Review

Bacitracin in the Food Enzyme Subtilisin: Low Levels Do Not Pose an Antimicrobial Resistance Risk

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Bacitracin in the Food Enzyme Subtilisin: Low Levels Do Not Pose an Antimicrobial Resistance Risk

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Abstract

Food enzymes are essential to modern food processing, with many produced industrially through microbial fermentation. However, bacterial production strains can generate secondary metabolites, including trace amounts of antibiotics. Subtilisin, a widely used protease, is commonly produced using *Bacillus paralicheniformis*, a strain known to naturally synthesize bacitracin. This has raised concerns by the European Food Safety Authority (EFSA) regarding the potential contribution of subtilisin preparations to antimicrobial resistance (AMR).This review evaluates published evidence and quantitative data to assess whether to assess whether trace levels in subtilisin preparations pose an AMR risk. First, analytical data indicate that any residual bacitracin in the final enzyme preparation is extremely low and further diluted during food processing, resulting in dietary exposure well below that from permitted sources such as meat and dairy. Second, these concentrations fall far below established thresholds for selective pressure. Third, there is no robust evidence of cross-resistance to medically important antibiotics or of transferable resistance elements associated with bacitracin. Finally, bacitracin resistance genes are already widespread in the environment, including in drinking water, further diminishing the relative impact of trace exposures from food enzymes. Taken together, the evidence does not support the view that trace bacitracin in subtilisin poses a meaningful risk for the development or spread of AMR.

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Bacitracin is a polypeptide antibiotic composed of a mixture of structurally related cyclic peptides [1]. These peptides act primarily against Gram-positive bacteria by disrupting cell wall and peptidoglycan synthesis. Specifically, bacitracin inhibits bacterial cell wall formation by blocking the dephosphorylation of C55-isoprenyl pyrophosphate (also known as bactoprenol pyrophosphate), a lipid carrier responsible for transporting peptidoglycan precursors across the inner membrane [2]. Owing to its antimicrobial properties, bacitracin is widely used in consumer products such as ophthalmic and topical ointments, as well as in cosmetics, for the treatment of minor skin infections.

Subtilisin is a serine protease commonly produced by *Bacillus* species and widely used as a food enzyme for protein hydrolysis [3]. When subtilisin is produced using *Bacillus paralicheniformis*, trace amounts of bacitracin may be co-produced. This has prompted the European Food Safety Authority (EFSA) to evaluate whether the presence of bacitracin in such enzyme preparations poses a significant antimicrobial resistance (AMR) risk.

Bacillus paralicheniformis was formally recognized in 2015 as a species distinct from *B. licheniformis*, based on its ability to produce secondary metabolites such as bacitracin, fengycin, and a lantipeptide [4,5]. As a result, EFSA requires that subtilisin preparations derived from *B. paralicheniformis*, within the broader *Bacillus subtilis* group, undergo additional safety qualifications, including confirmation via whole-genome sequencing (WGS) that the production strain does not synthesize bacitracin.

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EFSA has issued several scientific opinions concerning the safety of subtilisin produced by *Bacillus paralicheniformis* [6–8]. In one assessment involving strain AP-01 (Nagase [Europa] GmbH), bacitracin was not detected in any of the tested enzyme batches. However, the EFSA

panel considered the limit of detection too high to definitively rule out the presence of bacitracin at concentrations potentially relevant to the development of AMR, particularly due to concerns about cross-resistance to colistin, a highest priority critically important antimicrobial [8].

A similar rationale was applied in the evaluation of subtilisin produced by strain DP-Dzx96 (Genencor), in which bacitracin was detected in the enzyme preparation. As a result, EFSA determined that the product could not be considered safe for use in food processing [6].

Likewise, in a third case involving strain LMG S-30155 (ENMEX), EFSA identified genes involved in bacitracin biosynthesis and confirmed the presence of bacitracin in the enzyme preparation, leading to the same safety concern and conclusion [7].

Notably, this precautionary approach was consistently applied across all three cases, regardless of whether bacitracin was detected analytically or inferred from genomic data. However, this position warrants further scrutiny, as it appears to conflict with a substantial body of scientific evidence.

In the following section, I review the available evidence and data to assess whether trace levels of bacitracin in subtilisin preparations pose a meaningful AMR risk. This is supported by the following three key arguments:

1. Extremely Low Exposure Levels and Regulatory Comparisons

Subtilisin is not consumed directly but is used as a processing aid in food production. EFSA estimated dietary exposure to the enzyme based on Total Organic Solids (TOS) intake among high-consuming population groups. Although subtilisin preparations may contain trace amounts of bacitracin, the enzyme undergoes dilution by several orders of magnitude during food manufacturing. Notably, EFSA's risk assessment does not appear to have fully accounted for this dilution factor.

As a result, the levels of bacitracin potentially entering the food chain are substantially lower than the maximum residue limits established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [9] and by EU Regulation 37/2010 on pharmacologically active substances in foodstuffs of animal origin [10]. These international standards permit bacitracin residues in foods such as milk, meat, and eggs at concentrations significantly higher than those that could arise from the use of subtilisin in food processing. (See Table 1 for a comparison of bacitracin concentrations across regulatory thresholds and environmental sources).

2. Trace-Level Concentrations Are Below Selection Thresholds

The minimum inhibitory concentration (MIC) of bacitracin is approximately 2000 ng/ml [11]. In contrast, EFSA cites a 'Minimal Selective Concentration' (MSC) of 8 ng/mL, derived from a theoretical model that is not specific to bacitracin. This value lacks practical relevance for food safety assessments, as residual bacitracin levels in the final subtilisin preparations are at least 4000-fold lower than this MSC.

Moreover, polypeptide antibiotics like bacitracin are unlikely to induce secondary resistance when present at subinhibitory concentrations [12]. Consequently, the development of AMR under these conditions is highly improbable.

3. Lack of Evidence for Cross-Resistance or Transferability

EFSA has expressed concern that bacitracin may contribute to cross-resistance to colistin, referencing the emergence of plasmid-mediated colistin resistance (*mcr-1*) in *Enterobacteriaceae* [13]. However, the cited study reported *mcr-1* in the context of general antibiotic use in livestock and did not identify bacitracin as a selective agent. The high-dose therapeutic use of multiple antibiotics in

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animal production is not comparable to the trace bacitracin levels present in food enzyme preparations, making selection for *mcr-1* under these conditions highly improbable.

Over more than five decades of use as a feed additive and growth promoter, bacitracin has not been linked to increased resistance in clinically relevant pathogens such as *Clostridium perfringens*, staphylococci, or streptococci [14]. Furthermore, multiple studies have found no evidence of cross-resistance between bacitracin and medically important antibiotics used in human therapy [15–19]. Sub-therapeutic exposure to bacitracin has also been shown not to increase the frequency of multidrug resistance [20,21].

In addition, current data suggest that bacitracin resistance is not readily transferable via plasmids or other mobile genetic elements. Several investigations have failed to demonstrate such transferability [19,22], and some studies even indicate that bacitracin may inhibit plasmid transfer, thereby reducing the incidence of resistance to unrelated antibiotics [23,24].

Additional Considerations

Bacitracin also confers several secondary benefits, including reduced gut inflammation, enhanced absorption of vitamins and nutrients, inhibition of toxin production, increased phagocytic activity, and improved tolerance to physiological stress. Its low toxicity has supported its use in clinical settings, such as in skin grafting procedures [25], as well as in oral treatments for colitis and *Clostridium difficile*-associated diarrhea [26].

Further supporting the evidence outlined above, bacitracin occurs naturally in various environmental compartments, including groundwater, surface water, and soil—often at concentrations significantly higher than those found in subtilisin preparations [27,28]. Notably, no adverse health effects have been associated with such environmental exposure. This broader context reinforces the conclusion that trace levels of bacitracin in food enzyme preparations are unlikely to pose a risk to public health.

As illustrated in Figure 1, data on the abundance of bacitracin resistance genes (ARGs) in drinking water samples confirm their frequent detection without any documented association with increased AMR risk [27,28].

In summary, this manuscript demonstrates that:

- The levels of bacitracin present in subtilisin preparations do not raise safety concerns.
- Antimicrobial resistance (AMR) cannot develop at bacitracin concentrations below its minimum inhibitory concentration (MIC); levels in subtilisin are nearly 60-fold below the MIC and approximately 4000-fold below the minimal selective concentration (MSC) referenced by EFSA.
- Bacitracin does not induce cross-resistance with human medicinal antibiotics or other marketed antimicrobials.
- The transferability of bacitracin resistance via plasmids or other mobile genetic elements is unlikely.
- Environmental concentrations of bacitracin in groundwater and soil are significantly higher than those found in subtilisin preparations.
- Acceptable residue limits for bacitracin in meat, eggs, and milk, as established by regulatory bodies, are far higher than the trace levels present in food processed with subtilisin.
- Therefore, the likelihood that the use of subtilisin in food processing contributes to AMR development is considerably lower than other existing exposure pathways.

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Table 1. Comparative Bacitracin Levels in Food and Environmental Sources vs. Relevant Thresholds.

Source or Threshold	Bacitracin Concentration (ng/mL or ng/g)	Reference / Note
Subtilisin-processed food (estimated)	≤ 0.002	4000× below MSC; 60× below MIC
Minimal Selective Concentration (MSC)	8	Hypothetical threshold (EFSA)
Minimum Inhibitory Concentration (MIC)	2000	Effective bacterial inhibition [11]
EU MRL in milk (‡)	50,400	1.2 IU/mL × 42 (†)[9,10]
EU MRL in meat	29,400	0.7 IU/g × 42 [9,10]
EU MRL in eggs	201,600	4.8 IU/g × 42 [9,10]
Groundwater/Drinking water (natural presence)	Up to 10	Detected environmental levels [27,28]
Animal feed (therapeutic use)	100,000–200,000	Used as additive [14]

‡ MRL (Maximum Residue Limit): the highest legally permitted concentration of a veterinary drug residue in food, established to ensure consumer safety. † Conversion based on JECFA: 1 IU of bacitracin = 42 ng.

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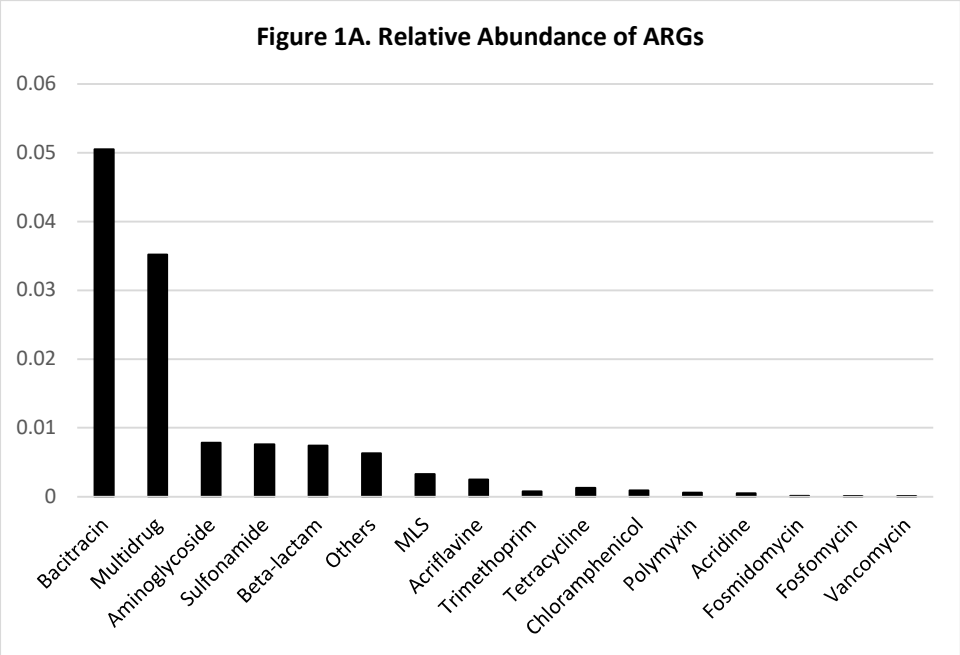

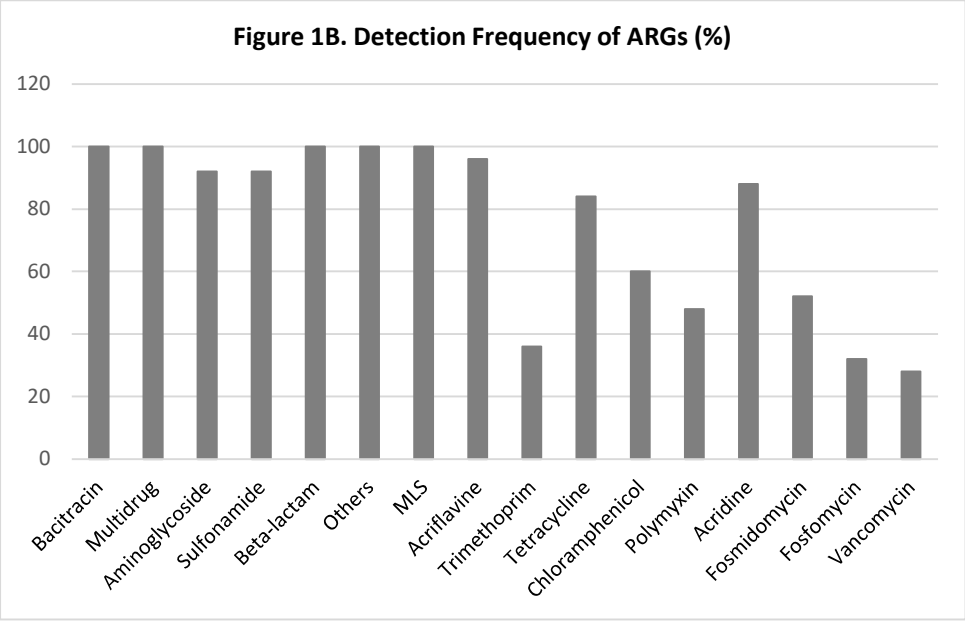

Figure 1. Abundance and Detection Frequency of Antibiotic Resistance Genes (ARGs) in Drinking Water Samples. Top (A): Average relative abundance of ARG types, expressed as copies per average bacterial cell (capc). Bottom (B): Detection frequency of each ARG type across 25 drinking water samples. Bacitracin resistance genes are the most abundant and universally detected, while other ARGs vary in both abundance and prevalence. Data adapted from [28], with modifications to the presentation and graph style.

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