



Teratogenicity and volatile composition evaluation of soymilk fermented with a *Deinococcus* member

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Abstract

The possibility of producing functional ingredients based on soymilk, fermented with a new identified strain *Deinococcus* sp. GKB-Aid 1995, was examined. Firstly, a prenatal developmental toxicity test in Sprague-Dawley rats was conducted to assess any potential human health risk. Next, owing to the unique odor that is significantly different from traditional fermented soymilk products, investigations on specific volatile compositions of GKB-Aid 1995 fermented soymilk are pivotal to the study. The results showed that there was no any detrimental effect on fetal and skeletal development under the highest dose of GKB-Aid 1995 fermented soymilk (3 g/kg body weight/day) during 20 days of the study. HS-SPME-GC-MS analysis indicated that a total of 35 identified volatile compounds were found in this new functional ingredient. In conclusion, smell of the GKB-Aid 1995 fermented soymilk, varied from the unpleasant odor of general soymilk products, has shown its possibility as a new source of functional ingredient.

Keywords: *Deinococcus* sp., fermented soymilk, fetal and skeletal development, volatile compounds

Introduction

Recently, the global functional food industry is striving to explore new ingredients and develop new applications to meet the demands of consumers. In 1995, a red pigment producing strain-*Deinococcus* sp. strain GKB-Aid 1995 was isolated from the soil in Taoyuan County of Taiwan and was found to be Gram-negative, aerobic, non-motile, and rod-shaped. Based on the bacterial identification by the 16S ribosomal RNA gene sequencing and the Vitek 2 colorimetric GN card, strain GKB-Aid 1995 was identified as a member of the *Deinococcus* species [1]. Several studies have demonstrated that the *Deinococcus* species had beneficial impacts in the field of environmental science and biotechnology [2,3]; however, no information is available on their uses as functional food ingredients. As several recent studies have demonstrated that carotenoid extracts from the *Deinococcus* species have potential anti-oxidant and anti-tumor activities [4,5], this study was conducted to investigate the potential application of strain GKB-Aid 1995 as a functional food additive or ingredient.

In a culture media comprised of soymilk and yeast extract,

the maximal growth rate of strain GKB-Aid 1995 from overnight was 10⁹ cfu/ml when fermented in a 20-ton bioreactor. The fermented product was shown with orange-red pigments in the nutrient broth and had a unique and strong aroma. Preliminary *in vitro* and *in vivo* studies indicated that the fermented product of strain GKB-Aid 1995 possessed potent antioxidant and anticarcinogenic effects when fermented with soymilk (unpublished data). Previous results showed that the fermented soymilk product with strain GKB-Aid 1995 was found without any genotoxic effect under the experimental conditions [1], and the no observed adverse effect level (NOAEL) of the product is greater than 10 mL/kg body weight in a 28 day animal study in Sprague-Dawley rats (unpublished data). Although genotoxicity and systemic toxicity have been investigated, the teratogenic effects of this new ingredient on the embryo at pregnancy have not been elucidated. Therefore, the first goal of the study was focused on examining whether the fermented soymilk caused teratogenic toxicity. In the meantime, the unique odor was proposed as being a typical characteristic of fermentating the strain. Thus, the second goal of this study was to investigate the

key odorants in the strain GKB-Aid 1995 fermented product. The results from this study may not only give further information about the product safety of fermented soymilk with strain GKB-Aid 1995 but also support its use as a potential functional food ingredient.

Materials and methods

Fermentation of soymilk with GKB-Aid 1995

One liter of broth composed of 2% sucrose, 1% peptone, 1% yeast extract, and soy milk was inoculated with an active culture of GKB-Aid 1995 and then incubated at 32°C for 24 h on a rotary shaker (120 rpm) for seed culturing prior to its scale-up production step. The scale-up of the fermentation process was performed using the same media agitated at 60 rpm with an aeration rate of 0.5 vvm at 32°C in the 20-ton fermenter for 24 h. At the end of the cultivation, the fermented broth was heated at 60°C, lyophilized and stored in a desiccator at room temperature. The freeze-dried powder was vigorously shaken with distilled water in order to get a final concentration of 100, 200 and 300 mg/mL for a constant dosing volume of 10 mL/kg bw /day at all dose levels to prevent the adverse effect of gavage.

Animal study

88 pregnant Sprague-Dawley rats at 8 weeks of age (BioLASCO Taiwan Co., Ltd., Ilan, Taiwan) were housed as one rat per autoclaved polyethylene cage in a controlled 12:12 h light and dark schedule with free access to water and a standard diet (MFG diet; Oriental Yeast Co., Ltd, Tokyo, Japan). This study was performed in accordance with Good Laboratory Practice (GLP) and approved by the Institutional Animal Care and Use Committee (IACUC:103-9k).

Prenatal developmental toxicity

This study was conducted under the guidance of Organization for Economic Co-operation and Development (OECD) 414 (Prenatal Developmental Toxicity Study) [6]. A total of 88 rats randomly divided across 4 test groups (three dosages test groups and one negative control group) were established in the present study. The negative control group received vehicle treatment (10 ml distilled water), while the low-, mid- and high- dose groups received GKB-Aid 1995 fermented soymilk in water at doses of 1,000, 2,000 and 3,000 mg/kg bw, respectively. These dose levels were selected according to the previous acute toxicity study (unpublished data) and were administered orally during the major embryonic organogenesis (Gestation days 6 to 15).

Clinical observation, body weight measurement and food intake were conducted daily during the study period. On the 20th day of pregnancy, the rats were euthanized with inhalation of CO₂, and their uterus and fetuses were delivered by caesarean sections. The number of viable fetuses, corpora lutea, implantation sites, stillbirths, resorption sites and the percentages of pre-implantation and post-implantation loss

were documented. All surviving fetuses were then individually weighed, sex distributed and examined for external abnormalities. After external examination, half of the offspring in each litter were fixed with 10 % neutral buffered formalin for organ examination while the remaining half were subjected to skeletal examination after 1 % KOH Alizarin staining.

Headspace solid-phase micro-extraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS) analyses of GKB-Aid 1995 fermented soymilk

For the analysis of volatile compounds in the GKB-Aid 1995 fermented soymilk, an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) that is interfaced with a mass spectrometer (5973A MSD, Agilent) equipped with a fused silica capillary column (60 mx0.25 mm, 0.25 mm film thickness, CP-Wax 52-CB, Chrompack, Middelburg, The Netherlands) was used. HS-SPME was used as the sampling method and was performed under optimal parameters used for highly efficient isolation of volatile compounds from fermented soymilk according to a previous study [7] with the following slight modifications. The prepared sample (50 g fermented liquid), after being blended with 20 mL of cold saline solution (10%, w/w), was added into a Schott bottle (100 mL, Duran, Mainz, Germany) and then placed in a water bath at 40°C and kept for 5 min for stabilization of the aliquot. Afterwards, adsorption of the volatile fraction of citrus peel using a DVB/CAR/PDMS fiber (2 cm length, 50/30 mm film thickness, Supelco, Bellefonte, PA) was performed. Extraction was carried out for 30 min at 40°C.

During headspace stabilization and extraction, the sample was agitated. After this step, the SPME fiber was removed from the bottle and placed into a GC injector heated at 220°C for 5 min for thermal desorption of the adsorbed compounds. Thermal desorption was carried out in the splitless mode of the injector. Separation of the desorbed compounds was achieved on the aforementioned capillary column. The column temperature program was as follows: started at 40°C, programmed to increase to 22°C at 5°C/min and held at 220°C for 20 min. The helium carrier flow rate was 1.0 mL/min. GC-MS transfer line temperature was 265°C. The MSD source and quadrupole temperatures were set to 230°C and 150°C, respectively. The analyte mass spectra were compared with those from the Wiley computer library. Some of the compounds were further identified with commercially available, authentic compounds. The amount of each compound was determined based on its peak area in the total ion chromatogram.

Statistical analyses

All data were expressed as mean±standard deviation (SD). Mean differences between the treatment and control group were analyzed using one-way ANOVA followed by Duncan's multiple range test with SPSS version 12.0 software (IBM, Armonk, NY). The gender ratio, external, organ and skeletal examination data were analyzed by utilizing the Chi-squared

test. A value of $p < 0.05$ was used as the statistical significance criterion.

Results and discussion

Previously, the genotoxic and systemic toxicity potential of GKB-Aid 1995 fermented soymilk have been investigated [1]. As a follow-up to the previous study, the present investigation was the first to evaluate the developmental toxicity potential of GKB-Aid 1995 fermented soymilk in animals exposed daily during major organogenesis. Treatment with GKB-Aid 1995 fermented soymilk during the gestation period showed no abnormal clinical signs, miscarriages or deaths in any of the pregnant rats. Average daily body weight was not significantly different among groups (Figure 1; $p > 0.05$, one-way ANOVA followed by Duncan's test). Similarly, no significant alterations in food intake were observed between control and treated groups (data not shown; $p > 0.05$, one-way ANOVA followed by Duncan's test).

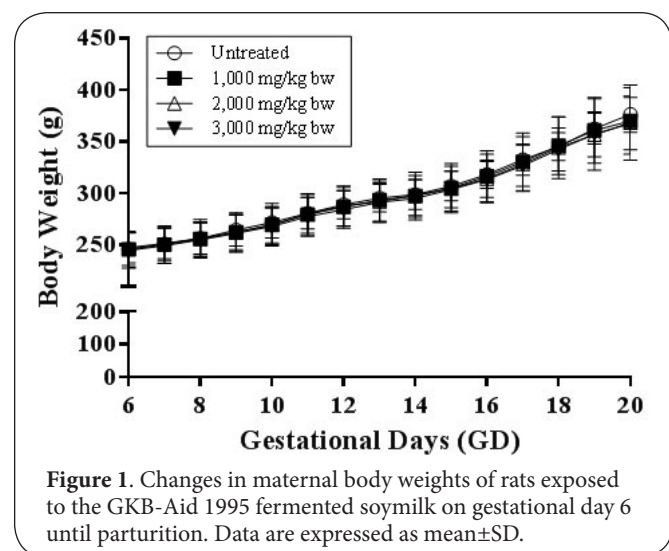


Figure 1. Changes in maternal body weights of rats exposed to the GKB-Aid 1995 fermented soymilk on gestational day 6 until parturition. Data are expressed as mean±SD.

After euthanizing, the uterus and the fetuses were drawn out from the pregnant rats for evaluation. Results showed no statistically significant changes in the litter size, mean uterus weight, fetal gender ratio, corpora lutea number, number of implantation sites, live or dead fetal number, fetal resorption number, pre-implantation loss and post-implantation death (Table 1). Although there was a significant increase in the mean body weight of medium and high dose groups compared to the control group ($p < 0.05$, one-way ANOVA followed by Duncan's test), this change was not considered to be treatment-related since all values were within the normal historical range [8].

Moreover, observations and examinations carried out under a dissecting stereomicroscope on 761 treated dams and 266 control group animals showed no external and visceral abnormalities. Regarding the development of the fetal skeleton, the most frequent skeletal alterations observed were unossified

Table 1. Effects of GKB-Aid 1995 fermented soymilk on fetal development.

	Negative control 0 mg/kg	Low dose 1000 mg/kg	Med dose 2000 mg/kg	High dose 3000 mg/kg
Number of fetuses	266	269	250	242
Mean uterus weight (g)	81.8±9.4	79.4±15.0	80.3±26.6	80.6±16.8
Mean weight of fetuses (g)	4.14±0.38	4.18±0.41	4.31±0.44*	4.38±0.41*
Fetus gender ratio ^a	0.94	0.95	0.97	0.89
Number of corpora lutea	15.0±1.9	15.6±2.3	15.9±2.4	15.3±3.0
Number of implantation sites	13.3±1.5	13.1±2.0	13.4±3.8	13.0±2.6
Number of live fetuses	266	269	250	242
Number of stillbirths	0	0	0	0
Number of resorption fetuses	14	20	17	18
Pre-implantation loss ^b	10.2±9.0	15.5±9.6	16.0±20.7	14.4±12.0
Post-implantation loss ^c	4.8±7.0	7.5±9.9	6.8±15.4	7.4±9.7

Data are expressed as mean±SD.

^aFetus gender ratio=Number of male rats÷number of female rats.

^bPre-implantation loss (%)=(number of corpora lutea-number of implantation sites)÷number of corpora lutea x 100 %

^cPost-implantation loss (%)=(number of implantation sites-number of live fetuses)÷number of implantation sites x100 %

*Significantly different from the negative control group ($p < 0.05$).

5th sternebrae and unossified 2nd or 5th metacarpus (Figure 2). The general occurrence of unossified 5th sternebrae was significantly lower in the mid- (9.8%) and high-dose (10.8%) groups compared to the control group (25.4%) ($p < 0.05$, χ^2 -test). In addition, the rate of unossified 2nd or 5th metacarpus was significantly less frequent in the high-dose group (12.6%) when compared to the control group (23.8 %) ($p < 0.05$, χ^2 -test). As there was no evidence of a dose-dependent increase in fetal malformation, which suggest that GKB-Aid 1995 fermented soymilk is a safe functional food ingredient with multi-bioactivities for women during pregnancy.

Typically, fermented foods have strong and distinct flavors due to the natural biosynthesis processing from microbial interactions. In this study, a total of 35 identified volatile compounds comprising terpenes (36.17%), pyrazines (24.82%), ketones (13.67%), alcohols (10.39%) and other volatiles (14.95%) were identified by GC-MS in GKB-Aid 1995 fermented soymilk (Figure 3). In terpenes, a significantly high proportion of limonene (33.17%) was determined in the fermented products. It was speculated that the characteristic of limonene being insoluble in water resulted in an easier adsorption onto SPME

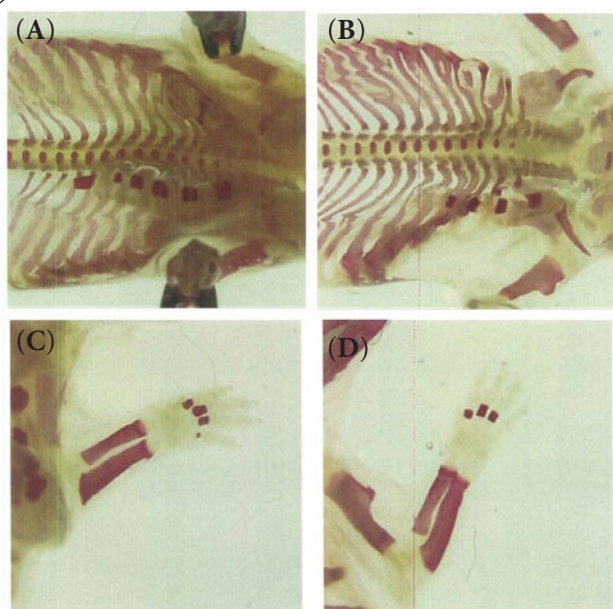


Figure 2. Representative pictures of fetal skeletal alternations from dams treated with water, or 1,000, 2,000 or 3,000 mg/kg GKB-Aid 1995 fermented soymilk on days 6 to 15 during pregnancy. (A) Normal sternbrae. (B) Unossified of 5th and 6th sternbrae. (C) Normal metacarpals. (D) Unossified 5th metacarpus.

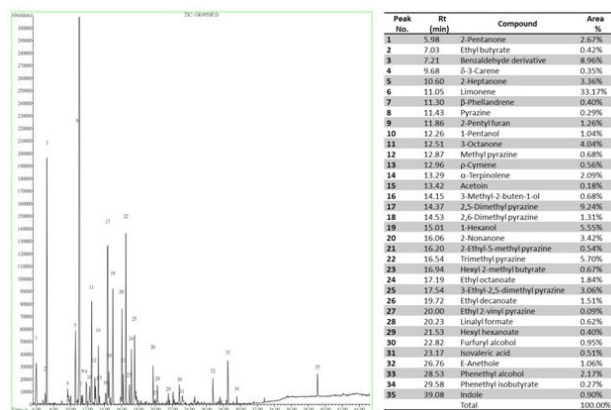


Figure 3. Total ion chromatogram of volatile components from GKB-Aid 1995 fermented soymilk by GC/MS analysis. Compounds are listed by peak number, corresponding to the table next to the figure.

fiber, especially with the addition of NaCl before GC/MS analysis sampling [9]. This limonene, produced from the hydrolyzed soybean, has contributed to the pleasant lemon-like odor in the fermented products.

The pyrazines, which are heterocyclic, nitrogen containing compounds with very low sensory detection thresholds (ppb to ppt levels), have drawn much attention to the product acceptance [10]. Surprisingly, a large proportion of pyrazines

(20.91%) was identified in the products, in which the particularly high amount of 2,5-dimethyl pyrazine (9.24%) was first to be reported in this study. The unique nutty and roasted odor characteristics of pyrazines resulted in a significantly different odor compared to traditional fermented soybean products, i.e., fermented stinky tofu, which contained sulfide compounds for more than 15 % of its contents but does not have any pyrazine compounds at all [11]. The biosynthetic formation of pyrazines may be attributed to the addition of acetoin, where this addition in fermenting medium resulted in the incorporation of the acetoin substance into 2,3,5-trimethylpyrazine [12]. Furthermore, the biosynthesis of acetoin might be formed from pyruvate via α-acetolactate [13]. Therefore, it may be plausible to enhance the pyrazine biosynthesis by the addition of precursors such as acetoin in order to make such products more attractive to consumers.

The third important components of the volatile compounds in the products are ketones, especially the methyl ketones, such as 2-pentanone, 2-heptanone, 3-octanone and 2-nonanone, totaling 13.49% of the contents in the identified compounds. The formation of ketones may have resulted from alkane utilization by microorganisms [14] or from oxidation of the carboxyl end of the carbon chain of fatty acids followed by decarboxylation [15]. The olfactometric profile of ketones was compared to that of a cheese-like odor [16].

Four alcohols were also identified in the headspace of the fermented products: 1-pentanol, 3-methyl-2-buten-1-ol, 1-hexanol, furfuryl alcohol and phenethyl alcohol. Most of them were detected in lower amounts in the sample, comprising 10.39% of the total contents. Phenethyl alcohol (2.17% of the contents) is one of the most important alcohol compounds for the flavor and fragrance industries due to its valuable rose-like odor [17]. It can be produced not only through bioconversion of L-phenylalanine but also by denovo synthesis via the shikimate pathway [17].

Lastly, the least in amount but the most in impact on odor reception were the isovaleric acid and indole components. Isovaleric acid (0.51%) was responsible for the acid-acrid odor and was recognized as a repulsive odor from most people. Similarly, indole (0.90%) can confer a disagreeable fecal odor at an extremely low threshold value of 0.3 ppb [18]. Indole can be produced in large quantities in both Gram-positive and Gram-negative bacteria, where indole has been identified as a signaling molecule involved in diverse functions for cell survival [19,20]. Even though a trace amount of isovaleric acid existed in the fermented products, its distinct unpleasant odors may affect the acceptance of the product. However, we firstly have found that a soymilk fermented with the newly identified strain presented no teratogenic toxicity as well as a unique odor resulting from the high content (20.91%) of pyrazines.

Conclusion

There were no detrimental reproductive and developmental

effects observed in rats attributable to GKB-Aid 1995 fermented soymilk administration. The no-observed-adverse-effect of GKB-Aid 1995 fermented soymilk is 3 g/kg bw/day, the highest dose level administered. Thirty-five metabolites were determined and reported for the first time in soymilk fermented with a *Deinococcus* member. Additional research on GKB-Aid 1995 fermented soymilk, including chronic toxicity and pre-clinical studies, may be included in future studies to further support the safety of its consumption.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	ICL	WPC	YCL	CCC1	CCC2
Research concept and design	✓	--	--	--	✓
Collection and/or assembly of data	--	✓	--	--	--
Data analysis and interpretation	✓	--	✓	--	--
Writing the article	✓	✓	--	✓	--
Critical revision of the article	--	--	--	✓	✓
Final approval of article	--	--	--	✓	✓
Statistical analysis	--	--	✓	--	--

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