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The Effect of Probiotic Bacteria on the Reduction of 3-Monochloropropane-1,2-Diol (3-MCPD) in Powdered Infant Formula

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Abstract

Background & Objectives: 3-Monochloropropane-1,2-diol (3-MCPD) is a chemical contaminant found in many food products, including infant formulas. Given the vital contribution of milk and its products to the human diet, particularly for children, the presence of 3-MCPD in dairy products poses a significant public health concern. Therefore, it is crucial to explore natural compounds for 3-MCPD removal. This study investigates the effect of probiotic microorganisms (*Lactobacillus plantarum, Lactobacillus murinus, and Yarrowia lipolytica*) on reducing 3-MCPD levels in infant formula containing various 3-MCPD concentrations.

Materials & Methods: *L. plantarum, L. murinus* bacteria**,** and *Y. lipolytica* yeast were prepared as active cultures. Subsequently, various 3-MCPD concentrations were added to the infant formula, and the effect of the bacteria and the yeast on 3-MCPD reduction was investigated using gas chromatography with flame ionization detection (GC-FID).

Results: *L. plantarum, L. murinus, and Y. lipolytica* demonstrated the ability to reduce 3-MCPD levels in the infant formula at different concentrations and contact times. *L. plantarum and Y. lipolytica* were found to be more effective in reducing 3-MCPD in the infant formula compared to *L. murinus*.

Conclusion: The results indicate that probiotic bacteria can effectively mitigate the toxic effect of 3-MCPD. These findings have potential applications in the food industry, particularly in dairy products.

Keywords: Infant formulas, 3-monochloropropane-1, 2-diol, Probiotics, Gas chromatography

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Introduction

3-Monochloropropane-1,2-diol (3-MCPD) is a contaminant that forms during food production, particularly in cases where foods high in salt and fats are processed at high temperatures. This compound exists in both ester and free forms. 3-MCPD fatty acid esters are contaminants that develop during food processing (1, 2).

The International Agency for Research on Cancer classifies 3-MCPD as a Group 2B carcinogen. Research has demonstrated that 3-MCPD has neurotoxic and carcinogenic effects and negatively impacts male fertility. The presence of 3-MCPD esters (3-MCPDEs) in food raises potential health concerns, as toxicological studies in rodents have shown that these fatty acid esters are substantially hydrolyzed to their free forms,

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3-MCPD and glycidol, in the gastrointestinal tract, resulting in toxicity (3). Consequently, removing or reducing these esters from food can mitigate their toxicity and positively impact human health. Achieving this goal requires the identification of effective, selective, and environmentally safe reduction methods (4). While physical and chemical removal methods are costly, inefficient, and may cause nutritional damage to products, biodegradation using microorganisms or enzymes presents an alternative and eco-friendly method for eliminating or reducing chemical contaminants in foods while maintaining their safety and quality (5).

Recent research has focused on utilizing probiotic microorganisms and fungi as biological approaches for eliminating chemical contaminants. Probiotic microorganisms have demonstrated the ability to effectively eliminate hazardous compounds such as fatty acid esters from food matrices, thereby contributing to public health promotion (6).

Among probiotics, the lactic acid bacteria (LAB) group, through the secretion of bioactive compounds, can serve as preservative agents. Consequently, the majority of probiotics belong to the LAB group (6). LAB comprise a functionally heterogeneous bacterial group associated with traditional dairy and fermented food products such as yogurt, milk, and cheese. *Lactobacillus* species (such as *Lactobacillus plantarum* and *Lactobacillus murinus*) are common LAB frequently consumed as probiotics.

Yarrowia lipolytica is a nonconventional yeast species that has garnered interest in fundamental and biotechnological studies over the last three decades. This yeast is not pathogenic to humans, and the Food and Drug Administration (FDA) has classified it as Generally Recognized as Safe (GRAS) for use in production and industrial processes (7).

Considering the diversity of microorganisms and their growth conditions, fat-producing yeasts represent good sources for producing triglycerides, surfactants, and unsaturated fatty acids. This is particularly important for producing unsaturated fatty acids for pharmaceutical applications and/or for enriching food products, such as infant formulas (8). Recent studies have shown that the production of foods containing fats and salts at high temperatures in the presence of chlorine increases the likelihood of 3-MCPD formation (9). Consequently, increased concentrations of 3-MCPD and its intake over a short time period may lead to tumor formation (10). As public health is a critical concern in the present era, it is necessary to safeguard the health of children, who are more vulnerable. Given the established permissible limits of 3-MCPD, especially for infant formulas used by different age groups from infants to young children, the concentration of this compound in infant formulas should be carefully measured and controlled. In light of these concerns, this study employed probiotic bacteria and yeasts that have been confirmed to be harmless to humans with the aim of lowering the 3-MCPD content in products such as infant formulas.

Materials and Methods

Bacterial and yeast strains

The probiotic bacteria *L. plantarum* (PTCC1058) and *L. murinus* (PTCC322), along with the yeast *Y. lipolytica* (ATCC18942), were used in this study. The bacteria were obtained as active cultures from the Microbial Bank in Isfahan, while *Y. lipolytica* was procured as an active culture from the Iranian National Center for Genetic and Biological Resources. All strains were stored at 4°C.

Culture

Culturing of *L. plantarum* and *L. murinus* was performed on MRS agar culture medium (Merck, Germany) from active plates. The inoculated plates were then incubated in an anaerobic jar at 37°C for 24 h. To purify *Y. lipolytica*, culturing was conducted on M-enterococcus (ME) agar from an active plate and incubated at 25°C for 72 h. After reaching the logarithmic phase in a shaking incubator (120 rpm) at 37°C (for bacteria)

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and 25°C (for yeast), the microorganisms were washed with phosphate buffer, centrifuged, and serial dilutions were prepared from the precipitate. To ensure the viability of the bacteria and yeast, cell cultures were performed on MRS agar and ME agar culture media, respectively, to obtain the related CFU using the following formula:

$$N = \frac{\sum C}{V \times 1/1 \times d}.$$

Extraction of 3-MCPD

3-MCPD was extracted from the infant formula using a combination of methyl tert-butyl ether, methanol, and hexane. To remove the aqueous medium, organic solvents diethyl ether and ethyl acetate (8:12 v/v) were added to the aqueous phase. Subsequently, derivatization was performed using saturated phenylboronic acid in diethyl ether (11, 12). The bacterial and yeast strains (in the logarithmic growth phase) were washed with phosphate buffer, and 10^9 CFU/mL of each were separately added to 5 mL of infant formula. Samples of infant formula alone and those containing the bacteria and yeast were then treated with 3-MCPD at concentrations of 20, 40, 60, and 80 µg/kg. The samples were placed in an anaerobic jar inside an incubator at 37°C (for bacteria) and 25°C (for yeast). Extraction was performed after 24, 48, and 72 h (13, 14).

Gas chromatography

To separate the aqueous phase, 1 mL methyl tert-butyl ether and 4 mL methanol were mixed with the infant formula, followed by the addition of 1 mL indicator A (0.2 g sodium hydroxide in 100 mL methanol). After 5 min, 1.5 mL indicator B (20 g sodium hydroxide in 90 mL water and 3 mL 25% sulfuric acid) was added. Finally, 1.5 mL n-hexane was introduced, and the solution was centrifuged at 2800 g. The aqueous medium was transferred to an organic solvent by adding 1.5 mL of the diethyl ether and ethyl acetate mixture to the aqueous phase, followed by centrifugation at 2800 g. After the samples separated into two phases, 1 mL of the supernatant from each sample was transferred to microtubes (15, 16).

Prior to derivatization, 0.5 mL of the diethyl ether and ethyl acetate mixture (8:12) was added to enhance the reaction between 3-MCPD and saturated phenylboronic acid. Subsequently, 50 µL of saturated phenylboronic acid in diethyl ether was introduced. The samples were placed in an oven at 50°C to evaporate the solvents. Before injection into the gas chromatograph, 100 µL of isooctane was added to each sample.

Statistical Analysis

Statistical analyses, including the determination of means, standard deviations (SDs), and p-values of the test data, were performed using the Chi-square test in SPSS. A p-value less than 0.05 was considered statistically significant.

Results

Activity of the yeast and bacteria

Culturing *L. plantarum*, *L. murinus*, and *Y. lipolytica* on MRS and ME agar produced milky white colonies, indicating the viability of the yeast and bacteria. *L. plantarum* and *L. murinus* entered the logarithmic growth phase after 2 h, reached their highest growth rate after 6 h, and finally entered the stationary phase after 7 h. The yeast *Y. lipolytica* entered the logarithmic growth phase after 10 h, reached its maximum growth rate after 30 h, and finally entered the stationary phase after 31 h.

Calibration curve of 3-MCPD

To plot the calibration curve, 3-MCPD concentrations of 20, 40, 60, and 80 µg/kg were prepared. The resulting calibration curve was represented by the equation y = 0.3838x + 2.0295, demonstrating the goodness-of-fit and linearity of the curve.

Gas chromatography

Figure 1 presents the chromatogram obtained using GC-FID. The area under the peaks was calculated and utilized in subsequent stages of analysis.

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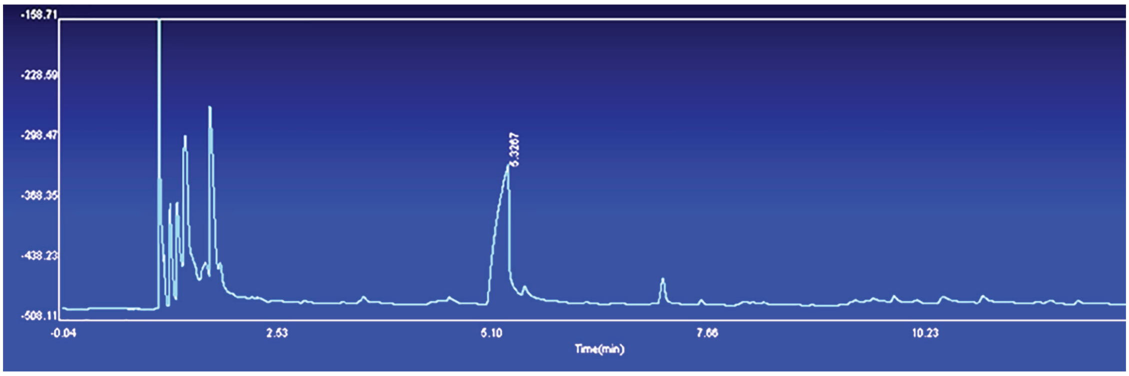

Figure 1. Chromatogram of 3-MCPD injection to GC-FID

Table 1. Effect of *L. murinus, L. plantarum* and *Y. lipolytica* in reducing 3-MCPD in milk powder matrix with four concentrations

	Variations of 3-MCPD Hours	20 µg/kg		40 µg/kg		60 µg/kg		80 µg/kg	
		3-MCPD conc.	Reduction (%)	3-MCPD conc.	Reduction (%)	3-MCPD conc.	Reduction (%)	3-MCPD conc.	Reduction (%)
L. murinus	24 h	9.56	52.20	20.50	48.75	32.42	45.97	44.74	40.07
	48 h	7.83	65.85	16.80	58.00	28.91	51.81	43.91	45.11
	72 h	3.46	82.70	9.66	75.85	28.13	53.11	40.71	49.11
L. plantarum	24 h	0	100	0	100	0	100	0	100
	48 h	0	100	0	100	0	100	0	100
	72 h	0	100	0	100	0	100	0	100
Y. lipolytica	24 h	11.21	43.95	13.70	65.75	18.64	68.94	22.25	72.19
	48 h	9.36	53.20	1.90	95.25	0.17	99.72	0	100
	72 h	2.33	88.35	0	100	0	100	0	100

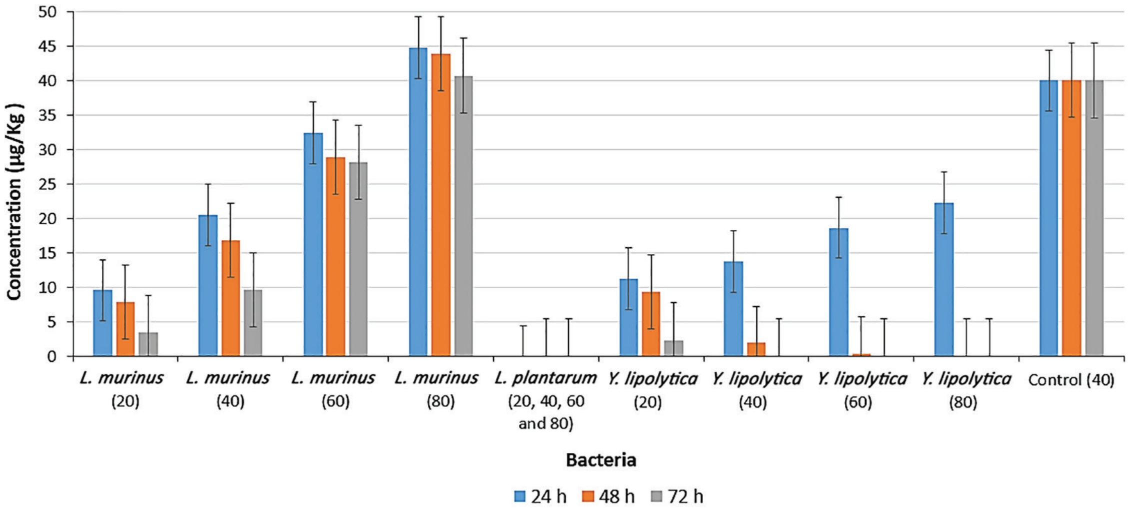

Chart 1. Effect of *L. murinus, L. plantarum, Y. lipolytica* in reducing 3- MCPD in milk powder matrix with four concentrations

Effect of bacteria and yeast in reducing MCPD-3

The effects of *L. murinus*, *L. plantarum*, and *Y. lipolytica* in reducing 3-MCPD in the milk powder matrix at concentrations of 20, 40, 60, and 80 µg/kg are presented in Table 1 and Chart 1.

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The results demonstrated that the activity of *L. murinus*, *L. plantarum*, and *Y. lipolytica* against 3-MCPD was most significant at a concentration of 40 µg/kg across all four tested concentrations.

Discussion

Infant formulas, as the primary substitute for breast milk, require a high degree of food safety. The contamination of these products with chloropropanols is, therefore, a matter of significant concern. Utilizing probiotic bacteria to remove this harmful compound from infant formulas not only enhances their safety but also improves their nutritional value for infants (17). The results of this study demonstrated the considerable efficacy of probiotic microorganisms in 3-MCPD removal. *L. murinus* effectively reduced 3-MCPD concentrations of 20, 40, 60, and 80 µg/kg over periods of 24, 48, and 72 h. *L. plantarum* achieved 100% reduction of 3-MCPD at all tested concentrations after 24, 48, and 72 h. Furthermore, *Y. lipolytica* exhibited significant reduction of 3-MCPD at all concentrations and time points. Among the tested microorganisms, *L. plantarum* demonstrated the greatest efficacy in decomposing 3-MCPD.

Our findings align with recent studies that have utilized probiotic bacteria for toxin reduction. Bel-Rhlid et al. (2004) reported on 3-MCPD reduction using *Saccharomyces cerevisiae*. Their bioassays, conducted under aerobic conditions at 28°C, demonstrated 68% decomposition of 3-MCPD at 27 mmol/L after 48 h, and 73% decomposition at 7.3 mmol/L after 72 h (18). Khanafari et al. (2007) found that *L. plantarum* removed 45% and 100% of Aflatoxin B1 from a liquid medium after 1 and 9 h, respectively (19). In a study on the efficacy of probiotics in eradicating *Helicobacter pylori*, Homan et al. (2015) discovered that specific doses of probiotics such as *Saccharomyces boulardii* and *Lactobacillus johnsonii* could reduce the bacterial load, though generally did not completely eradicate *H. pylori*. They concluded that *S. boulardii* is a standard treatment that likely increases the eradication rate (20). Navarrad et al. (2019) investigated the rate of Aflatoxin M1 removal by probiotic bacteria in milk. Their results indicated that decreasing aflatoxin concentration in milk from 0.75 ng/mL to 0.5 ng/mL enhanced the ability of *Animalis* to reduce aflatoxin concentration. Moreover, *Animalis* was more effective in reducing aflatoxin concentration in the contaminated culture medium after 120 min compared to 60 min (21). Similarly, our study found that increasing contact time led to further reduction in toxin concentration.

It is worth noting that limited access to yeast strains and a scarcity of information in this field were among the constraints of this study.

Conclusion

Given the widespread hazards caused by various toxins in Iran and other countries, extensive studies have been conducted on removing or reducing 3-MCPD concentration. This study investigated the effect of *L. plantarum*, *L. murinus*, and *Y. lipolytica* on different concentrations of 3-MCPD at various contact times. The results demonstrated that increasing contact time enhanced the efficacy of both bacteria and yeast in reducing 3-MCPD concentration. Conversely, while increasing toxin concentration in the infant formula decreased bacterial performance, yeast performance improved with higher toxin concentrations.

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Conflict of Interest

The authors declare no competing interests.

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Authors' Contribution

L.R. designed and supervised the study. E.S. performed sample collection and conducted experiments. E.S., L.R., and N.H. carried out statistical analysis, interpretation, and drafting of the paper. All authors have read and approved the manuscript for publication.

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