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# Safety considerations in the use of nonviable microbial cells as health-promoting agents in food and dietary supplements

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This review explores the safety issues related to the utilization of nonviable microbial cells in food and dietary supplements. It addresses potential risks associated with their consumption, drawing insights from probiotic research. Four categories of risks are outlined:

- Antibiotic resistance genes, which may persist even in nonviable cells;
- The presence of viable microbial cells, which can result from incomplete inactivation or contamination;
- Bioactive microbial cell components, which can influence immune responses;
- Detrimental enzymatic activities, relevant particularly when considering novel inactivation methods.

Human intervention trials involving nonviable microbes demonstrate a high safety profile, especially for established probiotics. Nonetheless, caution is warranted in vulnerable individuals. Furthermore, the use of nonviable microorganisms provides an opportunity to explore microbial species not commonly used as probiotics, referred to as 'next-generation probiotics.' As our understanding of nonviable microbes deepens, their potential benefits will likely lead to increased interest in various biotic product applications.

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## Introduction

A growing body of research, both *in vitro* and *in vivo*, is demonstrating the potential health benefits of microorganisms when consumed in foods and supplements, even after their inactivation [1,2]. Reportedly, the beneficial effects of nonviable microbes are primarily associated with their molecular cellular components, which can interact with host receptors at the level of the epithelium and mucosa-associated lymphoid tissue. These interactions result in modulation of host gene expression and immune response [1,3], leading to various beneficial effects such as inhibition of pathogen invasion, reduction of intestinal inflammation, reduction of incidence of cold, reduction of Helicobacter pylori gastric colonization, and improvement of bowel habits [2,4].

The formulation of foods and dietary supplements using nonviable microbial cells is gaining increasing importance in the industry, primarily because this approach eliminates the need to maintain a sufficient amount of live microbial cells until the end of the product's shelf life, which is a particular challenge for certain stress-sensitive microorganisms such as some bifidobacteria and lactobacilli.

The use of nonviable microorganisms also offers an interesting opportunity for the development of foods containing 'next-generation probiotics' (NGPs, e.g. *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, and *Bacteroides fragilis*; see Table 1 for definition) [1]. NGPs are microorganisms that miss a long history of safe use and therefore have not received safety approval from competent authorities, such as qualified presumption of safety (QPS) status from the European Food Safety Authority (EFSA), which allows their use in foods and food supplements. For example, *Akkermansia muciniphila*, a symbiont in the human gut, was not recommended by EFSA for inclusion in the QPS list due to safety concerns [5]. Nevertheless, a pasteurized biomass of *A. muciniphila* was approved as a novel food in Europe based on an EFSA opinion that consumption of $3.49 \times 10^{10}$ cells per day is safe for the target population [6].

Microbial cells are usually considered nonviable (dead) when "*the extent of injury is beyond the ability of a cell to resume growth*" [7]. Consequently, their administration is

**Table 1**

**Terms and their corresponding definitions adopted in this review. Initially, the term 'paraprobiotic' was proposed to describe products containing health-promoting nonviable microbes [1]. However, more recently, an alternative redefinition of the term 'postbiotic' has been suggested [50], leading to an ongoing debate on the most appropriate terminology to be used [51]. As there is currently no consensus regarding the preferred term, neither 'paraprobiotic' nor 'postbiotic' will be adopted in this article.**

| Terms | Definition for the purpose of this paper | Source |
|---|---|---|
| Nonviable microbial cells<br>Dead<br>Inactivated<br>Killed | Microbial cells with a state of damage or injury beyond their capacity to resume growth. | [52] |
| Probiotics | Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. | [53] |
| Next-generation probiotics (NGPs) | NGPs adhere to the standard definition of probiotics, however, they refer to microorganisms that lack a long history of safe use and have not been employed as agents for promoting health. These include human gut microbial symbionts with potential health-promoting properties that have been discovered in the last decades through metataxonomic and metagenomic studies of the human microbiome. | [54] |
| MAMPs | Also known as pathogen-associated molecular patterns (PAMPs), this term is generally used when referring to microbial molecules that elicit innate immune responses. More specifically, they are evolutionarily conserved microbe-derived molecules or parts of molecules with structures or chemical patterns unique to microbes. These patterns are perceived as nonself and are recognized by the PRRs of the innate immune system. MAMPs include bacterial LPS, peptidoglycan, flagellin, and yeast mannans. | [55–57] |
| Human gut resistome | Collection of all antibiotic resistance genes present in the intestinal microbiome. | [58] |

virtually free from the risk of causing infections. However, it would be inappropriate to consider the use of nonviable microorganisms as inherently free from any risk of adverse events. In this context, the purpose of this short review is to outline the potential safety concerns associated with the consumption of foods and dietary supplements fortified with significant amounts of nonviable microbial cells.

## Types of potential risks

Given the limited research on this topic, I have drawn the evidence on safety concerns related to nonviable microbes mainly from the literature on probiotic microorganisms. Thus, antibiotic resistance genes and bioactive properties of microbial cell components have been identified as the major potential safety risks associated with consumption of nonviable microbes. In addition, the inherent characteristics of industrial processes for inactivating microbial biomass suggest that incomplete inactivation of microbial cells and their enzymatic activities could pose additional safety risks. Therefore, I have categorized four potential risk areas (Figure 1), which are explained below.

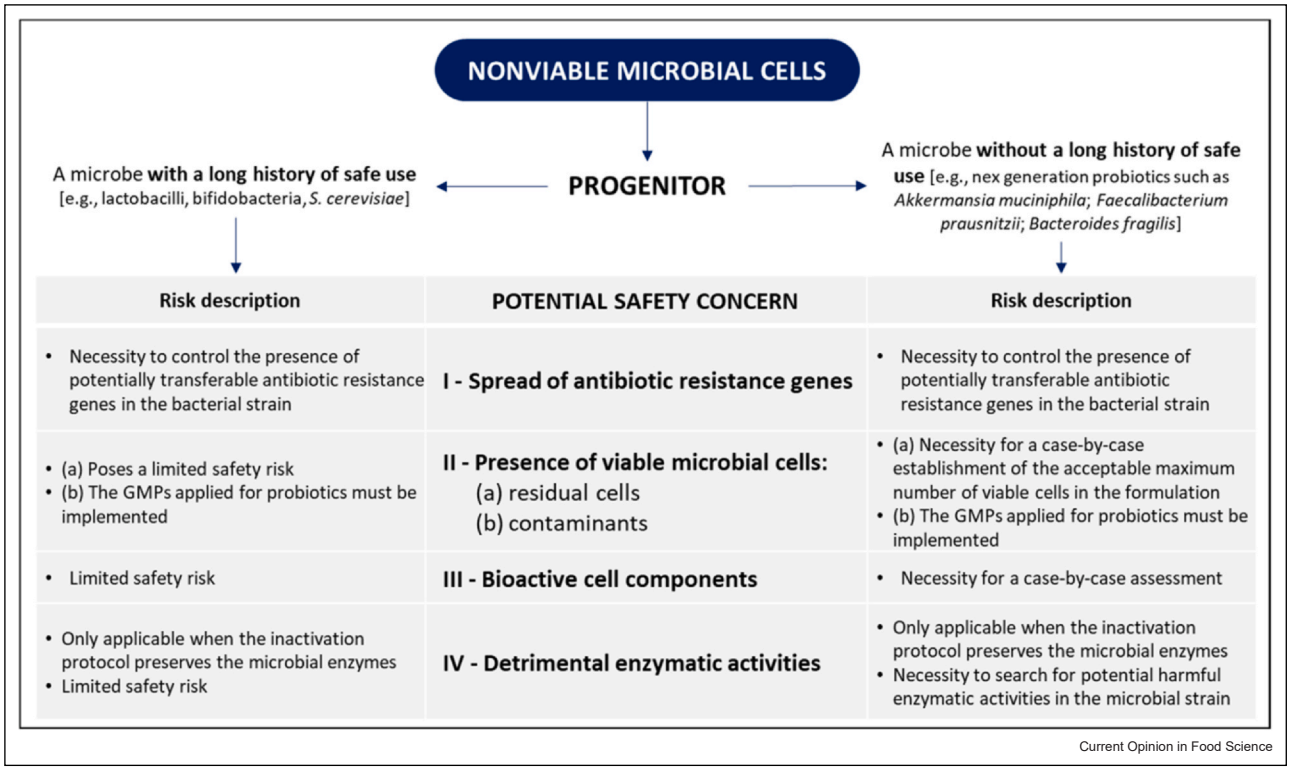
### Antibiotic resistance genes

The primary safety concerns associated with the administration of foods and dietary supplements containing nonviable microbial cells are related to the potential spread of antibiotic resistance genes. Probiotic bacteria may harbor intrinsic and acquired genetic elements that confer resistance to antibiotics [8]. Therefore, the large amounts of microbial cells in probiotic products may serve as reservoirs for antibiotic-resistant genes (ARGs), which may persist even after the microbial cells are inactivated. As a result, ingestion of microbial cells carrying transmissible ARGs, regardless of their viability, may expand the human gut resistome and facilitate the dissemination of ARGs through natural transformation into potentially harmful bacteria in the gut microbiota [9]. To counteract the emergence of antibiotic resistance, the recommendations and guidelines for the intentional use of microorganisms in food and feed [10] should also apply to the use of nonviable bacterial biomasses.

### The presence of viable microbial cells

Several studies have reported taxonomic inconsistencies between the microorganisms indicated on the label of probiotic products and those present in the formulation. However, these discrepancies mainly relate to the presence of microorganisms commonly used in food and as probiotics [11,12], so safety concerns can be considered limited. Nevertheless, contamination with potentially pathogenic bacteria has also been reported. For example, *Enterococcus faecium*, a species that includes strains used in probiotic formulations, but which also possess virulence factors and can cause disease, has been reported as the most common contaminant in dietary supplements [12–14]. In general, contamination with environmental and potentially pathogenic microorganisms has been reported to reach levels ranging from $10^2$ to more than $10^9$ CFU per dose [15–17]. If the level of contaminating microbial cells is high in some of these products, it is plausible that the contamination occurred during the fermentation step. Thus, if a product is based on inactivated microbial cells, contaminants would be killed during the intentional sterilization process of the

**Figure 1**




Summary of the potential safety concerns associated with the consumption of food and dietary supplements prepared with the deliberate addition of adequate amounts of health-promoting nonviable microbial cells.

microbial biomass. However, the possibility of contamination during subsequent steps after biomass preparation and inactivation cannot be excluded, for example, during mixing with excipients in the final formulation. This may have been the case with probiotic products associated with two clinical cases of severe infections in susceptible infants due to the presence of filamentous fungi *Absidia* (*Lichtheimia*) *corymbifera* and *Rhizopus oryzae* in the formulation [18,19]. Therefore, it would be advisable to apply the good manufacturing practices and quality control procedures specifically recommended for probiotics to products based on nonviable microbes [20,21].

Another potential source of viable cells may be incomplete killing of microbial cells. Industrial protocols commonly used to inactivate microbial biomasses, often based on heat treatments such as pasteurization and tyndallization, may not eliminate all cells within a biomass. Accordingly, agar plate counts of some commercial products purported to contain nonviable bacteria have shown residual viable probiotics of 100–1000 CFU per gram (personal communication). Assuming that the progenitor strain is a microorganism recognized as safe (e.g. a probiotic), such a limited number of viable cells is unlikely to pose a safety risk (with the possible exception of susceptible individuals, such as those with compromised immune systems). However, efforts should be made to minimize the presence of residual viable cells in products, especially if the progenitor is a microorganism without a long history of safe use. A relevant example in this regard is *Akkermansia muciniphila*, whose use as pasteurized biomass has been approved in Europe as novel food with the requirement that the number of viable cells be less than 10 CFU per gram of formulation (Commission Implementing Regulation EU 2022/168 of February 8 2022). Overall, similar to *A. muciniphila*, it would be critical to establish the maximum number of viable cells considered acceptable in each product based on nonviable microbial cells.

## Bioactive microbial cell components

Available scientific data suggest that the main route by which nonviable microbial cells can affect human health is through direct interaction with host cells in the gastrointestinal tract, leading to the induction of specific immune responses [1] through crosstalk mechanisms, which may occur mainly in the ileum [22]. In particular, crosstalk between inactivated microbial cells and host cells is mediated by the recognition of microbe-associated molecular patterns (MAMPs) by specific pattern recognition receptors (PRRs) on the surface of gut enterocytes and immune cells. This recognition can trigger cytokine production via cellular signal transduction,

influence lymphocyte priming, and modulate the inflammatory response [1]. Innate immunity can detect very subtle differences in microbial MAMPs, resulting in strain-dependent variations in the immune response. However, general considerations can be made.

MAMPs include molecules known to have potent immunostimulatory activities that can potentially induce septic shock by triggering strong inflammatory responses [23]. The best-known example in this context is lipopolysaccharide (LPS), which is present in the outer membrane of Gram-negative bacteria such as *Escherichia coli*, and is also known as endotoxin. It can induce strong inflammatory responses and is the most potent microbial mediator involved in septic shock during severe Gram-negative pathogen infections [24]. However, the best-known Gram-negative probiotic strain, *E. coli* Nissle 1917, has LPS modified in its lipid A- and O-antigen structure, which elicits a lower inflammatory response compared with conventional *E. coli* LPS [25–27]. Another Gram-negative bacterium with interesting probiotic properties used in dietary supplements is *Hafnia alvei*, but its LPS has been shown to induce high levels of the anti-inflammatory/regulatory cytokine interleukin-10 in dendritic cells [28]. The LPS of *Akkermansia muciniphila*, another Gram-negative bacterium used as pasteurized cells in dietary supplements, also has an LPS with significantly lower proinflammatory activity compared to that of *E. coli* in the epithelium [29]. Numerous other cellular components of microbial cells are known to have stimulatory (proinflammatory) effects, such as zymosan, which consists of protein–carbohydrate complexes found in yeast cell walls [30], and the cell wall components of Gram-positive bacteria, including murein, teichoic acids, and lipopeptides [31–33]. However, the immunogenic potency of these molecules appears to be much lower than that of LPS [34].

### Detrimental enzymatic activities

Even microorganisms that are generally considered safe may have potentially harmful enzymatic activities. For example, the 2002 FAO/WHO guidelines for probiotics [35] indicate that certain metabolic activities, such as D-lactate production and deconjugation of bile salts, must be tested for microorganisms to be used as probiotics.

D-Lactate production is common in many lactic acid bacteria, including *L. plantarum*, *L. acidophilus*, *L. reuteri*, and *L. delbrueckii*. Very few clinical cases of D-lactic acidosis associated with probiotic consumption have been reported, especially in young people and in the presence of short-bowel syndrome [36,37]. Nevertheless, debate has arisen regarding a possible causal relationship between probiotic consumption and D-lactic acidosis [38,39]. Furthermore, lactic acid bacteria have been found to possibly express other enzymatic activities with potentially deleterious effects, such as histidine decarboxylase (also found in bifidobacteria), which can lead to the production of the biogenic amine histamine [40], and β-glucuronidase [41,42], an enzyme that interferes with host detoxification processes and has been proposed as a marker for increased risk of colorectal cancer [43].

The theoretical risks associated with these enzymatic activities are largely mitigated when microbial biomass undergoes industrial heat treatment, as the commonly used processes (between 60 and 121°C for 5–60 min [3]) can inactivate the enzymes of microorganisms used as probiotics such as bifidobacteria and lactobacilli [44]. However, there is increasing interest in the potential use of gamma irradiation for inactivation of microbial cells on an industrial scale. Interestingly, irradiation doses of up to 10 000 Gy have been reported to complete the replicative ability of lactobacilli while preserving membrane integrity and cellular enzymatic activities [45]. Although the classification of these cells as nonviable may be questionable, their use still requires an evaluation of the safety implications related to the metabolic activities exhibited by the microbial strain.

## *In vivo* trials

A search of the PubMed database revealed 54 human intervention studies in which nonviable microbial cells were administered orally. Most of these studies focused on lactobacilli, whereas other studies examined inactivated bifidobacteria, *Lactococcus lactis*, *Streptococcus thermophilus*, *E. coli*, and mycobacteria (Supplementary Table 1). Collectively, these studies included over 5600 subjects, including children under two years of age, older individuals, and patients with tuberculosis, atopic dermatitis, obesity, irritable bowel syndrome, infant colic, allergic rhinitis, and patients undergoing supportive periodontal therapy. None of these studies reported serious adverse events that could plausibly be associated with the product administered. The only adverse events reported were mild and transient, such as nausea and epigastric pain. These effects were self-limiting and not significantly different from those observed in the placebo group. Additionally, no clinical case reports associated with oral administration of nonviable microbial cells have been reported in the literature.

## Conclusion

In summary, the utilization of nonviable microbial cells in food and dietary supplements offers a safer alternative compared with live microorganisms, as it eliminates the risk of infection associated with probiotic use [46–49]. However, some safety concerns that have emerged for probiotics are also applicable to the administration of nonviable microbes. In particular, the absence of acquired antibiotic resistance genes should be a mandatory

requirement, even when using microbial cells in an inactivated form.

Clinical trials conducted thus far indicate a remarkably high safety profile for products containing nonviable cells, particularly when derived from well-established probiotics such as lactic acid bacteria and bifidobacteria. Nonetheless, a case-by-case evaluation is essential when administering these nonviable cells to critically ill patients and susceptible individuals.

Additionally, the use of nonviable microorganisms opens avenues for exploring the potential of those microbes lacking a long history of safe use, such as NGPs. However, their inclusion in food and dietary supplements necessitates regulatory approval from competent authorities following strain-specific safety assessments, as exemplified by the recent approval of pasteurized *Akkermansia muciniphila* MucT as a novel food in Europe [6].

In the future, as our understanding of nonviable microbes and their potential benefits deepens, we can anticipate a growing interest in harnessing their unique properties for various applications in the realm of biotic products.

## Data Availability

No data were used for the research described in the article.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: SG carries out consultancy activities and has received funding for research activities from companies that produce and commercialize probiotic products.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cofs.2023.101105.

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