as having mutagenic properties with respect to chromosomal or chromatid change, if: (i) the number of chromosomal aberrations was significantly increased ($p \leqslant 0.05$) compared with the control, (ii) the increase observed was concentration-dependent, (iii) both duplicate cultures led to similar results; *(iv)* the increase did not occur in the severely cytotoxic range (mitotic index < 0.25), as it was known that high cytotoxicity causes artifacts, and (v) a reproducible increase in the number of cells with chromosomal aberrations was observed.

2.4. Ninety-day oral toxicity study in mice

This study was conducted by the Laboratory of Pharmacology (Hamburg, Germany) under GLP standards and according to EC 2001/59/EC B.26 as well as the OECD guideline No. 408.

Hundred Crl:NMRI mice (50 male and 50 female) supplied by Charles River Laboratories (Sulzfeld, Germany) were weighed and allocated by means of a computerized randomization program to each of the five test groups (10 males and 10 females per group). The approximate age of animals was 6–7 weeks at the initiation of dosing. The animals were kept singly in macrolon cages (type I) with granulated textured wood bedding at a room temperature of 22 °C ± 3 °C (maximum range) and a relative humidity of 55% ± 15% (maximum range). The cages were changed and cleaned once a week. Commercial ssniff R/M-H V1530 (ssniff Spezialdiäten GmbH, 59494 Soest, Germany) served as food. Food and water were offered *ad libitum*. Periodic analysis of the food and drinking water for contaminants were conducted based according to EPA/USA rules (Proposed Health Effects Test Standards for Toxic Substances Control Act Test Rules, Federal Register 44, 27334–27375, May 1979) on a routine basis by LUFA-ITL (Landwirtschaftliche Untersuchungs- und Forschungsanstalt, Institut für Tiergesundheit und Lebensmittelqualität GmbH, 24107 Kiel, Germany). Certificates of analysis of the composition and for contaminants were provided by the manufacturer. The test item-vehicle mixtures were freshly prepared each day. Based on the determined bacteria concentration (see "test items"), lyophilized test items were

resuspended to final concentrations of 2×10^6, 2×10^7, and 2×10^8 CFUs of *B. xylanisolvens* DSM 23964 per ml 0.8% aqueous hydroxypropylmethylcellulose gel (referred to as "vehicle"), or 2×10^{11} pasteurized *B. xylanisolvens* DSM 23964 per ml under anaerobic conditions. A daily amount of 0.5 ml of the test item-vehicle mixtures was dosed orally by gavage to each mouse. One group of mice (control group) received the vehicle without bacteria. Daily cage-side observations focused on skin/fur, eyes, mucous membranes, respiratory and circulatory systems, motor activity, and behavioral pattern. Additionally, once before the first exposure (to allow for within-subject comparisons) and once a week thereafter (1, 2, 4, 8 and 24 h after administration), detailed clinical observations were made on all animals. Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies or bizarre behavior were also recorded. The body weights were recorded at the time of allocation of animals to groups, on the day of commencement of treatment, and subsequently in weekly intervals. All surviving animals were killed by exsanguination under light ether anesthesia after at least 90 days of dosing. Blood samples were collected at termination for hematological and clinical chemical examinations (Tables 5 and 6). The following instrumentation was used for hematological and biochemical analysis: for hematology ADVIATM 120 (Siemens diagnostics GmbH, Fernwald, Germany), for coagulation AMAX Destiny (Trinity Biotech GmbH, Lemgo, Germany), and for biochemistry KONELAB 30i (Thermo fisher scientific, Dreieich, Germany). All animals were subjected to gross necropsy and the weight of the main organs was recorded (Table 7). Histopathological examination of the main organs and tissues (Table 8) was performed on all animals of groups 1, 4 and 5.

Data on all functional tests were analyzed by Student's *t*-test. Data on hematological and clinicochemical parameters, body weight, food consumption, and organ weight were evaluated for treatment-related differences by Dunnett's multiple comparison tests. Histological changes were examined by Fischer's exact probability test.

2.5. In vivo pathogenicity of B. xylanisolvens DSM 23964 (abscess formation)

This study was conducted by the Laboratory of Pharmacology (Hamburg, Germany). Twenty-six male Swiss Webster mice (Taconic Europe A/S, Denmark), weighing between 35.5 and 52.6 g, were divided in seven groups (Table 1). The approximate age of the animals was 19 weeks at the time of administration. The animals were kept singly in macrolon cages (type I) with granulated textured wood bedding at a room temperature of 22 °C ± 3 °C (maximum range) and a relative humidity of 55% ± 15% (maximum range). Five gram of fresh feces from six untreated CD rats (male) were collected, suspended in 50 ml of sterile PBS and sterilized in an autoclave. After centrifugation (15 min at 750g), the supernatant was collected, autoclaved again, tested for sterility, and stored at −20 °C until use. *B. fragilis* RMA 6791 (positive control), and the test item *B. xylanisolvens* DSM 23964 were cultured in Wilkins–Chalgren broth medium for 24–48 h at 37 °C under anaerobic conditions. Shortly before the start of the experiments the cultures were centrifuged. The bacterial pellets were washed with 0.85% NaCl solution, and collected and stored at 37 °C under anaerobic conditions. The mice received intraperitoneally 200 µl of a test item-mixture solution (see test items) containing bacteria at a concentration ranging from 5×10^6 to 1×10^9 CFUs/ml (Table 1). Following application, the remaining suspensions were tested to ensure the microbiological purity of the test solutions at the time of injection. For this purpose, the undiluted bacterial suspensions were plated on appropriate agar plates and incubated 24–48 h at 37 °C under aerobic and anaerobic conditions. A sample from each plate was scraped and submitted to DNA extraction using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. Using the isolated DNA, multiplex species-specific PCRs were performed as described by Liu et al. (2003). For B. xylanisolvens, the primers and conditions described for the species *Bacteroides* ovatus revealed a single and *B. xylanisolvens-specific* band of about 820 bp. The multiplex PCR assay detected 300–500 cells. Animals were dissected after 7 days and examined for intra-abdominal abscesses. Two abscesses per animal of each group were removed under sterile conditions. Abscesses were punctured, and the abscess contents submitted to DNA extraction. Species-specific PCR as described above was applied for detecting the administrated bacteria.

Table 1
Experimental groups for intraperitoneal abscess formation.

Group	Bacterial strain	Dose (CFU/ml)	Number of animals Dissection on day 7
1	Negative control	–	4
2	*B. fragilis* RMA 6971	1.0×10^9	4
3	*B. xylanisolvens* DSM 23964	1.0×10^9	4
4	*B. fragilis* RMA 6971	1.5×10^8	4
5	*B. xylanisolvens* DSM 23964	1.5×10^8	4
6	*B. fragilis* RMA 6971	5.0×10^6	3
7	*B. xylanisolvens* DSM 23964	5.0×10^6	3

3. Results

3.1. Viability assay

The viability of *B. xylanisolvens* DSM 23964 after lyophilization and rehydration was analyzed in several independent experiments. The lowest identified survival rate indicated a minimum concentration of 4×10^9 CFU/g viable bacteria. This concentration was considered as the "available concentration".

3.2. In vitro mutagenicity study (Ames test)

This test was performed to detect any toxic or mutagenic effects of the *B. xylanisolvens* DSM 23964. Five doses of viable bacteria ranging from 0.28 to 28.5 mg/plate or one dose of 59 mg of pasteurized bacteria/plate were applied in two independent experiments, each carried out with and without metabolic activation. No signs of cytotoxicity and no increase in revertant colony numbers as compared with control counts were observed for any concentration of the five test strains with and without metabolic activation, and also in plate incorporation as well as in pre-incubation mode, respectively (Table 2).

3.3. In vitro assessment of the clastogenic activity (in vitro chromosomal aberration assay)

The top concentration of *B. xylanisolvens* DSM 23964 employed in the study was 2.8 mg viable or 5.9 mg pasteurized bacteria/ml sterile 0.8% aqueous hydroxypropyl-methylcellulose, which were considered to be the maximum reasonable concentration. As shown in Table 3, in the absence of metabolic activation, the mean incidence of chromosomal aberrations (excluding gaps) observed in the negative control was 1.0% or 0.5% after a 4-h or 24-h exposure, respectively. None of the concentrations of *B. xylanisolvens* DSM 23964, either viable or pasteurized, produced any statistically significant increase in aberrant cells from 0.5% to 2.5% after 4-h or 24-h exposure. In contrast, the positive control presented a 10.5% or 17.5% increase in aberrant cells after a 4-h or 24-h exposure, respectively. As presented in Table 4, in the presence of metabolic activation, the mean incidence of chromosomal aberrations

Table 2
Summary of reverted colonies of *Salmonella typhimurium* (Ames test) with and without metabolic activation with S9.

Test item	TA 98		TA 100		TA 102		TA 1535		TA 1537	
	(+S9)	(−S9)	(+S9)	(−S9)	(+S9)	(−S9)	(+S9)	(−S9)	(+S9)	(−S9)
Plate incorporation										
DSM 23964 (past.)[a]	37 ± 3.6	35.3 ± 4.5	157 ± 5.6	155.7 ± 5.7	270 ± 16.7	269 ± 4.6	23.7 ± 2.3	27.3 ± 11	8.3 ± 2.1	8.7 ± 2.3
DSM 23964 (viable)[b]	39.7 ± 4.2	34 ± 1	156.7 ± 6	159 ± 15.7	266 ± 21.7	267 ± 13.1	16.7 ± 3.2	29.7 ± 0.6	9.7 ± 0.6	6.7 ± 2.3
Negative control [c]	33.3 ± 4.9	32.7 ± 5.7	169 ± 11.4	160.3 ± 12.1	267 ± 13.1	280 ± 20	22 ± 4	31 ± 3.6	9.7 ± 0.6	9 ± 1
Positive control	451 ± 11[d]	685 ± 7.5[f]	748.3 ± 3.2[e]	774 ± 25.1[g]	1172 ± 7[d]	1185 ± 3.5[h]	266.7 ± 3.8[e]	262 ± 7.2[g]	194 ± 15.7[d]	194 ± 17.3[i]
Preincubation										
DSM 23964 (past.)[a]	30.3 ± 2.5	35.3 ± 4.5	132.3 ± 9.2	155.7 ± 5.7	264 ± 2.6	269 ± 4.6	16.3 ± 3.8	27.3 ± 11	4.7 ± 0.6	8.7 ± 2.3
DSM 23964 (viable)[b]	26.3 ± 1.5	28 ± 1	117.3 ± 6.7	130.7 ± 4	252.3 ± 14.5	269 ± 4	12.7 ± 2.1	20 ± 2	5.3 ± 3.1	6.3 ± 1.5
Negative control [c]	38 ± 6	31 ± 4.4	147.3 ± 4.2	152.7 ± 11.6	285.3 ± 5.5	288.3 ± 3.5	17.3 ± 3.1	20 ± 2	8.3 ± 1.5	8.7 ± 0.6
Positive control	441 ± 12[d]	689 ± 2.1[f]	1050 ± 11.3[e]	1020 ± 12.7[g]	1042 ± 46.8[d]	1145.7 ± 6.8[h]	379.7 ± 2.1[e]	410.3 ± 8.1[g]	177 ± 12.5[d]	166.3 ± 4[i]

Data are presented as Mean of revertant colony numbers ± SEM.
[a] DSM 23964 (past.) at 59 mg/plate.
[b] DSM 23964 (viable) at 25 mg/plate.
[c] Hydroxypropylmethylcellulose at 100 µl/plate.
[d] 2-Amino-anthracene at 2 µg/plate.
[e] Cyclophosphamide at 1500 mg/plate.
[f] 2-Nitrofluorene at 10 µg/plate.
[g] Sodium-azide at 10 mg/plate.
[h] Methyl methane sulfonate at 1300 µg/plate.
[i] 9-Aminoacridine at 100 µg/plate.

Table 3
Summary of *in vitro* chromosome analysis in human peripheral lymphocytes without metabolic activation.

Test item	Concentration (mg bacteria/ml)	1st Experiment 4 h Exposure			2nd Experiment 24 h Exposure		
		Mitotic index [a]	Metaphases scored	Aberrant cells (%)	Mitotic index	Metaphases scored	Aberrant cells (%)
DSM 23964 (viable)	0.085	1.15	200	1.5	0.95	200	2.0
	0.29	0.91	200	1.0[d]	1.02	200	1.0
	0.85	0.91	200	1.5[d]	1.22	200	2.5[d]
	2.8	1.09	200	2.0	0.76	200	1.0
DSM 23964 (past.)	5.9	0.98	200	1.5	1.03	200	0.5
Positive control[b]		0.76	200	10.5*	0.72	200	17.5[d,*]
Negative control[c]		1.00	200	1.0	1.00	200	0.5

[a] Number of metaphases/1000 cells.
[b] Mitomycin C (0.1 µg/ml in 1st experiment; 0.2 µg/ml in 2nd experiment).
[c] 0.8% Aqueous hydroxypropylmethylcellulose.
[d] Tetraploidy (excluded from evaluation).
* Significantly different from negative control ($p \leqslant 0.05$).

Table 4
Summary of *in vitro* chromosome analyses in human peripheral lymphocytes with metabolic activation.

Test item	Concentration (mg bacteria/ml)	1st Experiment 4 h Exposure			2nd Experiment 4 h Exposure		
		Mitotic index [a]	Metaphases scored	Aberrant cells (%)	Mitotic index	Metaphases scored	Aberrant cells (%)
DSM 23964 (viable)	0.085	1.06	200	1.5	1.10	200	1.5
	0.29	1.00	200	0.0	0.99	200	1.5
	0.85	1.06	200	0.5[d]	1.09	200	1.0
	2.8	0.92	200	0.5	0.98	200	1.0[d]
DSM 23964 (past.)	5.9	0.92	200	1.0	1.10	200	0.5
Positive control[b]		0.54	200	13.5*	1.05	200	16.5*
Negative control[c]		1.00	200	0.5[d]	1.00	200	0.5

[a] Number of metaphases/1000 cells.
[b] Cyclophosphamide (10 µg/ml in 1st experiment; 20 µg/ml in 2nd experiment).
[c] 0.8% Aqueous hydroxypropylmethylcellulose.
[d] Tetraploidy (excluded from evaluation).
* Significantly different from negative control ($p \leqslant 0.05$).

Table 5
Summary of hematological values in the mice subchronic 90-day oral toxicity study.

Females	HgB (mmol/L)	RBC (10^{12}/L)	WBC (10^9/L)	Retic (%)	PCT (10^9/L)	Neut (10^9/L)	Lym (109/L)	Mono (10^9/L)	Eos (10^9/L)
Group 1[a]	10.20 ± 0.48	10.22 ± 0.33	5.02 ± 2.64	25.40 ± 3.21	1275.4 ± 183.6	0.996 ± 0.92	3.892 ± 2.7	0.036 ± 0.02	0.058 ± 0.026
Group 2[b]	9.82 ± 0.79	9.84 ± 0.65	2.60 ± 0.68	27 ± 2.24	1122.6 ± 158	0.328 ± 0.042	2.17 ± 0.59	0.022 ± 0.016	0.056 ± 0.042
Group 3[c]	10.46 ± 0.3	10.2 ± 0.44	4.9 ± 2.55	27.2 ± 10.35	1394.2 ± 235.3	0.586 ± 0.35	4.128 ± 2.18	0.068 ± 0.036	0.094 ± 0.055
Group 4[d]	10.08 ± 0.33	9.86 ± 0.43	4.98 ± 1.53	26.40 ± 4.83	1349.6 ± 218.7	0.432 ± 0.2	4.332 ± 1.433	0.024 ± 0.009	0.162 ± 0.119
Group 5[e]	10.26 ± 0.26	10.06 ± 0.19	5.28 ± 2.35	29.8 ± 3.35	1261 ± 160.3	0.352 ± 0.124	4.730 ± 2.18	0.044 ± 0.043	0.086 ± 0.042
Females	LUC (10^9/L)	Baso (10^9/L)	Hct (%)	PT (s)	APTT (s)	MCV (fL)	MCH (pg)	MCHC (g/l)	
Group 1	0.016 ± 0.018	0.010 ± 0.007	48.8 ± 1.6	7.52 ± 0.4	24.42 ± 2.45	47.48 ± 1.06	16.08 ± 0.59	338.8 ± 6.9	
Group 2	0.006 ± 0.005	0.006 ± 0.005	47.6 ± 2.9	7.80 ± 0.44	24.98 ± 4.94	48.26 ± 1.4	16.08 ± 0.31	333 ± 11.9	
Group 3	0.020 ± 0.014	0.004 ± 0.005	50.2 ± 1.3	7.24 ± 0.4	24.12 ± 3.07	49.28 ± 1.9	16.56 ± 0.6	336.3 ± 2.25	
Group 4	0.012 ± 0.008	0.010 ± 0.007	47.8 ± 1.3	7.46 ± 0.18	23.44 ± 1.33	48.64 ± 1.87	16.5 ± 0.52	339.6 ± 9.25	
Group 5	0.022 ± 0.016	0.008 ± 0.008	48.6 ± 0.9	7.62 ± 0.26	21.1 ± 1.85	48.22 ± 0.86	16.43 ± 0.5	340.5 ± 7.77	
Males	HgB (mmol/L)	RBC (10^{12}/L)	WBC (10^9/L)	Retic (%)	PCT (10^9/L)	Neut (10^9/L)	Lym (10^9/L)	Mono (10^9/L)	Eos (10^9/L)
Group 1	9.82 ± 0.38	9.96 ± 0.38	6.58 ± 3.03	23.80 ± 2.39	1670.4 ± 162.4	0.778 ± 0.389	5.566 ± 2.546	0.106 ± 0.078	0.086 ± 0.044
Group 2	10.06 ± 0.62	10.18 ± 0.50	2.82 ± 0.96	26.20 ± 2.59	1372.8 ± 148.9	0.478 ± 0.239	2.202 ± 0.736	0.032 ± 0.030	0.060 ± 0.046
Group 3	9.98 ± 0.42	10.08 ± 0.62	5.02 ± 3.07	25.60 ± 3.91	1426.6 ± 234.1	0.818 ± 0.442	3.942 ± 2.531	0.040 ± 0.042	0.162 ± 0.204
Group 4	9.72 ± 0.50	9.98 ± 0.61	3.28 ± 1.82	26.40 ± 1.34	1538.6 ± 99.9	0.504 ± 0.354	2.692 ± 1.683	0.022 ± 0.013	0.050 ± 0.019
Group 5	9.94 ± 0.30	10.44 ± 0.27	5.78 ± 2.38	26.20 ± 2.95	1481.0 ± 218.5	0.626 ± 0.430	4.844 ± 2.105	0.058 ± 0.033	0.200 ± 0.193
Males	LUC (10^9/L)	Baso (10^9/L)	Hct (%)	PT (sec)	APTT (sec)	MCV (fL)	MCH (pg)	MCHC (g/l)	
Group 1	0.028 ± 0.019	0.008 ± 0.004	47.8 ± 1.6	7.78 ± 0.28	23.14 ± 2.06	47.98 ± 0.82	15.89 ± 0.24	331.23 ± 3.7	
Group 2	0.008 ± 0.008	0.004 ± 0.005	47.8 ± 2.5	7.98 ± 0.35	24.24 ± 2.45	47.10 ± 0.5	15.95 ± 0.4	339.02 ± 5.2	
Group 3	0.016 ± 0.015	0.006 ± 0.005	47.8 ± 2.2	7.84 ± 0.21	23.56 ± 2.35	47.64 ± 1.11	15.98 ± 0.5	334.8 ± 3.2	
Group 4	0.010 ± 0.012	0.002 ± 0.004	47.6 ± 1.8	7.54 ± 0.4	23.62 ± 1.2	47.66 ± 1.35	15.72 ± 0.54	329.8 ± 5.1	
Group 5	0.018 ± 0.015	0.006 ± 0.005	48.6 ± 1.1	7.94 ± 0.18	23.76 ± 0.96	46.62 ± 0.96	15.4 ± 0.63	330.5 ± 9.1	

Abbreviations: APTT, activated partial thromboplastin time; Baso, basophilic granulocytes; Eos, eosinophilic granulocytes; Hct, haematocrit value; HgB, haemoglobin content; LUC, large unstained cells; Lym, lymphocytes; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; Mono, monocytes; Neut, neutrophilic granulocytes; PCT, platelets; RBC, red blood cells; Retic, reticulocytes; fl, femtoliter.

[a] Control.
[b] DSM 23964 (viable) 1×10^6 CFU/animal/day.
[c] DSM 23964 (viable) 1×10^7 CFU/animal/day.
[d] DSM 23964 (viable) 1×10^8 CFU/animal/day.
[e] DSM 23964 (past.) 1×10^{11} cells/animal/day.

Table 6
Summary of clinical biochemistry values in the mice subchronic 90-day oral toxicity study.

Females	Albumin (g/L)	Globulin(g/L)	Bil.(μmol/L)	Chol.(mmol/L)	Creat.(μmol/L)	Glu.(mmol/L)	Protein(g/L)	Triglyc. (mmol/L)	BUN (mmol/L)
Group 1[a]	29.90 ± 0.76	24.50 ± 1.67	4.52 ± 0.65	2.79 ± 0.31	23.8 ± 1.6	6.00 ± 1.27	54.4 ± 1.8	0.77 ± 0.14	6.98 ± 1.27
Group 2[b]	31.02 ± 0.66	26.78 ± 2.98	4.08 ± 0.31	3.05 ± 0.56	22.4 ± 1.9	6.76 ± 1.04	57.8 ± 3.1	0.88 ± 0.23	6.20 ± 0.97
Group 3[c]	30.14 ± 1.34	26.86 ± 4.8	4.12 ± 1.02	3.06 ± 0.76	23.0 ± 2.1	6.95 ± 2.31	57.0 ± 5.2	0.72 ± 0.08	5.42 ± 1.5
Group 4[d]	31.06 ± 1.51	24.94 ± 2.66	4.14 ± 1.02	3.66 ± 0.28	24.4 ± 3.5	7.92 ± 2.46	56.0 ± 3.9	0.70 ± 0.135	6.26 ± 3.32
Group 5[e]	30.82 ± 0.75	24.38 ± 1.65	4.36 ± 0.49	3.20 ± 0.56	22.2 ± 3.8	5.756 ± 1.32	55.2 ± 1.6	0.87 ± 0.27	7.71 ± 2.01
Females	Ca^{++}(mmol/L)	Cl^-(mmol/L)	K^+(mmol/L)	Na^+(mmol/L)	ALAT(u/L)	AP(u/L)	ASAT(u/L)	LDH(u/L)	
Group 1	2.45 ± 0.11	106.0 ± 2.8	3.49 ± 0.28	147.8 ± 4.9	43.4 ± 15.6	120.6 ± 28.0	103.4 ± 23.8	265.6 ± 83.7	
Group 2	2.63 ± 0.11	108.4 ± 1.5	3.74 ± 0.63	151.4 ± 1.1	24.2 ± 4.1	136.4 ± 38.9	67.4 ± 9.3	159.0 ± 35.7	
Group 3	2.51 ± 0.06	108.8 ± 1.1	3.76 ± 0.39	150.8 ± 1.8	29.4 ± 5.9	112.4 ± 12.4	77.4 ± 25.8	192.4 ± 69.8	
Group 4	2.59 ± 0.12	108.6 ± 1.5	3.76 ± 0.61	151.4 ± 1.1	34.0 ± 17.7	119.2 ± 21.9	62.4 ± 10.5	184.4 ± 83.5	
Group 5	2.56 ± 0.15	108.2 ± 1.3	3.91 ± 0.19	150.0 ± 1.9	33.0 ± 15.0	156.6 ± 52.5	149.6 ± 93.8	241.6 ± 103.7	
Males	Albumin(g/L)	Globulin(g/L)	Bil.(μmol/L)	Chol.(mmol/L)	Creat.(μmol/L)	Glu.(mmol/L)	Protein(g/L)	Triglyc. (mmol/L)	BUN (mmol/L)
Group 1	29.56 ± 0.44	25.84 ± 1.63	4.78 ± 0.48	4.226 ± 0.64	24.2 ± 2.9	7.866 ± 0.94	55.4 ± 1.7	0.866 ± 0.15	8.37 ± 1.38
Group 2	28.92 ± 0.75	25.08 ± 1.55	5.36 ± 0.86	4.094 ± 0.49	22.0 ± 2.4	6.468 ± 0.95	54.0 ± 1.6	0.802 ± 0.16	8.21 ± 3.03
Group 3	29.62 ± 1.10	28.18 ± 5.20	5.14 ± 0.49	3.802 ± 0.37	25.8 ± 3.3	6.392 ± 1.25	57.8 ± 5.5	0.840 ± 0.11	11.04 ± 2.84
Group 4	29.30 ± 0.98	27.70 ± 2.79	4.58 ± 0.71	4.176 ± 0.47	21.2 ± 1.6	6.238 ± 0.89	57.0 ± 3.2	0.878 ± 0.22	8.02 ± 1.25
Group 5	28.46 ± 1.13	25.94 ± 1.73	5.08 ± 0.86	3.776 ± 0.77	23.4 ± 3.4	7.638 ± 0.54	54.4 ± 2.1	0.790 ± 0.365	9.42 ± 2.29
Males	Ca^{++}(mmol/L)	Cl^-(mmol/L)	K^+(mmol/L)	Na^+(mmol/L)	ALAT(u/L)	AP(u/L)	ASAT(u/L)	LDH(u/L)	
Group 1	2.60 ± 0.11	109.4 ± 0.5	3.84 ± 0.35	151.8 ± 1.8	33.2 ± 18.6	79.4 ± 4.4	105.8 ± 105.5	284.0 ± 183.6	
Group 2	2.50 ± 0.11	109.2 ± 1.6	4.00 ± 0.4	150.6 ± 3.0	33.4 ± 9.0	87.6 ± 18.8	72.2 ± 22.9	240.4 ± 64.7	
Group 3	2.61 ± 0.105	109.6 ± 2.4	3.82 ± 0.29	151.8 ± 2.6	28.6 ± 6.2	90.4 ± 4.1	67.6 ± 14.2	168.4 ± 14.1	
Group 4	2.53 ± 0.09	109.4 ± 1.7	4.16 ± 0.49	152.8 ± 2.3	30.8 ± 6.0	81.4 ± 17.0	69.0 ± 8.2	214.0 ± 58.3	
Group 5	2.48 ± 0.14	109.0 ± 1.6	3.91 ± 0.3	151.2 ± 1.3	32.4 ± 6.3	85.6 ± 16.3	70.8 ± 21.2	216.6 ± 65.5	

Abbreviations: ALAT, alanine aminotransferase; AP, alkaline phosphatase; ASAT, aspartate aminotransferase; Bil, bilirubin; BUN, blood urea nitrogen; Chol, cholesterol; Creat, creatinine; Glu, glucose; LDH, lactate dehydrogenase; Triglyc, triglycerides.

[a] Control.
[b] DSM 23964 (viable) 1×10^6 CFU/animal/day.
[c] DSM 23964(viable) 1×10^7 CFU/animal/day.
[d] DSM 23964 (viable) 1×10^8 CFU/animal/day.
[e] DSM 23964 (past.) 1×10^{11} cells/animal/day.

Table 7
Summary of relative organ weights (g/kg b.w.).

Organs		Group 1[a]	Group 2[b]	Group 3[c]	Group 4[d]	Group 5[e]
Males						
Adrenal	Left	0.171 ± 0.062	0.183 ± 0.205	0.200 ± 0.100	0.128 ± 0.043	0.182 ± 0.063
	Right	0.160 ± 0.083	0.158 ± 0.088	0.198 ± 0.075	0.132 ± 0.051	0.171 ± 0.073
Brain		12.74 ± 0.824	14.297 ± 1.771	14.01 ± 1.096	13.525 ± 1.822	14.378 ± 2.154
Epididymis	Left	1.808 ± 0.716	1.830 ± 0.634	1.768 ± 0.313	1.659 ± 0.519	1.820 ± 0.182
	Right	1.461 ± 0.265	1.843 ± 0.483	1.956 ± 0.312	1.617 ± 0.369	2.009 ± 0.384*
Gonads	Left	3.594 ± 0.431	4.152 ± 0.860	3.532 ± 0.301	3.326 ± 0.536	3.807 ± 0.272
	Right	3.603 ± 0.390	4.244 ± 0.561*	3.742 ± 0.410	3.397 ± 0.457	3.779 ± 0.543
Heart		4.985 ± 0.588	5.440 ± 0.695	5.396 ± 0.828	5.182 ± 0.507	5.330 ± 0.620
Kidney	Left	8.218 ± 0.912	8.763 ± 1.008	8.649 ± 0.719	8.654 ± 1.114	8.469 ± 1.164
	Right	8.108 ± 0.915	8.571 ± 1.391	8.895 ± 0.686	8.570 ± 1.061	9.204 ± 1.216
Liver		48.52 ± 4.09	50.76 ± 4.63	50.08 ± 4.40	50.43 ± 4.33	46.85 ± 6.05
Lungs		6.935 ± 0.851	7.199 ± 1.307	7.253 ± 0.775	7.349 ± 1.239	7.316 ± 1.266
Spleen		2.626 ± 0.707	2.447 ± 0.727	2.996 ± 0.982	2.759 ± 0.979	2.726 ± 0.594
Thymus		1.337 ± 0.296	1.007 ± 0.260	1.181 ± 0.425	1.140 ± 0.232	1.391 ± 0.348
Females						
Adrenal	Left	0.353 ± 0.098	0.549 ± 0.380	0.396 ± 0.063	0.320 ± 0.085	0.354 ± 0.156
	Right	0.358 ± 0.164	0.491 ± 0.334	0.356 ± 0.119	0.318 ± 0.072	0.308 ± 0.101
Brain		18.18 ± 2.024	18.063 ± 2.026	17.710 ± 1.681	17.651 ± 3.098	17.433 ± 1.806
Gonads	Left	0.473 ± 0.133	0.627 ± 0.272	0.552 ± 0.184	0.367 ± 0.166	0.436 ± 0.123
	Right	0.447 ± 0.183	0.551 ± 0.254	0.507 ± 0.156	0.406 ± 0.171	0.453 ± 0.189
Heart		5.343 ± 0.602	5.229 ± 0.624	5.254 ± 0.587	5.478 ± 0.819	5.610 ± 0.643
Kidney	Left	7.696 ± 1.145	7.328 ± 0.719	7.086 ± 0.908	6.778 ± 0.883	6.706 ± 0.438
	Right	7.775 ± 0.817	7.342 ± 0.802	7.016 ± 0.895	6.840 ± 0.986	6.908 ± 0.519
Liver		52.55 ± 7.12	49.19 ± 6.74	51.78 ± 4.14	48.90 ± 4.29	56.66 ± 6.07
Lungs		8.739 ± 0.976	7.912 ± 0.709	8.267 ± 1.080	7.906 ± 0.585	8.439 ± 1.304
Spleen		3.358 ± 0.725	3.280 ± 0.946	3.186 ± 0.609	3.425 ± 1.099	3.475 ± 0.790
Thymus		1.606 ± 0.321	1.714 ± 0.559	1.928 ± 0.456	1.776 ± 0.320	1.575 ± 0.394
Uterus		5.582 ± 1.429	6.510 ± 3.073	5.495 ± 2.362	6.394 ± 5.836	5.218 ± 1.310

Data are presented as mean (g/kg b.w.) ± SEM.
[a] Control.
[b] DSM 23964 (viable) 1×10^6 CFU/animal/day.
[c] DSM 23964(viable) 1×10^7 CFU/animal/day.
[d] DSM 23964 (viable) 1×10^8 CFU/animal/day.
[e] DSM 23964 (past.) 1×10^{11} cells/animal/day.
* Statistically significant ($p < 0.01$) difference with group 1 (control).

(excluding gaps) observed in the negative control was 0.5% after a 4-h exposure. Again, none of the concentrations of *B. xylanisolvens* DSM 23964, either viable or pasteurized, produced any statistically significant increase in aberrant cells, resulting in 0% to 1.5% in two independent experiments. The positive control presented 13.5% and 16.5% aberrant cells after a 4-h period in two experiments, respectively. For all *B. xylanisolvens* DSM 23964 concentrations tested, no item-related endoreduplication was noted in the experiments with or without metabolic activation. Furthermore, no signs of cytotoxicity were noted at any tested concentration of *B. xylanisolvens* DSM 23964 in the experiments with and without metabolic activation (data not shown).

3.4. Ninety-day oral toxicity study in mice

No mortality occurred during the study. None of the mice treated or untreated exhibited any clinical sign on cage-side observation. The results of the functional battery observations and motor activity tests also did not reveal any test item-related changes (data not shown). Food and water consumption as well as body weight gain did not differ between the treated groups (Fig. 1). The hematological examination revealed no statistically significant differences between treated groups and the control group (Table 5). Noteworthy, the number of leucocytes identified in group 2 animals and the respective parameters of the differential blood count were relatively low compared to the control group and other treatment groups. However, all values were still in the normal range, and the difference to the control group was not statistically significant. The clinical biochemical parameters also remained unaffected by the treatments. The observed decreased plasma ASAT concentrations in females of group 4 were considered to be fortuitous since there was no evidence of a dose–response relationship, and since there were no corresponding changes observed in male mice (Table 6). The ophthalmological examination revealed no pathological observations for any group of mice. Organ weights and their physical appearance were not affected by the treatment (Table 7). The observed statistically significant difference between males of group 2 and group 5 and the control group for the right epididymis and right gonad, respectively, were considered to be incidental. No dose–response relationships were detected, and in each case statistical significance could only be identified for one of the pair organs. Furthermore, the histopathological examination of standard organs and tissues revealed no treatment-related changes (Table 8). All microscopic findings noted were within the normal range for animals of this strain and age.

3.5. In vivo pathogenicity of B. xylanisolvens DSM 23964 (abscess formation)

The bacterial viability, concentration and purity of the injected test mixture solutions were determined retrospectively on the remaining material after injection. The bacterial viability and concentration were confirmed through plating on agar plates under anaerobic incubation conditions (data not shown). Multiplex PCR confirmed the presence of the desired bacterial strain and

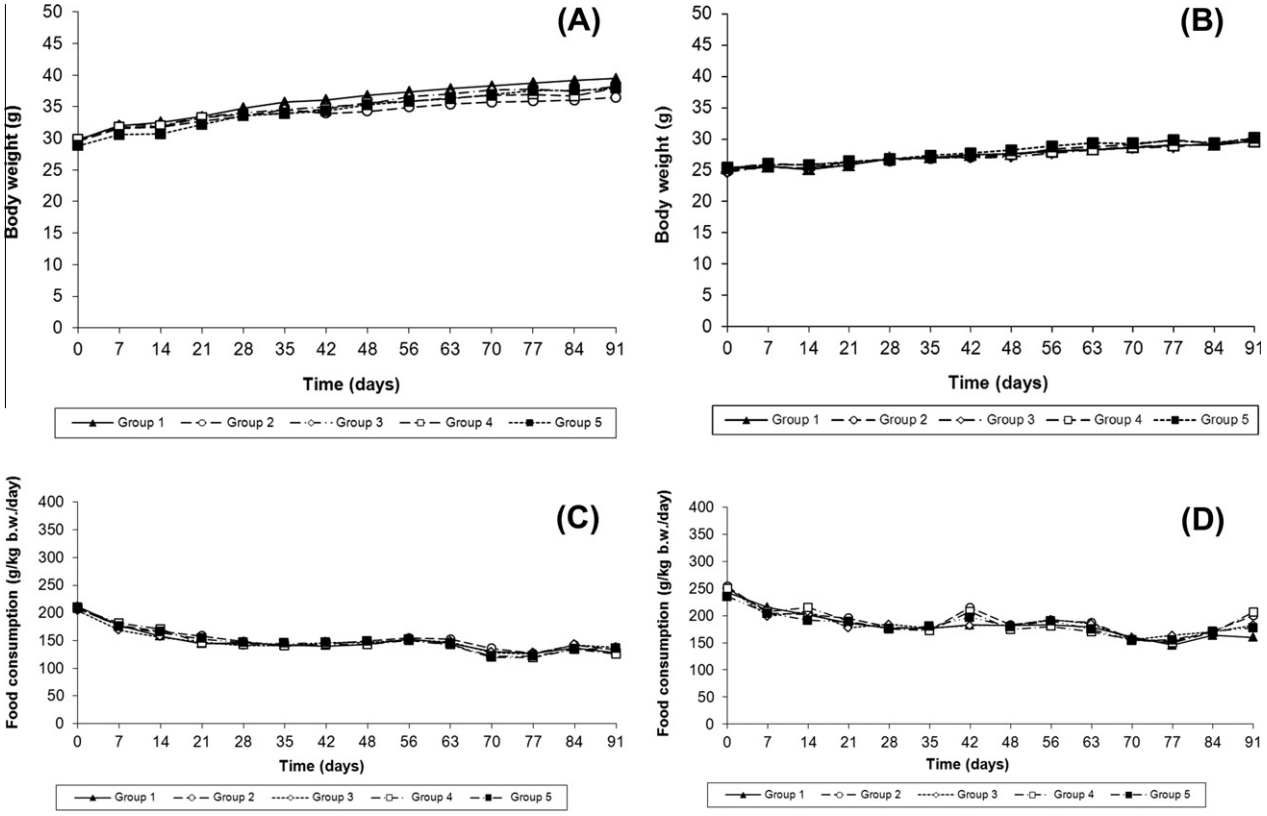

Fig. 1. Body weight and food consumption of mice during the 90 day oral toxicity study. (A) Body weights of male Crl:NMRI mice. (B) Body weights of female Crl:NMRI mice. (C) Food consumption of male Crl:NMRI mice. (D) Food consumption of female Crl:NMRI mice. Mean values per group: Group1 (0), Group2 (1×10^6), Group3 (1×10^7), Group4 (1×10^8) CFU's of *Bacteroides xylanisolvens* DSM 23964/animal/day. Group5 (1×10^{11}) *B. xylanisolvens* DSM 23964 pasteurized/animal/day. Differences are not statistically significance according to Dunnett's test.

the absence of cross contamination with the other strain (Fig. 2). Common contaminations could be excluded through plating of each test item on an appropriate agar plate and incubation under aerobic conditions.

In two separate experiments, mice injected with a high dose of *B. xylanisolvens* DSM 23964 did not develop more or larger abscesses than the negative control (barium sulfate + sterile rat feces). In contrast, high concentrations of *B. fragilis* RMA 6971 induced the formation of more and larger abscesses (data not shown). After 7 days, 2 abscesses per animal were taken under sterile conditions, punctured, and the contents submitted to DNA extraction. The presence of *B. xylanisolvens* DSM 23964 or *B. fragilis* RMA 6791 in the abscesses was evaluated by means of species-specific PCRs. As shown in Fig. 3, *B. fragilis* RMA 6971 could be detected in all abscesses isolated from groups 2, 4 and 6 injected with 5.0×10^6 to 1.0×10^9 CFUs of *B. fragilis* per ml, respectively. In contrast, independent of the bacterial concentration injected, *B. xylanisolvens* DSM 23964 could not be detected in any of the analyzed abscesses, suggesting that the strain was either absent or present at an amount below the detection limit of our species-specific PCRs. These results indicate that *B. xylanisolvens* DSM 23964 does not induce the formation of abscesses, and that it is quickly eliminated by the immune system after i.p. injection.

4. Discussion

We have recently isolated a novel *B. xylanisolvens* strain (DSM 23964) and demonstrated it to be free from any virulence factors known for the genus *Bacteroides* (Ulsemer et al. 2011, in press). The present study aimed to complete the safety assessment of *B. xylanisolvens* DSM 23964 through toxicological and pathological studies.

As no guidelines explicitly indicate the doses of probiotic bacteria to be tested, the genotoxicity and toxicity of a high daily dose of pasteurized or viable *B. xylanisolvens* DSM 23964 cells were assessed. Although lyophilized pasteurized bacteria could be produced to a final concentration of 4×10^{12} cells per gram, technical limitations prevented the use of such a concentration for viable cells. Indeed, as reported for many other microorganisms (Berner and Viernstein, 2006; Miyamoto-Shinohara et al., 2000), the lyophilization and/or rehydration process significantly affected the viability of our strain, limiting the post-rehydration concentration to 4×10^9 CFUs/g. Noteworthy, the integrity of the cell was not affected (data not shown).

Both the Ames test and the *in vitro* chromosomal aberration assay demonstrated that neither the viable nor the pasteurized form of the strain DSM 23964 exert any mutagenic or genotoxic effects.

Furthermore, a 90-days sub-chronic oral gavage study in mice with doses of up to 1×10^8 CFUs live bacteria/animal/day or 1×10^{11} pasteurized bacteria/animal/day demonstrated the absence of any toxic effect of strain DSM 23964. Indeed, extensive clinical observations, hematological data, clinical chemistry parameters, as well as *post mortem* macroscopic and histopathological examinations of organs revealed no adverse effects in either female or male mice for both viable or pasteurized bacteria of strain DSM 23964. Taking into account a mean body weight of

Table 8
Summary of histopathological findings.

Organ	Male			Female		
	Gr.1[a]	Gr.4[b]	Gr.5[c]	Gr.1[a]	Gr.4[b]	Gr.5[c]
Adrenal (I)	9	10	10	10	10	10
Foamy cells, zona reticularis	2	2	1	8	10	10
Subcapsular hyperplasia	–	–	1	3	6	6
Adrenal (II)	9	9	10	10	10	10
Capillary ectasia	–	1	–	7	10	10
Foamy cells, zona reticularis	2	3	3	7	5	7
Subcapsular hyperplasia	2	4	–*	–	–	–
Duodenum.	9	10	10	10	10	10
Subepithelial oedema	–	–	–	–	1	–
Epididymis (II)	10	9	10	10	10	10
Interstit.lymphocytic infiltr.	1	–	2	–	–	–
Eye (I), incl. optic nerve	10	10	10	10	10	10
Limbus: Mixed cell infiltr.	1	1	1	1	1	–
Retrobulbar,mixed cell infilt.	2	3	1	1	1	–
Destruction	1	–	–	–	1	–
Eye (II), incl. optic nerve	10	10	10	10	10	10
Limbus: Mixed cell infiltr.	–	1	–	–	1	2
Retrobulbar,mixed cell infilt.	–	–	1	–	1	2
Destruction	–	–	1	1	1	0
Gall bladder.	10	10	8	10	10	10
Mixed cell. infiltration	–	–	–	1	–	–
Subepithelial lymph.infiltr.	3	3	3	2	2	4
Epithelial vacuolisation	–	1	–	–	1	–
Heart (3 sections)	10	10	10	10	10	10
Mixed cell infiltration	2	1	–	–	–	–
Granulation tissue	1	1	1	–	–	–
Lympho-histiocytic infiltr.	1	–	–	–	1	–
Brown.pigm.endo.cells/macroph.	–	1	–	–	–	–
Heart (scarlet R).	10	10	10	10	10	10
Fatty infiltraion	–	–	–	3	3	1
Jejunum.	9	10	10	10	10	10
Granulocytic infiltration	–	–	–	–	1	–
Kidney (I) and ureter	10	10	10	10	10	10
Congestion	–	1	–	1	–	–
Hyaline tubular casts	–	2	–	1	1	2
Lympho-histiocytic infiltr.	5	4	5	3	2	4
Basophilic tubular cells	1	1	2	–	1	–
Kidney (II) and ureter	10	10	10	10	10	10
Congestion	–	1	–	1	–	–
Mineralization	1	–	–	1	–	–
Hyaline tubular casts	1	1	–	1	1	–
Lympho-histiocytic infiltr.	5	5	9	5	4	6
Basophilic tubular cells	–	1	–	–	–	–
Kidney I (scarlet R)	10	10	10	10	10	10
Fatty infiltr., tubuli	4	3	4	5	5	3
Larynx	10	10	9	8	9	10
Subepi.lympho-histioc.infiltr.	–	–	–	–	1	–
Liver.	10	10	10	10	10	10
Lympho-histiocytic infiltr.	6	3	3	5	6	5
Mixed-cellular infiltration	1	1	3	1	–	–
Brownish pigment,macrophages	–	–	1	1	–	–
Glycogen incr./plant c.struct.	6	6	4	5	2	6
Necrosis	–	–	–	1	–	–
Centril./diff.hepat.hypertr.	10	10	10	9	9	10
Liver (scarlet R)	10	10	10	10	10	10
Fatty infiltr., single cells	–	4	1	–	–	–
Diffuse fatty infiltration	4	3	5	4	8	5
Peripheral fatty infiltration	4	2	3	–	–	–
Centrilobul. fatty infiltr.	2	1	1	6	2	5
Lungs (w.bronchi/bronchioles)	10	10	10	10	10	10
Foreign body bronchopneumonia	–	–	–	–	1	–
Haemorrhage	1	1	1	3	1	1
Lymph node (cervical)	8	8	5	8	7	7
Haemorrhage/erythrophagocytos	–	–	–	1	–	–
Sinus histiocytosis	8	6	5	8	7	7
Lymphoid hyperplasia	8	7	5	7	7	6
Plasmocytosis	1	–	–	–	–	–
Yellow–brown pigm.in macroph.	7	5	5	8	7	6
Lymph node (mesenteric	10	10	10	10	9	10
Haemorrhage/erythrophagocytos.	–	–	1	–	–	–
Sinus histiocytosis	10	10	10	10	9	9
Lymphoid hyperplasia	10	10	10	10	9	10
Granulocytic infiltration	–	–	–	–	–	1

Table 8 (*continued*)

Organ	Male			Female		
	Gr.1[a]	Gr.4[b]	Gr.5[c]	Gr.1[a]	Gr.4[b]	Gr.5[c]
Yellow–brown pigm.in macroph.	3	3	4	5	4	3
Ovary I (incl. oviduct)	–	–	–	10	10	10
Follicular cysts	–	–	–	–	–	1
Pancreas	10	10	10	10	10	10
Acinar atrophy and fibrosis	–	–	–	–	–	2
Pituitary	10	10	10	10	9	10
Cysts in adenohypophysis	–	–	–	1	–	–
Preputial gland	0	1	1	–	–	–
Abscess	–	1	1	–	–	–
Prostate	10	10	10	–	–	–
Lymphocytic infiltration	2	3	4	–	–	–
Salivary glands (3 sections)	10	10	10	10	10	10
Lympho-histiocytic infiltr.	3	3	1	4	4	4
Stomach.	10	10	10	10	10	10
Mixed cell.infiltr.,subepith	–	–	–	–	1	1
Ulcer glandular mucosa	1	–	–	–	–	–
Thymus.	10	10	10	10	10	10
Haemorrhage	1	–	–	–	–	–
Lymphoid hyperplasia	1	–	1	–	–	–
Cystic transformation	–	–	–	–	–	1
Thyroid (I)	10	9	8	10	10	10
Follicular cyst	1	–	–	–	–	–
Lymphocytic infiltration	–	–	–	–	1	–
Chronic inflammation	–	1	–	1	–	–
Macrofollicular structure	–	–	1	–	–	–
Thyroid (II)	10	7	7	7	10	9
Lymphocytic infiltration	–	–	–	–	1	–
Macrofollicular structure	–	–	1	1	–	–
Trachea	10	9	7	8	9	9
Subepi.lympho-histioc.infiltr.	1	2	1	–	1	2
Urinary bladder	10	10	10	9	10	10
Granulocytic infiltration	–	–	–	–	–	1
Proteinaceous content	1	1	2	–	–	–
Lympho-histiocytic infiltr.	–	–	1	3	1	1
Chronic cystitis	–	–	1	–	–	–

For each group:
- The number assigned to each organ indicates the number of organ analyzed.
- The numbers assigned to each finding indicates the number of the analyzed organs that presented the corresponding finding.
Further organs that were analyzed but did not show any finding: aorta abdominalis, bone marrow, mammary gland, nerve (sciatic), oesophagus, ovary (II), skin, testicles, uterus and vagina.

[a] Control.
[b] DSM 23964 (viable) 1×10^8 CFU/animal/day.
[c] DSM 23964 (past.) 1×10^{11} cells/animal/day.
* significantly different from Group 4 ($p \leqslant 0.05$).

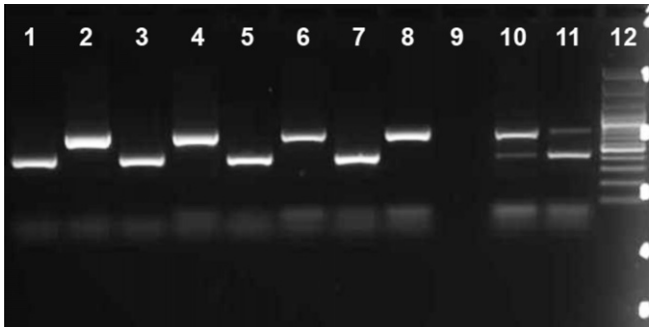

Fig. 2. Detection of potential contamination in injected solutions by multiplex species-specific PCR. Lanes: 1, positive control (*B. xylanisolvens* DSM 23964); 2, Solution 2 (1×10^9 *Bacteroides fragilis* RMA 6791/ml); 3, Solution 3 (1×10^9 *B. xylanisolvens* DSM 23964/ml); 4, Solution 4 (1.5×10^8 *Bacteroides fragilis* RMA 6791/ml); 5, Solution 5 (1.5×10^8 *B. xylanisolvens* DSM 23964/ml); 6, Solution 6 (5×10^6 *Bacteroides fragilis* RMA 6791/ml); 7, Solution 7 (5×10^6 *B. xylanisolvens* DSM 23964/ml), 8, positive control (*Bacteroides fragilis* RMA 6791); 9, Solution 1 (control group). Contamination controls: lane 10, mixture of 90% *Bacteroides fragilis* RMA 6791 and 10% *B. xylanisolvens* DSM 23964; lane 11, mixture of 10% *Bacteroides fragilis* RMA 6791 and 90% *B. xylanisolvens* DSM 23964. Lane 12, 1 kb DNA ladder (Fermentas).

about 30 g for all mice, these results revealed a NOAEL greater than 3.3×10^9 CFUs or 3.3×10^{12} pasteurized bacteria per kg body weight, corresponding to doses of 2.3×10^{11} CFUs or 2.3×10^{14} pasteurized bacteria, respectively, for an average 70 kg human being. The value obtained for viable strain DSM 23964 cells was within the range of typical values for *Lactobacillus* probiotic strains (Jia et al., 2011; Yakabe et al., 2009). The NOAEL value obtained for the pasteurized form of strain DSM 23964 would actually represent more than the total amount of bacteria estimated to inhabit the human colon.

Although we have previously demonstrated that the strain *B. xylanisolvens* DSM 23964 does not contain any of the virulence factors described for the genus *Bacteroides* (Ulsemer. et al. 2011.), its *in vivo* pathogenicity had not been previously examined. A well accepted model to study the pathogenicity of potential opportunist pathogens like *B. fragilis* is the "intraperitoneal abscess formation model" (McConville et al., 1981; Onderdonk et al., 1984; Thadepalli et al., 2001), in which viable microorganism(s) or substances thereof are intraperitoneally injected in mice together with sterilized rat feces extracts, to some extent mirroring a bacterial invasion under extremely unfavorable and unlikely conditions. Using freshly produced viable cells, it was demonstrated that, in contrast to a known clinical isolate of *B. fragilis*, the injection of up to 4.6×10^9 CFUs of *B. xylanisolvens* DSM 23964 per kilogram body weight did not have any pathological effect, and that *B. xylanisolvens* DSM 23964 was indeed quickly eradicated by the immune system.

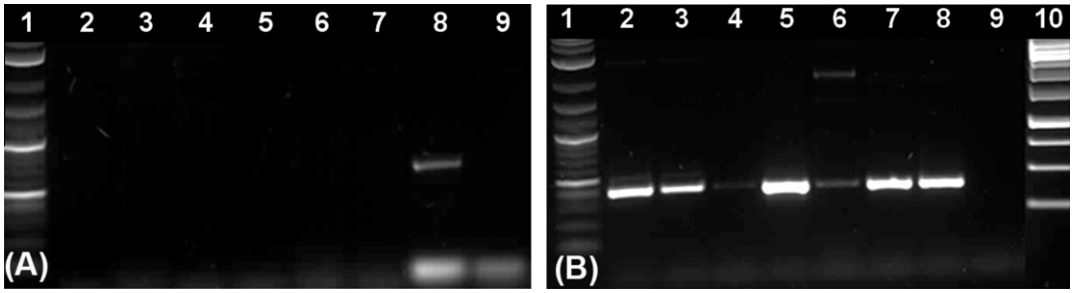

Fig. 3. Species-specific PCR of isolated DNA from punctured Abscesses. (A) Detection of *B. xylanisolvens* in abscesses of animals, which injected with *B. xylanisolvens* DSM 23964. Lanes: 1, 100 bp DNA ladder (Fermentas); 2 and 3, Group 3 (4.6×10^9 *B. xylanisolvens* DSM 23964/kg bw); 4 and 5, Group 5 (6.9×10^8 *B. xylanisolvens* DSM 23964/kg bw); 6 and 7, Group 7 (2.3×10^7 *B. xylanisolvens* DSM 23964/kg bw); 8, positive control (*B. xylanisolvens* DSM 23964); 9, negative control (water). B) Detection of *Bacteroides fragilis* in abscesses of animals, which injected with *Bacteroides fragilis* RMA 6791. Lanes: 1, 100 bp DNA ladder (Fermentas); 2 and 3, Group 2 (4.6×10^9 *Bacteroides fragilis* RMA 6791/kg bw); 4 and 5, Group 4 (6.9×10^8 *Bacteroides fragilis* RMA 6791/kg bw); 6 and 7, Group 6 (2.3×10^7 *Bacteroides fragilis* RMA 6791/kg bw), 8, positive control (*Bacteroides fragilis* RMA 6791); 9, negative control (Water); 10, 1 kb DNA ladder (Fermentas).

5. Conclusion

In summary, the strain *B. xylanisolvens* DSM 23964 was shown (i) not to possess any of the virulence factors previously described for the genus *Bacteroides* (Ulsemer et al., 2011 submitted), (ii) not to carry any mutagenic or clastogenic activities, either in its viable or in its pasteurized form, (iii) not to exert any toxicological effect, either in its viable or in its pasteurized form, and at concentrations up to 3.3×10^{12} pasteurized bacteria/kg body weight, (iv) not to present any pathogenic properties *in vivo*, and (v) to be sensitive to the defense system of the host.

The results of these studies support the safety of use of *B. xylanisolvens* DSM 23964 in both its viable and its pasteurized forms in food.

Conflict of interest statement

The studies were sponsored by the Glycotope GmbH. All authors except Jost Leuschner are employees of the Glycotope GmbH.

Acknowledgments

We thank Professor Arne Rodloff (Institute for Medical Microbiology, University of Leipzig, Germany), for kindly providing the strain *Bacteroides fragilis* RMA 6791, and for his support in the establishment of the intraperitoneal model. We are grateful to Heike Schmidt, Sigrid Wiese, Sabine Schulze and Anja Alfandega for technical assistance and Albert Bär for critical reading of the manuscript and helpful discussions.

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