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Tiny but mighty: bacterial membrane vesicles in food biotechnological applications

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Abstract

Membrane vesicle (MV) production is observed in all domains of life. Evidence of MV production accumulated in recent years among bacterial species involved in fermentation processes. These studies revealed MV composition, biological functions and properties, which made us recognize the potential of MVs in food applications as delivery vehicles of various compounds to other bacteria or the human host. Moreover, MV producing strains can deliver benefits as probiotics or starters in fermentation processes. Next to the natural production of MVs, we also highlight possible methods for artificial generation of bacterial MVs and cargo loading to enhance their applicability. We believe that a more in-depth understanding of bacterial MVs opens new avenues for their exploitation in biotechnological applications.

Introduction

Production of extracellular membrane vesicles (MVs or EVs) is a conserved phenomenon in Eukarya, Archaea and Bacteria [1,2•]. Being lumen-containing spheres enclosed by lipid-bilayers, MVs are identified in different sizes ranging from 20nm to 500nm in diameter, and with various cargo components, including proteins, DNA, RNA, signalling molecules, etc. Moreover, MVs are considered to have essential functions in cellular life, i.e., cell-to-cell communication, competition, survival or stress response of cells [3•–6].

In bacteria, MV production was first observed in Gram-negatives over fifty years ago, and there have been an increasing number of studies performed on these MVs in recent decades [2•]. Gram-negative bacterial MVs are often referred to as outer-membrane vesicles (OMVs), since they are derived from the outer membrane of the bacteria via budding processes [7]. In contrast, due to the presence of a thick cell wall in Gram-positive bacteria, MV production was expected to be absent. This assumption resulted in a three-decade delay in the discovery of Gram-positive MVs compared to their Gram-negative counterparts, although in recent years the evidence of MV production in Gram-positive bacteria accumulated rapidly [2•,3•].

Recent advances in the study of MV production in Gram-positive bacteria are particularly interesting for the food biotechnological field. The Gram-positive lactic acid bacteria (LAB) are key players in various food and feed fermentation processes as starter cultures, probiotics and producers of vitamins [8,9]. Among these, evidence has so far been collected for MV production from *Lactococcus lactis* (S. Alexeeva *et al.*, unpublished) and *Lactobacillus plantarum* [10••]. *Bacillus subtilis* was shown to secrete MVs as well [11], and certain strains of *B. subtilis* are involved in the production of fermented soybeans known as “natto” while boosting this food product with high levels of vitamin K2 [12]. Among Gram-positive Actinobacteria, species of the genus *Bifidobacterium* were associated with probiotic effects as well as with MV production [13••].

To date, comprehensive studies have been mostly focusing on MVs produced by pathogenic bacteria [14•,15–22]. One of the best-identified functions of MVs is indeed associated with pathogenesis, where they serve as the vehicles for delivering virulence factors and toxins to the host cells [3•,5,22]. As MVs are able to trigger responses of the host immune system, the application of vaccines against meningitis was realized using OMVs derived from the Gram-negative bacterium *Neisseria meningitidis* [23]. The potential of vaccine development based on Gram-positive MVs was recognized as well: vaccination with MVs derived from *Clostridium perfringens*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis* and *Staphylococcus aureus* were demonstrated to be effective in mice models against infections by respective bacteria [24-27]. However, the possibility of applying microbial MVs in other areas of biotechnology

remains largely unexplored. In this paper, we provide an overview on current understanding of microbial MVs, with a focus on potential applications of MVs from food-associated bacteria.

Natural functions and biotechnological applications of bacterial MVs

The composition of various MVs has been studied and has supported the formulation of hypotheses regarding their natural function. Although so far the studies of Gram-positive MVs are not as extensive, the available data already suggest that Gram-positive MVs have similar roles to their Gram-negative counterparts [28]. This knowledge served as a basis for us to propose potential applications of microbial MVs in food biotechnology, in which Gram-positive bacteria are intensively involved (Figure 1).

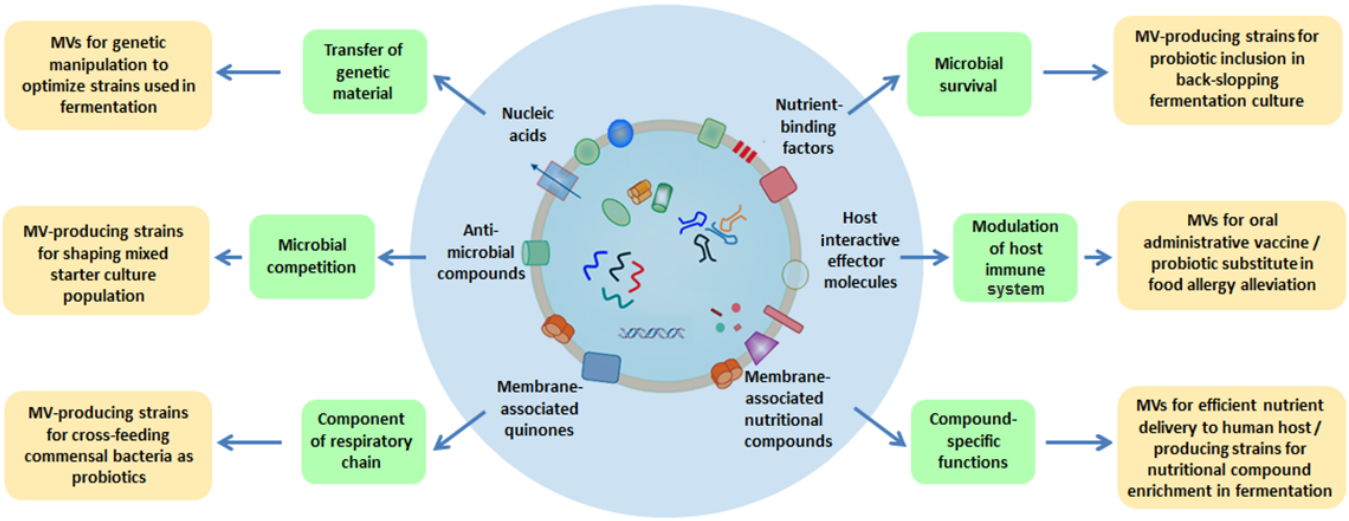

Figure 1. Microbial MV cargos, functions and potential applications. *Examples of MV cargos (blue circle), corresponding natural function of the MV carrying the cargo (green box) and proposed applications of the MVs or MV-producing strains (orange box) are presented. MV scheme modified from EVpedia* [29••,30].

Nucleic acids, including chromosomal DNA, extracellular DNA, plasmids, phage DNA, rRNA, tRNA, mRNA and intragenic RNA species are frequently found as cargo in bacterial MVs (Figure 1) [16,31–34]. MVs protect the nucleic acids from degradation by nucleases and are proposed to facilitate horizontal gene transfer in the microbial community. Gene transfer by MVs within or between species was demonstrated in various genera of Gram-negative bacteria and certain Gram-positive species [35,36]. As only a number of genes were retrieved from the MVs, the incorporation of nucleic acids into MVs might be a selective mechanism instead of a random process [20], although extra assessments are needed for a conclusive statement. In this regard, MVs may provide the opportunity to deliver designed or natural (large) DNA or RNA molecules to recipient bacteria in a controlled manner. Similarly, membrane-derived vesicles have been considered as novel tools for delivering therapeutic genome editing elements (i.e., CRISPR/Cas cassettes) to human cells [37]. Interestingly, natural MV-mediated gene transfer is not considered to result in genetically modified organisms (GMOs) under current legislation (Directive 2001/18/EC [38]), and is therefore especially interesting for food biotechnological applications. MV producing strains with favourable traits could be used to naturally transfer nucleic acids to recipient bacteria, providing them resistance to environmental stresses or bacteriophages, enhanced productivity etc., thus optimizing strains used in starter cultures and other fermentation processes (Figure 1).

In addition to nucleic acids, MVs can also carry other cargo that as well plays a role in microbial competition, survival and fitness increase in a microbial community (Figure 1). Antimicrobial compounds and bacteriolytic enzymes including autolysins and extracellular bacteriolytic enzymes, carried by MVs of particular bacterial species are suggested to exert an inhibitory effect on sensitive co-existing bacteria or fungi [14•,39,40]. MVs derived from several species were found to contain factors such as iron-binding

proteins, contributing to increased survival of bacteria under nutrient limiting conditions [41,42]. A few cases also revealed a protective effect of MVs that carry enzymes which degrade certain antimicrobial compounds [3•,43,44]. This type of protection can also benefit other bacterial species in the same community. Moreover, bacterial cross-feeding through trafficking of membrane-associated compounds, i.e., vitamin K2 (menaquinone), has been observed between *L. lactis* and another species [45]. In bacteria, menaquinones function as electron carriers in the membrane-embedded respiratory electron transport chain [46]. As a result of menaquinones cross-feeding, the respiratory chain was completed in the recipient bacteria resulting in stimulated growth [45]. Although the cross-feeding mechanism was not completely elucidated in this particular case, MVs may indeed serve as vehicles for microbial cross-feeding of compounds, especially the hydrophobic, conceivably membrane-associated ones.

These ecological roles of MVs highlight the interests of using MV producing bacterial strains in fermentation starter cultures or as probiotics. In stably-maintained, multi-strain starter cultures, strains producing MVs with an inhibitory effect on co-cultured bacteria may suppress the undesirable dominance of one single strain which in term prevents clonal sweeps of strains present at low abundance, thereby ensuring compositional diversity of the starter cultures. Compositional diversity of a microbial community is considered to be the basis of the functional stability (robustness) of the mixed starter culture in face of environmental fluctuations [47]. Moreover, the bacteriolytic enzymes carried by MVs may play a role in bacterial lysis and improved aroma formation during cheese ripening, which adds to the interest of applying MV-producing strains in starter cultures. It may also be desirable to include probiotic strains into well-established fermentation cultures generated by back-slopping. However, newly added strains in such complex microbial communities are often excluded shortly after addition to the culture, a process which is explained by the competitive exclusion principle [48]. The proposed role in competition and survival served by MVs suggests that probiotic strains producing MVs outstanding in these features may establish themselves more easily in complex fermentation cultures. The role of MVs as vehicles for cross-feeding to commensal bacteria also addresses the probiotic value of the MVs and their producing strains, as they may promote the fitness of certain commensals in the gut microbiota.

Besides microbe-microbe interactions, MVs also play a role in the interaction between gut-inhabiting bacteria and their vertebrate host. Bacterial extracellular MVs have been shown to interact with the plasma membrane of host cells and deliver effector molecules (Figure 1) [49,50]. In pathogenic bacteria, these effector molecules are often associated with virulence and can trigger host immune responses. Based on this phenomenon, the potential of developing vaccines from bacterial MVs has indeed been recognized. Moreover, it was demonstrated that MVs produced by the probiotic *Bifidobacterium longum* can penetrate through intestinal epithelial cells of the host and alleviate food allergy responses in a mouse model [13••]. The family 5 extracellular solute-protein carried by the MVs selectively induced apoptosis of mast cells without compromising T-cell immune response. Another study showed that *L. plantarum*-derived MVs up-regulated the expression of host defence genes and provided the host with protection against pathogenic bacteria in *Caenorhabditis elegans* and human cells [10••]. These findings shed new lights on the extra probiotic value that MVs add to the producing bacteria, and even suggest the possibility of using MVs as a substitute for bacteria to achieve certain probiotic effects. Given the fact that MVs offer protection and effective transport of functional molecules, and are able to penetrate intestinal epithelial cells and subsequently interact with the host immune system, the feasibility to vaccinate human or livestock via oral administration of bacterial MVs seems high. Besides, MVs may also serve as vehicles for efficient delivery of nutritional compounds to the host (Figure 1). For instance, vitamin K2 (menaquinone) is uniquely produced by bacteria and functions in the human body as a carboxylase co-factor for maturation of proteins involved in many vital physiological processes [51]. The natto fermentation bacterium *B. subtilis*, certain strains of lactic acid bacteria and propionibacteria are among the producers of vitamin K2 [12,52,53], which offers the opportunity for natural vitamin K2 fortification of fermented food products. Menaquinones are hydrophobic compounds which accumulate in the bacterial cell membrane. Although experimental evidence is still required, incorporation of these compounds in the MVs can be speculated. In that case, MVs could have advantages over the whole-cell bacteria in terms of delivering vitamin K2 to the host, as the intestinal absorption may be achieved without the need to first overcome the bacterial cell envelope barrier and solubilize the vitamin via the lipid digestion route. Therefore, MVs carrying nutritional compounds could be an ideal formulation to include in fermented foods or food supplements to further promote the health benefits.

MVs may indeed incorporate cell membrane-associated compounds that are of interest for human health (e.g. vitamin K2, certain fatty acids) or for favourable food product traits (e.g. hydrophobic aromatic

compounds) (Figure 1). In this context, bacterial MV production might provide chances for *in situ* enrichment of fermented foods or for biotechnological production methods.

Methods of bacterial MV production

With many potential applications of bacterial MVs proposed, efficient production of MVs with the desired functionality could be one of the major challenges in practice. Indeed, isolation and characterization of MV producing bacterial strains, understanding of the mechanisms governing natural MV production, identification of MV components and interpretation of MV functions are prerequisites to successful applications of naturally secreted bacterial MVs or the producing strains (Figure 2A). Studies have been performed to reveal conditions that encourage the secretion of MVs from Gram-negative bacteria [22,54], and the influencing factors for MV production from Gram-positive bacteria are being recognized gradually [11,17,28]. However, it should be realized that artificial MV production evades the limitations determined by the biological properties of bacteria in terms of MV yield and opens doors to even more opportunities (Figure 2B-D). Artificial MV production discussed in this context can be achieved at different levels: the artificial creation of bacterial cell-derived vesicles and the artificial loading of functional compounds into the MVs. Cell disruption due to osmotic pressure (after enzymatic removal of cell walls) or mechanical force (treatment with French pressure cell or sonication) [55] generates membrane fragments that spontaneously form vesicles by hydrophobic interaction. When the desired compounds are naturally accumulated in the bacterial cell membrane or intracellular space, artificial MVs can be directly collected after cell disruption: the membrane associated compounds are carried by the vesicle membrane, and the intracellular compounds are engulfed in the lumen (Figure 2B). When the desired compounds are not naturally present in the bacteria, artificial loading of membrane-associated or lumenal compounds in addition to cell disruption is required to generate the MV with desired effect (Figure 2C and D). Notably, different compounds can be loaded in combination to the MVs to achieve synergy.

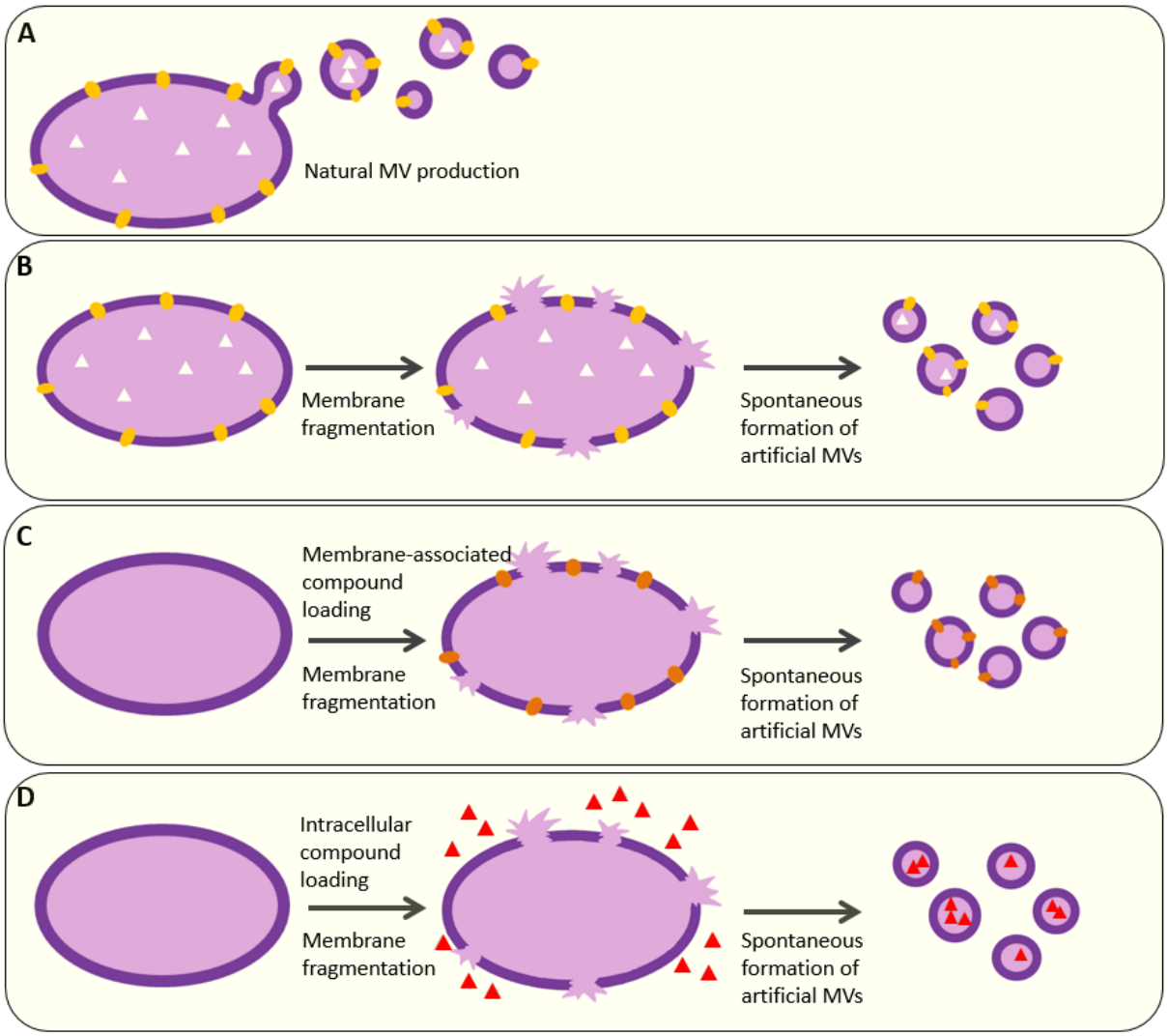

Figure 2. Scheme of different MV production methods. *A) Natural MVs produced by bacteria with desired lumenal cargos (white triangles) or membrane-associated cargos (yellow ovals). B) Artificial MVs produced by fragmenting bacteria with desired lumenal or membrane-associated cargos. C) Artificial MVs produced by fragmenting bacteria and artificially loading membrane-associated cargos (orange ovals). When hydrophobic compounds are added to the biomass, they adsorb or incorporate into the membrane. D) Artificial MVs produced by fragmenting bacteria and artificially loading lumenal cargos (red triangles). When water-soluble compounds are added to the biomass, they get encapsulated upon resealing of the membrane fragments. Bacterial cell membrane is depicted as thick purple line.*

Concluding remarks

In this opinion paper, we propose diverse applications of bacterial MVs in the field of food biotechnology based on their natural functions or properties. These applications include genetic engineering tools for strain optimization, agents for shaping starter culture communities, substitutes for probiotics, orally administered vaccines, delivery vehicles for nutritional compounds to the host and the use of natural enrichment of valuable membrane-associated compounds in fermented foods or food supplements. Future applications will also profit from the information contained on EVpedia, a community web source for prokaryotic and eukaryotic extracellular vesicles research [29••,30]. We foresee a bright future for applications of these tiny, but mighty, nano-sized MVs.

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