In vitro antibacterial mechanism of action of crude Garlic (*Allium sativum*) clove extract on selected probiotic *Bifidobacterium* species as revealed by SEM, TEM and SDS-PAGE analysis

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Abstract

There has been much research on the effects of garlic (*Allium sativum*) on numerous pathogens, but very few, if any, studies on its effect on beneficial, probiotic bifidobacteria. We have recently shown that garlic exhibits antibacterial activity against bifidobacteria. The mechanism by which garlic kills bifidobacteria is yet to be elucidated. This study sought to determine the mechanism of action of garlic clove extract on selected *Bifidobacterium* species using scanning and transmission electron microscopy and SDS-PAGE analysis. SEM micrographs revealed unusual morphological changes such as cell elongation, cocci-shaped cells with cross-walls and distorted cells with bulbous ends. With TEM observed changes included among others, condensation of cytoplasmic material, disintegration of membranes and loss of structural integrity. SDS-PAGE analysis did not reveal any differences in whole cell protein profiles of untreated and garlic clove extract treated cells. The current study is the first to reveal the mechanism of action of garlic clove extract on probiotic *Bifidobacterium* species. The results indicate that garlic affect these beneficial bacteria in a manner similar to that exhibited in pathogens. These results therefore further highlight that caution should be taken especially when using raw garlic and probiotic bifidobacteria simultaneously as viability of these bacteria could be reduced by allicin released upon crushing of garlic cloves, thereby limiting the health benefits that the consumer anticipate to gain from probiotics.

Keywords: Garlic; *Allium sativum*; *Bifidobacterium*; scanning electron microscopy; transmission electron microscopy; probiotic

Introduction

Bifidobacteria are Gram-positive, anaerobic, non-sporeforming bacteria that form a significant part of the intestinal microflora in the gastrointestinal tracts of healthy humans. Occurrence of these bacteria within the GIT is an indication of good intestinal health [14, 22]. Bifidobacteria are used in conjunction with lactobacilli in probiotic products due to their numerous health benefits which include prevention and treatment of allergies, gastrointestinal problems and immunodeficiencies [13, 22]. Bifidobacteria are also believed to reduce blood cholesterol, deconjugate bile acids as well as reduce traveler's diarrhea and antibiotic-associated diarrhea [14]. In order to confer the purported health benefits, these bacteria need to be available in sufficient amounts in probiotic-containing foods and products. It is worth noting that at the same time that probiotics are prescribed to consumers for health benefits, there are herbs such as garlic, which are also recommended for the same reasons.

Garlic (*Allium sativum*) has been used worldwide for many centuries as a spice and herbal medicine and is believed to treat and prevent various diseases [6]. It is a strong antibacterial agent and inhibits both Gram-positive and Gram-negative bacterial growth, which includes *Bacillus*, *Brusella*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Shigella, Staphylococcus*, *Salmonella* as well as *Helicobacter pylori* [9, 12]. The main component of garlic responsible for its antibacterial activity is allicin [19]. The enzyme allinase converts alliin into this volatile compound, once garlic is damaged by crushing or cutting [12, 26]. Most antimicrobial agents are able to modify bacterial cell membranes and this leads to cell leakage and autolysis thereby preventing growth and causing cell death [19].

Garlic has been found to have a morphological effect on various bacterial cells, resulting in changes to the outer surfaces, internal properties as well as behavior of the cells [6, 15, 18, 23]. Although there has been much research on the effect that garlic has on pathogenic bacteria, there are a very few studies on its effect on beneficial, probiotic bifidobacterial cells. We have recently shown that garlic does exhibit antibacterial effects

against probiotic bifidobacteria [7]. The aim of the current study was to determine the mechanism of action of garlic on selected *Bifidobacterium* species using SEM, TEM and SDS-PAGE.

Materials and Methods

Bacterial cultures

Bifidobacterium lactis Bb12 (CHR- Hansen, Denmark), *B. bifidum* LMG 11041 and *B. longum* LMG 13197 (LMG Culture collection, Belgium) were used as test cultures.

Preparation of inoculums

Bifidobacterium cultures were grown in MRS-cys-HCL broth and incubated at 37°C for 48 h in anaerobic jars with Anaerocult A gaspacks and Anaerocult C test strips (Merck KGaA, Germany) for confirmation of anaerobic conditions. Bacterial cell suspensions were adjusted to a 0.5 McFarland standard (approximately 1 x 10^8 cfu ml^{-1}).

Preparation of garlic clove extract

Fresh garlic cloves were purchased from a local supermarket in Pretoria and used within two weeks. The garlic clove extract was prepared as described by Bakri and Douglas [5], with slight modifications. Fresh cloves were separated and peeled and then 10 g was weighed and crushed using a mortar and pestle. This was suspended in 5 ml sterile distilled water. The suspension was then centrifuged at 1677 *g* for 5 min and then filtered through a 0.22 µm Ministart syringe filter (Sigma-Aldrich, USA). The weight of the insoluble material of the garlic clove was subtracted from the weight of the original material to get the final concentration of garlic in solution, which was determined to be 60.7% (w/v). The concentration of allicin, the major active compound in the extract, was determined spectrophotometrically by reaction with 4–mercaptopyridine as described previously [7]. The sterile extract was used within 30 min of its preparation.

Treatment of bifidobacteria with garlic clove extract

Garlic clove extract was added to a total volume of 1 ml of each *Bifidobacterium* broth culture, in triplicate. The final allicin concentration in each culture was equivalent to the minimum bactericidal concentration of 198.7, 99.4 and 39.8 µg ml^{-1} for *B. lactis* Bb12, *B. bifidum* LMG 11041 and *B. longum* LMG 13197,

respectively, as previously determined [7]. Cultures were then incubated at 37°C for 6 h followed by centrifugation for 10 min at 12044, 9 *g*. Cells were resuspended in 1 ml ¼ strength Ringer's solution.

Scanning electron microscopy (SEM)

The samples of *Bifidobacterium* spp. were observed under the scanning electron microscope before and after 6 h of garlic treatment. A modification of the method by Lai-King et al. [17] was used. Cells suspended in ¼ Ringer's solution were harvested by filtering through a 0.2 µm filter membrane. The cells were fixed to the membrane using 2.5% gluteraldehyde in 0.075 mol $^{-1}$ phosphate buffer (pH 7.4) for 1 h. They were then washed three times with 0.15 mol $^{-1}$ phosphate buffer, and dehydrated in a graded alcohol series (25, 50, 75, 90 and 100% ethanol). This was followed by critical-point drying for 24 h and the filters were mounted onto SEM specimen stubs and coated with gold. The samples were observed under a JEOL 780 and JEOL JSM-5800LV SEM (JEOL, Tokyo, Japan).

Transmission electron microscopy (TEM)

Garlic clove extract treated and untreated bacterial cultures (1 ml each) were centrifuged at 1677 g for 2 min. The pellet was then fixed using 2.5% gluteraldehyde in 0.075 mol $^{-1}$ phosphate buffer (pH 7.4). It was then rinsed three times in 0.15 mol $^{-1}$ phosphate buffer and fixed in 0.5% osmium tetroxide. The sample was rinsed with distilled H_2O and dehydrated in a graded series of ethanol (50, 70, 90 and 100%) before being infiltrated with Quetol epoxy resin and allowed to polymerize for 39 h at 60 ^{o}C. Ultrathin sections were cut and stained with aqueous uranyl acetate. The sections were then counterstained with lead acetate and rinsed in distilled H_2O. Monitor sections of 0.5 µm were cut and stained in Toluidine blue. The samples were then viewed using a JEOL JEM-2100F microscope (JEOL, Tokyo, Japan).

SDS-PAGE analysis

Garlic clove extract treated and untreated samples (1 ml each) were transferred to Eppendorf tubes and cells were harvested by centrifugation at 1677 *g* for 2 min. The pellet was subsequently washed three times with phosphate-buffered saline. The sample was then sonicated for 15 s at 30 W while on ice. Then 40 µl of sample buffer was added and the sample was heated at 100°C for 10 min and cooled on ice. 20 µl of the lysed cell product was run per lane on a 10% SDS gel at 100 V for 170 min. The gels were stained overnight using a colloidal Coomassie blue stain ((BioRad, USA) and destained in distilled H_2O for 1 h.

Results

Scanning electron microscopy micrographs revealed changes to the outer surface of *Bifidobacterium* cells exposed to garlic clove extract for 6 h (Fig. 1). The surfaces of all untreated cells were smooth and intact (Fig. 1a, d and g). *Bifidobacterium bifidum* LMG 11041 and *B. longum* LMG 13197 cells not treated with garlic clove extract had a uniform rod-shaped appearance (Fig. 1a and d) while *B. lactis* Bb12 cells were pleomorphic (Fig. 1g). After exposure of bifidobacteria to garlic clove extract there was an increase in highly distorted cells. Swollen cells, those with dumb-bell shaped appearance as well as those with bulbous ends were observed (Fig. 1b, c, h and i). *Bifidobacterium bifidum* LMG 11041 cells became coccoid shaped with cross-walls (Fig. 1b and c) and there was an increase in coccoid than rod shaped cells for *B. lactis* Bb12 cells (Fig. h and i). Pore formation and cell lysis was also apparent after garlic clove extract treatment (Fig. 1e, f and i). Disintegration of some cells and presence of debris in the vicinity of treated cells was also evident (Fig. 1e and h). *Bifidobacterium* treated cells appeared longer than untreated cells.

Transmission electron microscopy micrographs revealed intact cells with uniform cytoplasmic appearance and well defined walls and membranes for untreated bifidobacteria (Fig. 2a, d and g). However various morphological and intracellular changes were evident upon treatment of cells with garlic clove extract. Treatment of bifidobacteria with garlic clove extract resulted in aggregation or condensation of cytoplasmic contents, loss of structural integrity of the membranes and cell wall lysis with presence of debris evident in the lysed cells' surrounding environment (Fig. 2b, c, e, f, h and i).

SDS-PAGE analysis was also performed to determine whether there were any differences in protein profiles between untreated *Bifidobacterium* cells and those that were exposed to the garlic clove extract. There were no major differences in protein profiles of untreated and garlic extract treated bifidobacteria, except that a single band positioned between 130 and 250 kDa was fainter in *B. longum* LMG 13197 treated than in untreated cells (data not shown).

Discussion

The differences in appearances of treated and untreated bifidobacteria cells suggested that garlic clove extract affected the structure of these bacteria. The morphological characteristics of all untreated cells as observed in the current study were similar to those published by researchers elsewhere. Lv et al. [21] reported smooth rod

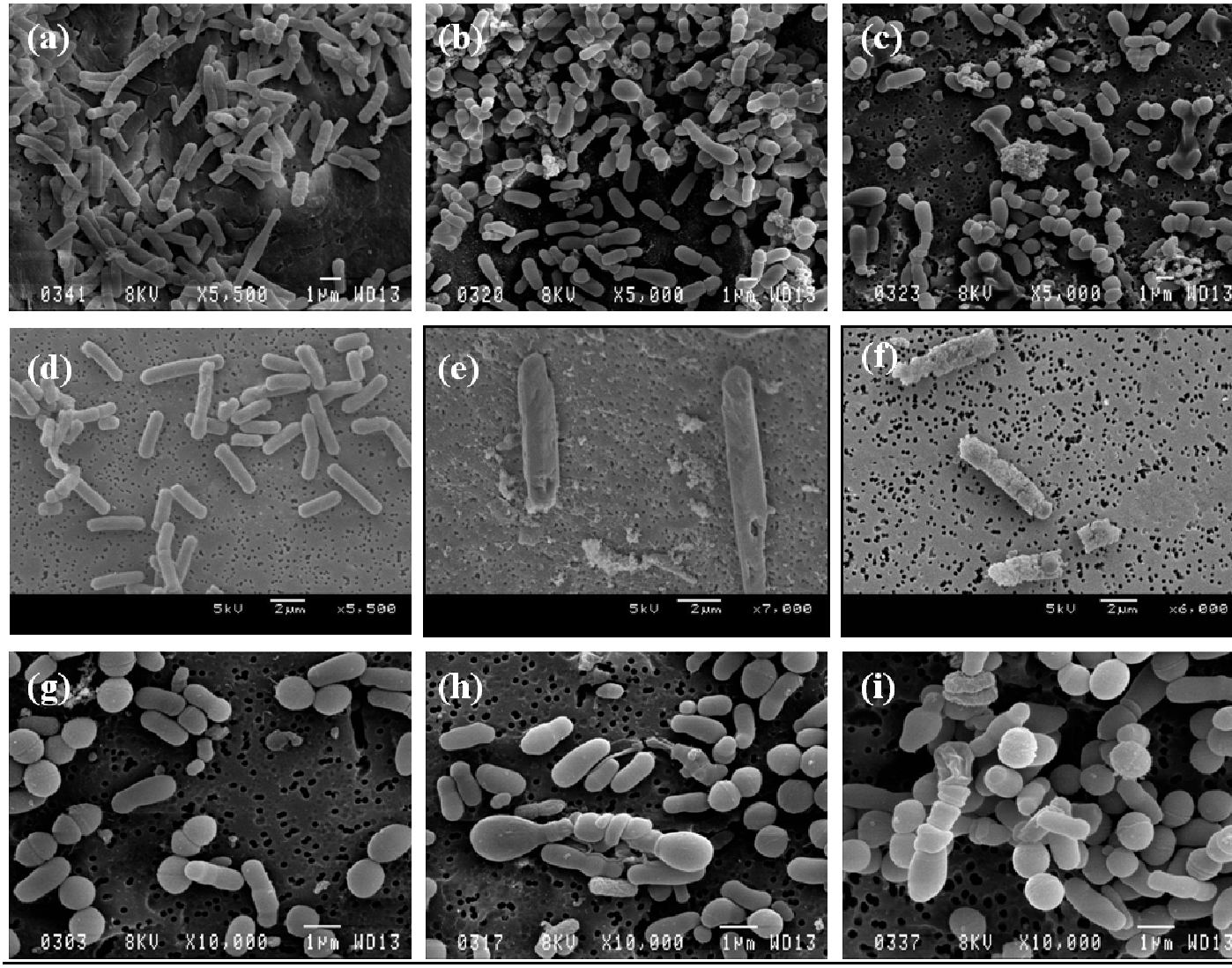

Fig. 1 Scanning electron micrographs of Bifidobacterium bifidum LMG 11041, untreated (a); treated (b, c); B.longum LMG 13197, untreated (d) and treated (e, f) and B. lactis Bb12, untreated (g) and treated (h, i) cells.

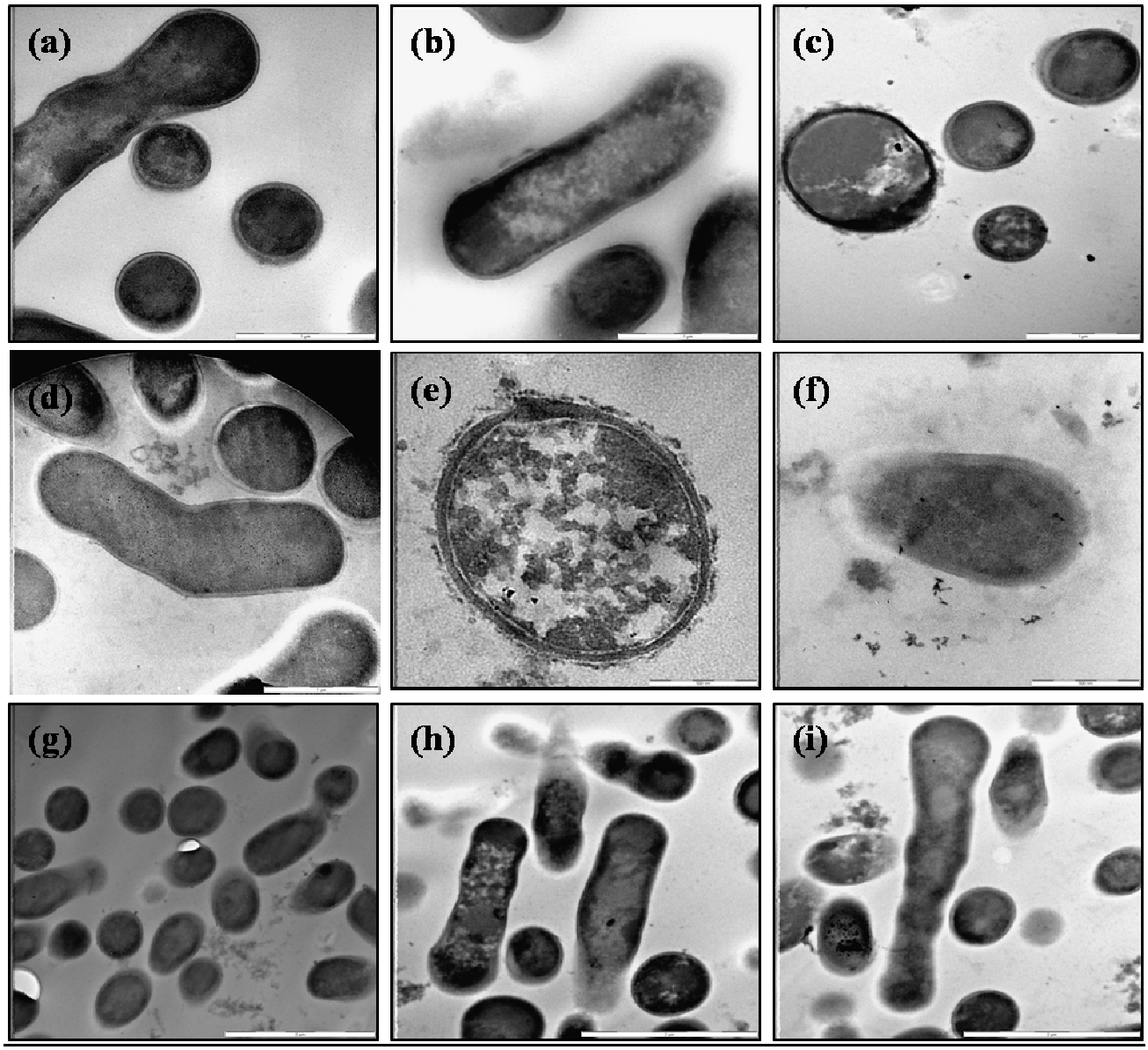

Fig. 2 Transmission electron micrographs of Bifidobacterium bifidum LMG 11041, untreated (a); treated (b, c); B. longum LMG 13197, untreated (d) and treated (e, f) and B. lactis Bb12, untreated (g) and treated (h, i) cells.

shaped cells for untreated bifidobacteria whereas a pleomorphic culture of untreated bifidobacterium cells was reported by [22, 24]. These researchers [22, 24] observed coccoid as well as branched, filamentous and swollen cells with cross-walls. Novik et al. [22] reported that during their death phase or periods of stress, bifidobacteria form coccoid and club-shaped cells. The observed cocooid shaped cells could also be attributed to cells entering death phase due to presence of allicin and other antimicrobial compounds present in the garlic clove extract. Cell wall lysis was also reported for *Listeria monocytogenes* treated with garlic shoot juice [15]. Cell wall degradation was proposed to be due to weakening of the peptidoglycan layer due to garlic extract exposure [15]. Changes caused by treatment with garlic clove extract were also reported to occur as a result of exposure of bacteria to other types of antimicrobial compounds. Lv et al. [21] previously documented cells with usual structures for untreated cells but those with increased permeability, disrupted membrane integrity, incomplete and deformed shapes as well as rupture, lysis and loss of cell walls for selected bacterial food pathogens treated with essential oils. An increase in size of bacteria after treatment with garlic was reported for *Pseudomonas aeruginosa* cells [2]. Cells elongate in order to increase their surface-to-volume ratio in response to changes in the environment [16].

Smooth intact cells with uniform cytoplasmic appearance for untreated bacteria and those with uneven cytoplasmic consistency and changed external structure for garlic clove treated cells as showed by TEM, were previously reported [21]. Exposure of lactic acid bacteria to bile acids induced similar internal morphological changes [27]. Treatment of *Salmonella hadar* cells with aqueous garlic extract resulted in loss of membrane integrity [6]. Deformation of cells, condensation of cellular material and presence of significant amounts of cytoplasmic material and membrane fragments have been reported for *Campylobacter jejuni* cells treated with garlic and garlic-derived organosulfur compounds [20]. Recently, Hatoum et al. [11] also reported TEM micrographs showing cell membrane perforation, cell lysis and leakage of cellular material in *Listeria monocytogenes* due to antimicrobial effects of compounds released by yeast cells. Cell wall lysis as well as reduction in cytoplasmic volume due to its aggregation has also been observed in bifidobacteria exposed to nisin [14]. Loss of cell contents evident from the SEM and TEM micrographs indicates irreversible damage to the cytoplasmic membranes. It is possibly as a result of accumulation of antimicrobial agent in the cell membrane, which is associated with an increase in permeability of the cell. The cells ultimately die due to leakage of cytoplasmic contents and impairment of enzyme systems [21].

Bacterial protein profiles are a reflection of its genome and therefore whole protein content plays a crucial role in comparative studies of bacteria [1]. Allicin kills bacteria through partial inhibition of DNA and protein synthesis and total inhibition of RNA synthesis [4]. Absence of visible differences in the whole cell proteins of treated and untreated bacteria may suggest that synthesis of the proteins is not affected, but that protective enzymes are inhibited in the presence of garlic [21]. It may also suggest a short exposure time of bifidobacteria to garlic as it has been indicated that an increase of incubation time of bacteria in the presence of antimicrobial compound led to presence of additional protein bands on SDS-PAGE gels [10, 25]. However, in the current study we did not increase the incubation time as our intention was to keep exposure period close to the retention time of food in the gastrointestinal tract. Reduction in the intensity of protein bands has been associated with inhibition of synthesis and expression of the protein in question by the toxic action of garlic or other plant extracts [25]. It was envisaged that should garlic have proteolytic activity on proteins, this would be indicated by disappearance of some protein bands and (or) appearance of additional bands on the gel. Disappearance of bands has been attributed to degradation of proteins by antimicrobial agents [3, 8]. Appearance of new bands could be due to production of proteins such as chaperones and signal transduction cascades that help bacteria to survive stressful condition [10], or in case of bands with low molecular weight, accumulation of smaller peptides from degraded high molecular weight proteins [3].

Conclusion

The current study is the first to reveal the mechanism of action of garlic clove extract on probiotic *Bifidobacterium* species. The results indicate that garlic clove extract damages or kills these beneficial bacteria in a manner similar to that exhibited in pathogens. These results therefore further highlight that caution should be taken especially when using raw garlic and probiotic bifidobacteria simultaneously as viability of these bacteria could be reduced by allicin released upon crushing of garlic cloves, thereby limiting the health benefits that the consumer anticipate to gain from probiotics.

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Conflict of interest All authors have no conflict of interest to declare.

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