Innovative application of postbiotics, parabiotics and encapsulated *Lactobacillus plantarum* RM1 and *Lactobacillus paracasei* KC39 for detoxification of aflatoxin M1 in milk powder.

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SUPPLEMENTARY FILE

- The research work focused on the topic of food safety as we assess the contamination level of infant formulae and milk powder with AFM_1 that considered a food safety issue specially for infants.
- The possible application of the bioactive compounds of probiotic bacteria for detoxification of aflatoxins (AFM1 and AFB1), in addition to the novel use of bioactive components; Cell Free Supernatants (postbiotic), acid-dead cells (parabiotic) and the encapsulated-cells of *L. plantarum* RM1 and *L. paracasei* KC39 which were evaluated for their antifungal activity against the toxigenic mold strains in addition to their impact on AFB_1 and AFM_1 reduction in reconstituted milk powder.

In conclusion; the application of bacterial bioactive compounds specifically the postbiotics in the dairy industry are suggested as superior to probiotics for aflatoxins control.

Highlights of research findings

- Contamination of infant formulae and milk powder with AFM_1 with levels exceeding the Egyptian and European standards
- Postbiotics were the most effective bioactives reduced AFM_1 (89.8%)
- AFB_1 reduction percentages ordered ascending; parabiotics < encapsulated < postbiotics for *L. plantarum* RM1, while KC39 was the contrary
- The antifungal activity and detoxification ability of the bioactive compounds of *L. paracasei* KC39 were higher than that of *L. plantarum* RM1
- Application of bacterial bioactive compounds specifically the postbiotics in the dairy industry are suggested as superior to probiotics for aflatoxins control

Material & methods

Collection of samples

Total number of fifty random samples of infant cow's milk-based formulae (25) and full-fat milk powder (17 unpacked and 8 packed) were collected from Local markets in Egypt.

Determination of AFM_1 content in the examined samples

Prevalence of AFM_1 in the examined samples was carried out using an indirect enzyme-linked immune-sorbent assay (ELISA) test kit, BioFront Technologies, Commonwealth Blvd., Tallahassee, USA according to **(Sani et al., 2012)** with modifications. Dry milk samples were

reconstituted (9.1 g/100 mL double-distilled water), stirred and homogenized, then centrifuged (3500 g/10 min/10°C), the creamy layer was removed completely. AFM_1 standard solutions (100µL/well) and the test samples (100µL/well) in duplicates were added to the wells of microtiters' plate and proceeded as the manufacturer's instructions. Sample was considered positive for AFM_1 if the level exceeded the minimum detection limit for the assay (0.005ng L^{-1}), and it was diluted with sample diluent solution and re-analyzed for a concentration greater than 1000 ng L^{-1}.

Reduction of the AFs using the bioactive compounds of Lactobacillus plantarum RM1 and Lactobacillus paracasei KC39

Preparation of the probiotic cell pellets

The new strains of *Lactobacillus plantarum* RM1 and *Lactobacillus paracasei* KC39, isolated from the fermented Rayeb and karesh cheese, respectively by **(Shehata et al., 2018 and 2019)** were activated on MRS broth media at 37 ℃ /24 h, then, transferred to Lab-fermenter (Jupiter stirred mini-fermenter 4L, Solaris Biotech., Porto Mantovano, Italy) containing MRS-broth and incubated at 37 ℃ /24 h and the yield of each strain was separately centrifuged at 4100Xg /5 ºC/30 min. to obtain the cell-pellets.

Preparation of RM1 and KC39 postbiotics

The cell-free supernatant "CFS" was prepared according to the methodology described by **Shehata et al. (2018 and 2019)**. The solution earned over *L. plantarum* RM1 and *L. paracasei* KC39 bacterial-pellets centrifugation were known as postbiotics. It was collected, purified and sterilized by an individual sterile-membrane (0.22 µm), then it was lyophilized by a Dura-Dry MP freeze-dryer (FTS System, USA) to yield dry-pure powder.

Preparation of RM1 and KC39 parabiotic

The treated probiotic cell-pellets were prepared according to **El-Nezami et al. (1998)** with modification. Hydrochloric acid (1M) was used for acidification of the media that made the bacterial cells to die. The bacterial concentrations were $2.1x10^{11}$ and $1.7x10^{11}$ CFU mL^{-1} media for RM1 and KC39, respectively. The endpoint was identified at pH 2.8, which indicated the cell death. Tubes were incubated (37ºC/ 1h/ pH=2.8) then re-centrifuged (4100 Xg/15 min) to earn the cell-pellets. These pellets that contain the acid dead-bacterial cells were handled as parabiotics, it was washed 3 times using 5mL of the phosphate buffer saline solution (pH=7.2), then lyophilized and stored under aseptic conditions (2 ºC/ sealed amber bottle).

Preparation of the encapsulated bacterial cells

Bacterial strains were encapsulated by wall material consists of maltodextrin and whey protein (1:2), according to the method designated by **Abdel-Razek et al. (2018)** with bacterial cell concentrations of $2.1x10^{11}$ and $1.7x10^{11}$ CFU mL^{-1} for RM1 and KC39, respectively. Maltodextrin solution containing Arabic gum and tween 80 as emulsifiers or surfactant (0.5% of each). About 10 mL of phosphate buffer solution was used to dissolve the bacterial pellets (6 grams), then it was injected to maltodextrin solution using micro syringe. The previous mixture was added slowly to whey protein concentrate solution (WPC80) to form the wall material contained the bacterial cells. The encapsulation technique was done using the Ultra-Turrax homogenizer T18 basic (IKA, Wilmington, USA), operating speed 18,000 rpm/min, for 20 min) and then lyophilized.

Determination of the antifungal activity of bacterial treatments

The antifungal activities of the postbiotics, parabiotics, and the encapsulated probiotics were assessed against four toxigenic fungal strains *(A.flavus* ITEM 698, *A. parasiticus* ITEM 11, *F. moniliforme* KF 488, and *Penicillium chrysogenum* ATCC 10106), which were obtained from ISPA, Bari, Italy using the agar well diffusion method according to **Badr et al. (2020).** The

toxigenic fungal strains were activated in sterile tween-water solution at a concentration of 10^5 spores mL^{-1}, and then 0.1ml of each fungal strain was spread on Potato dextrose agar media (PDA), in triplicates. The lyophilized CFS, Acid dead, and the encapsulated cells were suspended in PBS solution ($2.1x10^{11}$ and $1.7x10^{11}$ CFU mL^{-1}, of RM1 and KC39, respectively), and added to each well of PDA. The plates were incubated at 30°C/ 48 h for estimating the antifungal impact. The inhibition zones were determined for each fungal strain and the antifungal activity was expressed as activity units per milliliter (AU mL^{-1}).

Estimation of the effect of postbiotics, parabiotics and the encapsulated probiotics on AFB_1 secretion in a liquid media

Impact of the bacterial treatments on AFB_1 reduction in a liquid media was done according to the methodology described by **Shehata et al. (2019**) with modifications. The spores of *Aspergillus flavus* ITEM 698 were inoculated into yeast extract sucrose (YES) broth media at a concentration of 10^5 spores mL^{-1} and incubated for 18 h. Postbiotics, parabiotics and the encapsulated cell pellets were inoculated separately (1 mg mL^{-1} media), then re-incubated (96 h/30ºC). AFB_1 secreted in the liquid media was determined using ELISA method according to **Sani et al. (2012).** Additionally, the reduction in fungal mycelial weight was compared to the control one and the antifungal efficacy (AE) was calculated as a percentage by the following equation:

$$\mathbf{AE\% = [(MFWc- MFWt)/ MFWc] *100}$$

Where

AE: the antifungal efficacy of the treatment.

MFWc: dried mycelia-weight of control fungal growth.

MFWt: dried mycelia-weight of treated fungal growth.

The effect of postbiotics, parabiotics and encapsulated probiotics on AFM_1 reduction

The reduction in AFM_1 concentration owing to each treatment was determined in a model of reconstituted milk powder according to **Negera and Washe (2019)** with some modification**.** 9.1 g milk powder was reconstituted in phosphate buffer saline (100 mL/ pH 7.3), to which AFM_1 standard toxin solution (250 ng) and the different treatments (postbiotics, parabiotics & the encapsulated cells) (1 mg mL^{-1} media) were added separately and incubated at (pH 7.3/37 ºC/ 24h). By the end of the incubation time, tubes were centrifuged (4100 Xg/ 20 ºC/8 min) to recover the AFM_1. AFM_1 residue was determined using ELISA method according to **Sani et al. (2012)**. Toxin inhibition was calculated as a ratio of inhibition by the following equation:

$$\textbf{\% TRR = [Toc – Tot /Toc] *100}$$

Where

TRR: the toxin reduction ratio achieved by the treated components.

Toc: toxin concentration in the control buffer solution.

Tot: toxin concentration in a buffer solution with the different treatments.

Statistical analysis

The data were statistically analyzed using SPSS Version 17.0 software. An independent sample *t*-test was conducted to compare the AFM_1 levels in infant milk and milk powder samples, *Mann–Whitney U* test was used when data were not normally distributed, and it was used for comparing the effect of packaging on milk powder samples as well as for comparing the mean AFM_1 level in infant formulae and milk powder samples. In addition to assessing the impact of using postbiotics, parabiotics and the encapsulated cells on the reduction of AFM_1 and AFB_1. Significance was set at P-value < 0.05.