



Evaluation of mutagenicity and genotoxicity of food additive maltitol (E 965 i)

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ABSTRACT

Sugar alcohols, including maltitol, are widely used food additives. Maltitol closely resembles sucrose both structurally and functionally. In 2023, the European Food Safety Authority called for genotoxicity data for maltitol. This study aimed to comprehensively evaluate the mutagenic and genotoxic potential of MALT using *in-silico* and *in-vitro* approaches. *In-silico* predictions were performed using ProTox, Toxtree, and VEGA platforms. Mutagenicity was assessed via bacterial reverse mutation assays (*S. typhimurium* (TA1537, TA1535, TA100, and TA98) and *E. coli* WP2 *uvrA*) up to 5000 µg/plate, both with and without metabolic activation. Genotoxicity was evaluated using an *in-vitro* micronucleus test on human peripheral blood lymphocytes, with cytotoxicity determined up to 2000 µg/mL, and the main study was conducted in two phases (4h ± S9 mix; 24h -S9 mix). *In-silico* models indicated that maltitol is generally safe, with some minor structural alerts that require experimental validation. The bacterial reverse mutation assays showed no significant increase in revertant colonies across all tested strains and conditions. Similarly, the *in-vitro* micronucleus test, performed at concentrations demonstrating lower cytotoxicity (<55 ± 5%), revealed no statistically significant induction of micronuclei. Maltitol was confirmed to be non-mutagenic and non-genotoxic under the experimental conditions tested.

1. Introduction

Sugar alcohols or polyols are carbohydrate derivatives with nutritive value that are used in various food industries because of their health benefits and distinctive properties. Compared to traditional sugars, they provide the same sweetness and lower caloric content, which is beneficial for individuals who are calorie-conscious or on weight management. They are found naturally in vegetables and fruits or are produced by hydrogenating sugars, which convert aldehyde or ketone groups to hydroxyl groups (Grembecka, 2015). Widely used sugar alcohols include xylitol, maltitol, sorbitol, erythritol, mannitol, lactitol, isomalt, and glycerol, each with specific applications in various industries (Rapaille et al., 2016). Recent developments in biotechnological methods have made the large-scale production of sugar polyols possible and sustainable. Maltitol synthesis is a prominent example (Saraiva et al., 2020).

Maltitol (4-O-alpha-D-Glucopyranosyl-D-glucitol) is a non-reducing

sugar alcohol called polyol and is a class of nutritive sugar alternatives. This bulk sweetener is produced from starch by hydrogenation of maltose. There are two forms of maltitol, crystalline (E-965i) and syrup (E-965ii). Among all polyols, maltitol has 75–90% of the sweetness of sucrose and similar qualities, like solubility, except its browning effect (Ding et al., 2019). Although, it has a sweet taste similar to that of sucrose, it is absorbed more slowly than sucrose, which reduces the insulin response. Maltitol has about 2.1–2.4 kcal/g of energy, a glycemic index of 35, which leads to a milder insulin response (insulinemic index of approximately 30) than sucrose which has an energy density of 4 kcal/g, a glycemic index of 65, and a significantly higher insulinemic index of approximately 80 (Kearsley and Deis, 2012; Livesey, 2012). Maltitol has various physicochemical properties, such as low hygroscopicity (which preserves crunchiness by limiting moisture absorption), high solubility, thermo-chemical stability (which reduces excessive browning), cooling effect, and sweet taste. Owing to these properties, maltitol is effectively

Abbreviations: EFSA, “European Food Safety Authority”; OECD, “The Organization for Economic Cooperation and Development”; BRMT, “Bacterial Reverse Mutation Test”; IVMNT, “*In-vitro* Micronucleus Test”; MNBN, “Micronucleated Binucleated cells”; PBL, “Peripheral Blood Lymphocytes”; SCE, “Sister Chromatid Exchange”; CA, “Chromosomal Aberrations”.

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utilized as a humectant, sweetener, stabilizer, emulsifier, bulking agent, and thickener in food and beverage formulations (Grembecka, 2015; Zumbe et al., 2001). This allows its widespread use in dietary applications, including baked goods, chewing gum, dairy products, sugar confectioneries, chocolates, and snack bars. Additionally, it can be utilized as a tabletop sweetener based on weight-for-weight, as 1g maltitol is equivalent to 1g sucrose in sweetness (Lee et al., 2002; Ruskonefourmestraux et al., 2003; Thabuis et al., 2010). Low-calorie polyalcohols, including maltitol, are also used in pharmaceuticals and oral care products such as toothpaste (Imfeld, 1994; Ito et al., 2015). Previous studies have shown that maltitol promotes the proliferation of *Lactobacilli* and *Bifidobacteria*, which are crucial markers of prebiotic action in the gut (Ruizojeda et al., 2019).

The JECFA (Joint FAO/WHO Expert Committee on Food Additives) does not specify a recommended acceptable daily intake (ADI) of maltitol since the FDA (Food and Drug Administration) has given polyols "Generally Recognized as Safe" (GRAS) status (JECFA, 1999). Children and adults can consume up to approximately 15 g and 40 g per 24 h, with no notable symptoms (Koutsou et al., 1996; Ruskonefourmestraux et al., 2003; Thabuis et al., 2010). However, excessive consumption of most polyols including maltitol, may induce laxative and gastrointestinal symptoms such as bloating, abdominal ache, and diarrhea. This is primarily due to incomplete absorption and subsequent fermentation in the gut (Storey et al., 1998). In addition to the gastrointestinal effects, some individuals may experience a laxative effect owing to the osmotic properties of maltitol, which can draw water into the intestines (Mäkinen, 2016). This can lead to loose stools or diarrhea, especially in those with sensitive digestive systems or those who are not accustomed to sugar alcohols (Krüger et al., 1992; Oku et al., 1991; Petkovic, 2019). Currently, the European Food Safety Authority (EFSA) reviews the toxicological data on sweeteners added to foods. In 2023, EFSA called for data on the genotoxicity of maltitol (EFSA, 2023).

In-silico toxicity prediction acts as an initial screening phase within the 3R (Reduction, Refinement, and Replacement of animal use) and NAM (New approach/non-animal methods) frameworks, offering quick insights into possible toxicological profiles while reducing the need for animal testing. Tools like ProTox, Toxtree, and VEGA are utilized to predict structural alerts and mechanistic toxicity endpoints, supporting an effective and ethical risk assessment process (de Wit et al., 2024; Tornqvist et al., 2014; Wood et al., 2025).

A previous study reported that maltitol is non-mutagenic to *S. typhimurium* and *E. coli*. It has also been suggested that maltitol is not responsible for the increase micronuclei (MN) in mouse bone marrow cells (Takizawa and Hachiya, 1984). However, different studies found that maltitol caused an increase in MN frequency, but not in a dose-dependent manner, and a rise in chromosome aberrations (CA) in a statistically not significant manner, but did not induce sister chromatid exchange (SCE) in peripheral blood lymphocytes (PBLs). Additionally, they reported that it was not teratogenic and did not cause CA in rats (bone marrow cells) (Canimoglu and Rencuzogullari, 2006, 2013). This study assessed the *in-silico* toxicity of maltitol using ProTox, Toxtree, and VEGA software, as well as evaluated mutagenicity and genotoxicity through the Bacterial Reverse Mutation Test (BRMT) and *In-vitro* Micronucleus test (IVMNT) on human peripheral blood lymphocytes (Messinger et al., 2020).

2. Material and method

2.1. *In-silico* toxicity prediction

The molecular structure and SMILES of maltitol were retrieved from the PubChem database (Fig. 1, PubChem CID-493591 (<https://pubchem.ncbi.nlm.nih.gov/>)). The toxicity of maltitol was predicted using three *in-silico* platforms. ProTox 3.0, which is comprehensive online platform for predicting the toxicological properties of small molecules (<https://tox.charite.de/protox3/>). ProTox applies fragment propensities, molecular

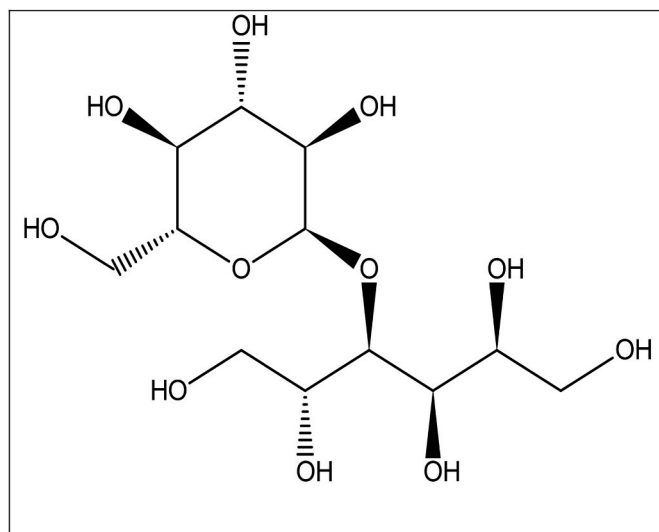


Fig. 1. Molecular structure of maltitol.

similarities, frequent features and machine learning (fragment similarity-based CLUSTER cross-validation) across 61 models to predict acute toxicity, organ toxicity, toxicological end points, molecular initiation events, metabolism, adverse outcome pathways (Tox 21), and toxicity targets (Banerjee et al., 2024). Toxtree v2.6.13 analyzes refined toxicity estimations using structure-activity-relationships/SAR (<http://toxtree.sourceforge.net>). Toxtrees can identify structural alerts (SA) for mutagenicity, carcinogenicity, skin irritation/sensitization, eye irritation, biodegradation and other characteristics such as protein or DNA binding alerts (Benigni et al., 2008; Contrera, 2013). VEGA is an open-source QSAR platform used to predict toxicological and physicochemical properties (<https://www.vegahub.eu/portfolio-item/vega-qsar/>). It assesses the prediction reliability through chemical similarity and facilitates read-cross assessment via structural analog identification (Danieli et al., 2023; Pradeep et al., 2021).

2.2. Bacterial reverse mutation assay

In this study, four histidine-deficient *S. typhimurium* strains (TA1535, TA1537, TA100, TA98) and one tryptophan-deficient *E. coli* strain (WP2 *uvrA* (pKM101)) were used to assess the mutagenic potential of maltitol (\pm S9 mix). The strains were acquired from the U.S.A. (Molecular Toxicology Inc.), and The Organization for Economic Cooperation and Development (OECD) guidelines were strictly followed for this study (OECD, 2020).

2.2.1. Solubility of test item

This study used maltitol (off-white powder) with a purity level of 99.8% manufactured by Sigma-Aldrich (CAS number: 585-88-6, batch number: 0000227217). Maltitol (50,000 μ g/mL) was completely dissolved in distilled water (DW) and did not precipitate when 100 μ L of this aliquot was mixed with top agar. The mixture was then poured onto a minimal glucose agar plate to check for precipitation. No precipitation was observed at the tested concentration *i.e.* 5000 μ g/plate. Therefore, it was selected as the highest concentration for the experiment. Distilled water (DW) used as the treatment vehicle.

2.2.2. Cell viability and genotype confirmation test

To obtain bacterial cultures with approximately $1 - 2 \times 10^9$ bacterial cells/mL (*E. coli* WP2 *uvrA* (pKM101)) and $1 - 3 \times 10^9$ bacterial cells/mL (*S. typhimurium*), the cryopreserved cultures were inoculated into a flask containing 10 mL sterile NB (nutrient broth N° 2) and incubated for 15 h at 37 ± 1 °C in thermostatic shaker (120 rpm). After incubation, the

optical density of all the strains was measured at 660 nm. The acceptable OD range was 0.3–0.5 for *E. coli* and 0.4–0.6 for *S. typhimurium*. Typically, samples exceeding these thresholds are diluted, whereas those below the required range are supplemented with additional culture. In this experiment, all OD values were within the specified range, as presented in the [supplementary data Table 1 and Table 2](#). *S. typhimurium* strains were evaluated for *uvrB* mutation by analyzing their sensitivity to ultraviolet light, R-factor resistance to ampicillin and tetracycline, dependence on histidine and biotin, and dependence on histidine. *E. coli* WP2 *uvrA* (pKM101) has been tested for tryptophan dependence, sensitivity to ampicillin (plasmid pKM101), and sensitivity to UV radiation (Ames et al., 1973; Green and Muriel, 1976; Maron and Ames, 1983; McCann et al., 1975).

2.2.3. Initial toxicity-mutation test

Maltitol was tested for initial toxicity-mutation test using all four tester strains at eight different concentrations (5000, 1500, 500, 150, 50, 15, 5, 1.5 µg maltitol/plate) ($\pm 5\%$ S9 mix).

For treatment, molten top agar (2 mL) which contains 0.5 mM histidine/biotin (for *S. typhimurium*), and plain top agar (2 mL) in tubes (for *E. coli*) were placed in a water bath at 45 ± 2 °C. For *E. coli*, 100 µL of tryptophan solution (100 µg/mL) was added to plain top agar tubes. The respective culture tubes were supplied with 500 µL of 0.2 M-phosphate buffer (-S9 mix) or 5%- S9 mix (+S9 mix).

Subsequently different concentrations of stock solutions of maltitol, positive controls (PC), and negative control (NC) were added (100 µL). The relevant bacterial cultures were then added to the tubes (100 µL), mixed, and poured on minimal agar plates. Duplicate sets were maintained for all concentrations as well as positive and negative controls. After incubation for 48 h at 37 ± 1 °C, the plates were examined to assess cytotoxicity by observing the state of the background bacterial lawn under a light microscope. The number of colonies was counted using a ProtoCol 3 automated colony counter (Version 1.3.14.0) (Kier et al., 1986). Following instrument-specific protocols, the plates were systematically loaded into the device, and colony counts were rapidly acquired, automatically saved, and exported for subsequent quantitative analyses.

2.2.4. Confirmatory mutation test

The plate incorporation method used for this test. The strains were treated with maltitol at 5000, 2500, 1250, 625, 312.5, and 156.25 µg/plate ($\pm 10\%$ S9 mix) in triplicates. After incubation for 48 h at 37 ± 1 °C, the number of revertant colonies was recorded using an automated colony counter. To validate the effectiveness of the S9 fraction, 2-amino anthracene was administered to TA100 without S9 mix.

2.3. In-vitro micronucleus test on human peripheral blood lymphocytes (IVMNT)

IVMNT was performed to evaluate the clastogenic and aneugenic potential of maltitol by assessing the occurrence of micronuclei in binucleated (BN) cells. This test is also renowned for its reproducibility and reliability in evaluating mutagenic potential. It is endorsed by the OECD and various regulatory bodies because of its nature (OECD, 2023; Parry and Sors, 1993).

2.3.1. Solubility: precipitation, osmolality, and pH tests

Maltitol was fully soluble in distilled water at 200,000 µg/mL, and was selected as the vehicle for the study. The pH, precipitation, and osmolality were assessed at 0 and 4 h after incubation. No precipitation occurred up to 2000 µg/mL, and the pH (± 1 unit) and osmolality (≥ 50 mOsm/kg H₂O) remained stable at concentrations of 62.5 to 2000 µg/mL. Thus, 2000 µg/mL was chosen as the highest concentration for the cytotoxicity test.

2.3.2. Culture preparation

For this experiment, two healthy male volunteers, who were non-alcoholic, non-smokers, and free from radiation, drugs, and chemical exposure were selected. A trained medical laboratory technician collected blood samples from the selected volunteers (a 28-year-old for the cytotoxicity test and a 30-year-old for the main study). The volume of 0.5 mL whole blood was cultured in 9.5 mL of complete medium containing centrifuge tube [RPMI-1640 with 2% PHA-M (Phyto hemagglutinin) and 20% FBS (Fetal Bovine Serum)] incubated for 48 h in a CO₂ incubator (5% CO₂) maintained at a temperature 37 ± 1 °C (Clare et al., 2006). The same procedure was used to prepare cultures for all phases of the experiment (Kirsch-Volders et al., 2011).

2.3.3. Cytotoxicity test

Blood was cultured in centrifuge tubes as described in section 2.3.2 and incubated for 48 h. Subsequently, all culture tubes were centrifuged at 1500 rpm ($\sim 450 \times g$) for 15 min. After removing the supernatant, each tube was supplemented with 8 mL of treatment media containing serum-free RPMI combined with maltitol (2000, 1000, 500, 250, 125, and 62.5 µg of maltitol/mL). For metabolic activation, 1% S9 mix was added, whereas parallel cultures received KCl as control in the absence of metabolic activation. Following a 4 h incubation, cultures were centrifuged, supernatants were discarded, and washed with 8 mL of serum-free RPMI to remove residual treatment media. Subsequently, 8 mL of RPMI containing 10% FBS and cytochalasin B (cyto-B, 6 µg/mL) was added, and cultures were incubated for further 20 h, completing 24 h incubation. The cells were harvested and processed for cytotoxicity (replicative index) evaluation (Galloway et al., 2011; Honma, 2011).

2.3.4. Main study

Based on cytotoxicity findings, maltitol was evaluated for induction of MN at concentrations of 2000, 1000, 500, 250, and 125 µg of maltitol/mL ($\pm 2\%$ v/v S9 mix). The blood cultures were prepared as per protocol mention in section 2.3.2. In the main study, the treatment conditions were divided into Phase I (4 h exposure, \pm S9 mix) and Phase II (24 h exposure, - S9 mix). In phase I, cultures were exposed to the designated concentrations of maltitol for 4 h in 8 mL of serum-free treatment medium containing either S9 mix or potassium chloride (KCl) to assess the influence of metabolic activation. Following the exposure period, the cultures were centrifuged at 1500 rpm ($\sim 450 \times g$) for 8 min, and the supernatant was removed. Subsequently, 8 mL of complete RPMI medium with 10% FBS and cytochalasin B (cyto-B, 6 µg/mL) was added and incubated for 24 h. In Phase II, cultures were treated with maltitol in a medium containing 10% FBS and cyto-B, followed by 24 h incubation. Subsequently, the cells were harvested, and the slides were evaluated for replicative index (RI) and micronucleus (MN) frequency.

For each phase of the experiment, all concentrations, positive controls, and negative controls were maintained in duplicate. In Phase I (+S9 mix), cyclophosphamide (30 µg/mL) was used as the positive control. Vinblastine (0.008 µg/mL) was used as the PC in Phase II (-S9 mix) (Lorge et al., 2006).

2.3.5. Harvesting of cells and slide preparation

In this step, cells were exposed to a chilled hypotonic (0.075 M) KCl solution and immediately centrifuged (1500 rpm/ $\sim 450 \times g$) for 8 min. After discarding the supernatant, the resulting cell pellet was resuspended in 8 mL of chilled fixative (methanol: glacial acetic acid; 3:1), and centrifuged again. Cells were subjected to three washes of chilled fixative. Approximately 0.5 mL of cells from each culture tube were then used to prepare slides. These slides were dried on a warming table, labeled, treated with a 5% Giemsa staining solution for 30 min, and mounted with dibutyl phthalate polystyrene xylene (DPX).

2.3.6. Scoring of slides

The relative cell cycle number per cell during cyto-B exposure in

treated cultures compared with that in negative controls was measured using the replicative index (RI), which assesses cell proliferation and cytotoxicity. Using a light microscope, a minimum of 500 cells per replicate (1000 cells per concentration) were counted. To calculate RI, the number of mono, bi, and multinucleated cells was recorded and added to the following equation (Kirsch-Volders et al., 2004; Lorge et al., 2008).

$$RI = \frac{\frac{[\text{Number of binucleate cells}]_T + 2 \times [\text{Number of multinucleate cells}]_T}{[\text{Total number of cells}]_T}}{\frac{[\text{Number of binucleate cells}]_C + 2 \times [\text{Number of multinucleate cells}]_C}{[\text{Total number of cells}]_C}} \times 100$$

Where, T = test chemical treated cultures, C = cultures treated with vehicle

$$\% \text{ Cytotoxicity} = 100 - \text{RI}$$

2.3.7. Scoring of micronucleus frequency and statistical analysis

The incidence of micronuclei was evaluated by screening 1000 BN per replicate (2000/concentration) and the number of micronucleated binucleated cells (MNBN). The proportions of MNBN at each test concentration was assessed against the proportion of negative controls. The identification of mono-, bi-, and MNBN is shown in Fig. 4. Statistical analysis of MNBN was conducted using the Fisher's exact test. A Chi-square trend analysis was also employed to assess dose-response relationships within the treatment group using GraphPad Prism (version No: 0.0.2).

3. Result

3.1. In-silico toxicity prediction

Maltitol displayed no significant organ or metabolic toxicity in the ProTox-3.0 assessment, and endpoints such as hepatotoxicity, neurotoxicity, respiratory toxicity and CYP interaction were classified as inactive. Moderate probabilities were predicted for nephrotoxicity (0.78) and cardiotoxicity (0.99), with borderline prediction for blood-brain barrier penetration. However, because of its large molecular size and low gastrointestinal absorption, its systemic exposure is minimal and experimental data do not support these predicted risks (Delre et al., 2022). Furthermore, maltitol is non-mutagenic, non-cytotoxic, non-carcinogenic and non-immunotoxic, as evidenced by the predicted probabilities of 0.90, 0.86, 0.89, and 0.99, respectively.

The ToxTree analysis classified maltitol as class I (low toxicity) using Cramer rules. All structural alerts for eye irritation, skin irritation, micronucleus assay, sensitization, DNA binding and protein binding yielded negative results. Ames mutagenicity analysis using ISS alerts showed no structural triggers for *S. typhimurium* mutagenicity; all 69 evaluated structural domains returned negative results. Likewise, assessment of carcinogenicity and mutagenicity via the Benigni/Bossa rules indicated negative findings for both genotoxic and nongenotoxic mechanisms, with no structural alerts identified. The application of the ILSI/Kroes TTC decision tree classified the substance as presenting negligible safety concern, with no genotoxic and carcinogenic alerts.

All six Ames mutagenicity models (CONSENSUS, CAESAR, ISS, SarPy-IRFMN, KNN-Read-Across, and aromatic amines) consistently predicted non-mutagenic classification with experimental validation and moderate-to-high reliability scores. Chromosomal aberration (CORAL v1.01.) and micronucleus assays (*in vitro* VERMEER, *in vivo* IRFMN) model predicted active status with low reliability due to applicability domain limitations such as moderate structural similarity, conflicting analog data and rare structural fragments. Therefore, experimental validation of genotoxicity is recommended. Multiple carcinogenicity models (CAESAR, ISS, IRFMN-ISSCAN-CGX, IRFMN-Antares, and oral/inhalation classification model) uniformly predicted non-carcinogenic or possible non-carcinogenic status with moderate reliability. Gender-specific rat carcinogenicity models have shown

mixed predictions with low reliability. Developmental and reproductive toxicity models predicted the non-toxicant status. Skin sensitization predictions were divergent (CAESAR/IRFMN-JRC: sensitizer; NCSTOX: non-sensitizer). The skin and ocular irritation models uniformly predicted no irritation. Endocrine disruption screening was inactive across all receptors.

3.2. Bacterial reverse mutation assay

3.2.1. Initial toxicity-mutation test

Up to a 5000 µg maltitol/plate concentration, all tester strains (5% S9 mix) exhibited a typical bacterial background lawn and showed no positive mutagenic effects. Linear regression indicated no significant impact on the tester strains, except for TA100 (-S9 mix). The average number of revertant colonies across selected concentration levels ranged from 122.00 to 137.00, compared to 128.50 in the negative control group. Nevertheless, the number of revertant colonies observed was within the range of the historical control data (Supplementary Data Table 3). Linear regression is a very sensitive tool, so even a small increase towards the highest concentration will be flagged. In this case, a very small number of colonies increased, which was less than or even ≥1-fold. This finding is not biologically significant (Claxton et al., 1987; Otobe et al., 2019). Fig. 2 explained a graphical representation of the mean revertant colonies/plate compared with the negative control.

3.2.2. Confirmatory mutation test

The experiment showed that maltitol did not cause a discernible increase in revertant colonies up to 5000 µg maltitol/plate for any tester strain (±S9 mix). The values observed for the negative control were consistent with the established historical control ranges. The mean revertant colonies/plates of all the bacterial strains at the tested concentrations are summarized in Fig. 3. The efficiency of the test system was demonstrated by positive controls, which displayed an increase in the revertant colony count (Supplementary data Table 4) (Kirkland, 1994).

3.3. In-vitro micronucleus test result

3.3.1. Cytotoxicity test

No notable alterations in pH or osmolality were detected across the range of maltitol concentrations tested. The observed cytotoxicity was -3.34, -2.82, -2.91, -3.42, -1.67, and -6.02 % in the absence of S9 mix. Whereas, In the presence of S9 mix at 2000 to 62.5 µg/mL observed cytotoxicity was 1.13, -3.56, -4.79, -5.91, -2.62, and -5.40 % (supplementary data Tables 5 and 6). The cytotoxicity test results indicated that 2000 µg maltitol/mL could be used as the highest concentration in the main study.

3.3.2. Main study

Maltitol did not noticeably affect the pH or osmolality at all concentrations. In Phase I, cytotoxicity observed was 6.58 % and 7.04 % at 2000 µg/mL without (-) and with (+) S9 mix, respectively. In Phase II, cytotoxicity observed was 10.08 % at the tested concentration of 2000 µg/mL. No cytotoxic effects (defined as 55 ± 5% inhibition of replicative index) were detected at concentrations up to 2000 µg/mL during Phase I and II. Therefore, the concentrations selected for MNBN scoring were: 2000, 1000, and 500 µg/mL. The cytotoxicity and replicative index summary are provided in the supplementary data in Tables 7 and 8. The micronucleus frequencies of the selected concentrations of Phases I and II are summarized in Table 1.

In Phase I (+S9 mix) and Phase II (-S9 mix) both Positive controls, cyclophosphamide (a clastogen), and vinblastine (an aneugen) produced a significantly high MNBN count respectively (Fenech and Morley, 1985).

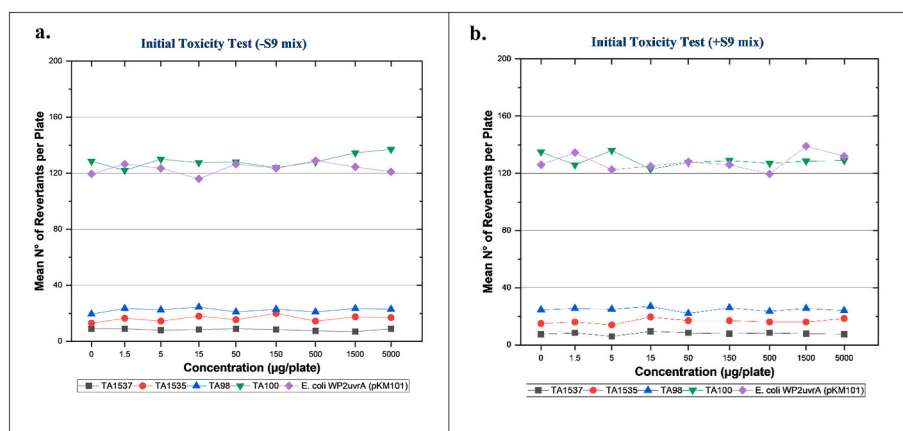


Fig. 2. Initial Toxicity-Mutation Test: a. Dose Response Curves for maltitol in absence of S9 mix, b. Dose-Response Curves for maltitol in presence of S9 mix.

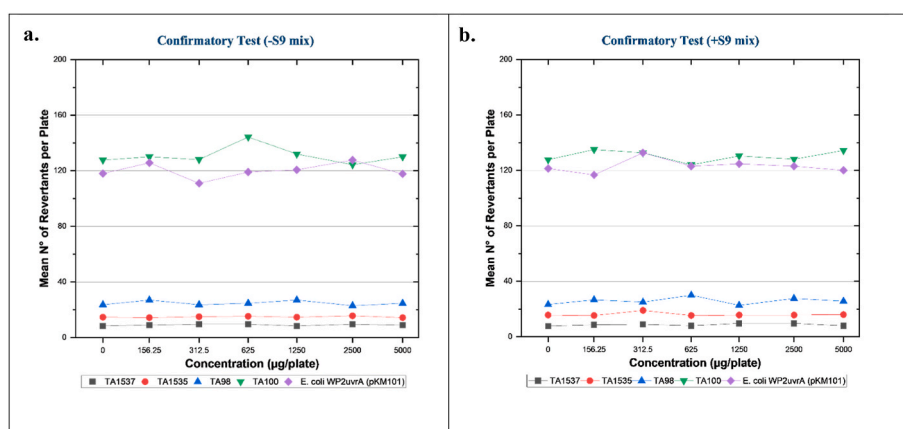


Fig. 3. Confirmatory Test: a. Dose-Response Curves for maltitol in absence of S9 mix, b. Dose Response Curves for maltitol in presence of S9 mix.

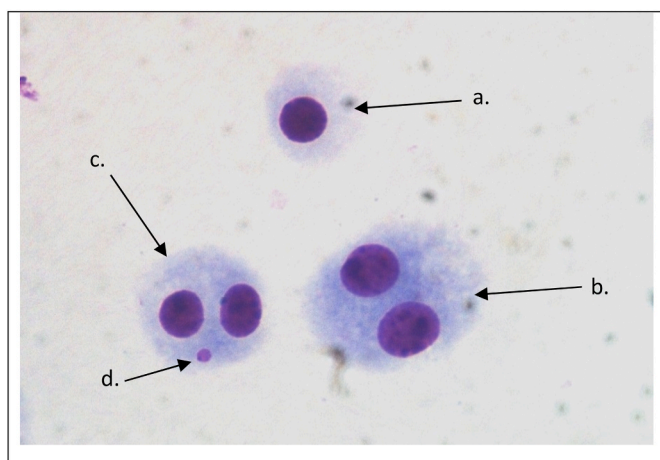


Fig. 4. Blood lymphocytes a. Mono nucleated cell, b. Binucleated cell (BN), c. Micronucleated binucleated cell (MNBN), d. Micronucleus (MN).

4. Discussion

In-silico toxicology assessment performed using ProTox, Toxtree, and VEGA indicated that maltitol is safe overall, with several model-derived flags requiring critical interpretation. However, ProTox-flagged cardiotoxicity (0.99), nephrotoxicity (0.78) and BBB permeability (0.75) probabilities represent false positive from applicability domain

violations rather than genuine hazards. The hERG-based cardiotoxicity models optimized for lipophilic pharmaceuticals, exhibit reduced specificity for hydrophilic polyols. However, nephrotoxicity and BBB permeability predictions fail to account for the minimal systemic bioavailability of maltitol (Banerjee et al., 2024). The VEGA platform predicted “active” genotoxicity for chromosomal aberration and micronucleus assays with low reliability scores, reflecting its limited applicability to food-grade polyols. VEGA genotoxicity models prioritize sensitivity over specificity, resulting false positive rates when evaluating compounds outside its pharmaceutical-focused training database (Danieli et al., 2023). From a mechanistic perspective, maltitol is a saturated sugar alcohol without electrophilic groups or structural features typically associated with DNA reactivity (Ashby and Tennant, 1991). It is only partially absorbed in the small intestine, and a substantial portion reaches the colon where it is fermented by intestinal microbiota. The absorbed fraction is converted to glucose and sorbitol and follows normal carbohydrate metabolism without formation of reactive intermediates (Krüger et al., 1992; Secchi et al., 1986). *In-silico* toxicity predictions for food additives are useful for initial screening but limited by inadequate training datasets, often yielding false positives that require experimental verification (Cronin et al., 2022; Frenzel et al., 2017).

The results of the BRMT indicated that maltitol did not elicit a mutagenic response in any of the *S. typhimurium* or *E. coli* WP2 *uvrA* (pKM101) strains tested, with up to 5000 µg/plate (\pm S9 mix). In strain TA100 (-S9 mix), the number of colonies increased, which is in the historical range of the negative control. A small increase in the number of colonies did not meet the evaluation criteria, which required a \geq 2-

Table 1
Summary of micronucleus analysis.

| Concentration of Maltitol | Phase I [Absence of Metabolic Activation] | | Phase I [Presence of Metabolic Activation (2% S9 mix)] | | Phase II [Absence of Metabolic Activation] | |
|---------------------------|---|-------------|--|----------------|--|-------------|
| | Mean ± SD | | Mean ± SD | | Mean ± SD | |
| | MNBN | % MNBN | MNBN | %MNBN | MNBN | %MNBN |
| NC (Distilled Water) | 5.00 ± 0.00 | 0.49 ± 0.00 | 5.00 ± 1.41 | 0.49 ± 0.14 | 4.50 ± 0.70 | 0.44 ± 0.06 |
| T3 (500 µg/mL) | 4.50 ± 0.70 | 0.44 ± 0.07 | 5.00 ± 0.00 | 0.49 ± 0.00 | 5.00 ± 1.41 | 0.49 ± 0.13 |
| T4 (1000 µg/mL) | 5.00 ± 1.41 | 0.49 ± 0.14 | 4.50 ± 2.12 | 0.45 ± 0.21 | 4.50 ± 2.12 | 0.44 ± 0.21 |
| T5 (2000 µg/mL) | 4.50 ± 0.70 | 0.44 ± 0.07 | 4.50 ± 0.707 | 0.44 ± 0.07 | 4.50 ± 0.70 | 0.44 ± 0.07 |
| PC | - | - | 34.00 ± 2.82 †† | 3.37 ± 0.26 †† | 34.50 ± 0.70 | 3.42 ± 0.05 |

Key: NC = Negative control, SD = Standard deviation, PC = Positive control [In Phase I (+S9 mix): Cyclophosphamide at 30 µg/mL; In Phase II (-S9 mix): Vinblastine at 0.008 µg/mL], MNBN = Micronucleated binucleate cells, - = Not Applicable.

†† = significantly higher than the negative control at 1% level ($p \leq 0.01$).

fold increase in revertant colonies in TA100 to be positive. A previous study reported that no detectable revertants were induced in *S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538, or *E. coli* WP2 *uvrA* (pKM101) at 0.5 to 50 mg maltitol/plate (\pm S9 mix). These findings align with our results and support the conclusion that maltitol does not exhibit mutagenic activity under the conditions employed (Takizawa and Hachiya, 1984).

According to the findings displayed in Table 1, the investigation concluded that maltitol did not raise micronuclei within human peripheral blood lymphocytes (PBL) at concentrations up to 2000 µg/mL (\pm 2% S9 mix). Based on the experimental conditions employed in this study, maltitol did not exhibit significant genotoxic effects on micronucleus formation. In contrast, a previous study reported an increased frequency of MN in a non-conc.-dependent manner up to 5 mg maltitol/mL, observed at 24 and 48 h in human PBL. However, this did not obey the evaluation criteria that require a dose-dependent increase for a positive result. Additionally, this study reported that maltitol did not induce sister chromatid exchange, but caused chromosomal aberrations in a statistically non-significant manner (Canimoglu and Rencuzogullari, 2006).

Another *in-vivo* experiment was performed by gastric intubation of 'Multi-Towa' (powder) and maltitol crystal (3.75-30 g/kg) in mice, and showed no increase in the frequency of micronucleated normo chromatic erythrocytes (MNNCE) in the bone marrow. Likewise, there was no statistically significant increase in CA or evidence of bone marrow toxicity in rats exposed to maltitol (2.5, 5, and 10 g/kg bw for 6, 12, and 24 h) intraperitoneally. Teratogenicity and embryotoxicity were also examined in this study, in which pregnant female rats were treated with maltitol intraperitoneally (1, 2, and 4 g/kg bw/day) throughout the first 7 days of gestation and embryos were collected at 19 days. They observed that maltitol was not teratogenic but at 4 g/kg bw (the highest dose) it decreased fetuses weight and induced growth retardation (Canimoglu and Rencuzogullari, 2013).

In addition to genotoxicity studies, the FAO/WHO documented that combined long-term carcinogenicity study identified the development of benign and malignant pheochromocytomas, along with an increased incidence of slight to moderate medullary hyperplasia, in female and male mice following maltitol administration at doses of 0–4.5 g/kg/day. Another study reported that maltitol administration induced hyperplastic alterations in the colonic and cecal mucosa of rats (Oku and Kwon, 1998). In contrast, one study reported that dietary maltitol (10 g/kg body weight) exerts a protective effect against dimethylhydrazine-induced tumorigenesis in the rat cecum and proximal colon (Tsukamura et al., 1998). There is limited literature available on the genotoxicity and mutagenicity of maltitol that adheres to OECD guidelines. Based on the available data, these findings suggest that maltitol is non-mutagenic and non-genotoxic at lower doses. Although maltitol is classified as GRAS, its consumption should be moderated to avoid potential side effects.

5. Conclusion

As per the call data on the genotoxicity of maltitol, studies should be conducted to assess three critical endpoints: gene mutation, clastogenicity, and aneugenicity (EFSA, 2023). In accordance with the EFSA guidelines, these endpoints were evaluated using a core set of two *in-vitro* assays, as performed in this study (EFSA Scientific Committee, 2011). The present study concluded that maltitol does not show any mutagenic and genotoxic effects at tested concentration.

CRedit authorship contribution statement

Anjali Patel: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Karishma Desai:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources. **Rajendra Nagane:** Writing – review & editing, Validation, Supervision, Resources. **Jagruti Barot:** Writing – review & editing, Visualization, Supervision, Resources, Methodology, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yrtph.2026.106094>.

Data availability

Data will be made available on request.

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