Good things come in small packages - delivery of vitamin K2 to human cells by extracellular vesicles from *Lactococcus cremoris*

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The "good things" - vitamin K2

Vitamin K2 (menaquinone, MK-n) is a lipophilic vitamin located the cell membranes of many species of bacteria and is essential for human health as a carboxylation co-factor. Beneficial effects in relation to human cardiovascular and bone health have also been associated with vitamin K2 intake.

The long-chain forms of vitamin K2 show high bioavailability for target tissues in the human body. However, the strong lipophilicity of long-chain vitamin K2 forms poses challenges to their uptake by target cells of the human host to achieve desired biological function.

The "small packages" - extracellular vesicles

Extracellular membrane vesicles (EVs) are nano-sized, lipid bilayer-enclosed spheres secreted by members of all domains of life. Bacterial EVs carry diverse cargo molecules, pointing to various roles of bacterial EVs in microbial ecophysiology, cellular signaling and communication, and interactions with the human host.

A wide range of species well-known as beneficial commensal bacteria produce EVs, including *Lactococcus cremoris*. The potential of applying these bacterial EVs in contribution to human health has gained attention in recent years.

Lactococcus cremoris has a long history of safe use in fermented foods and produces mainly long-chain vitamin K2 (in the form of MK-9 and MK-8), providing opportunities for vitamin K2 enrichment of food. In this study we used an *in vitro* assay to show that EVs produced by *L. cremoris* can deliver this lipophilic membrane-bound vitamin to human cells.

Lactococcal EVs production

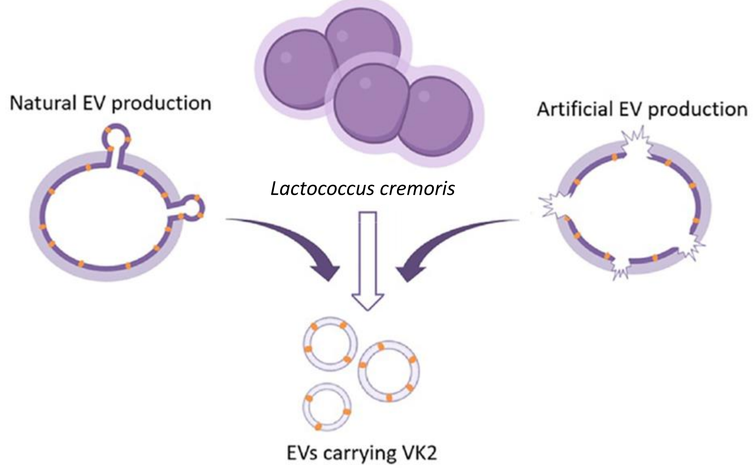


Figure 1. Extracellular vesicles (EVs) were produced from *L. cremoris* strains naturally or artificially. Vitamin K2 (orange ovals) accumulates in the cell membrane of this bacterium and is expected to be present in EVs.

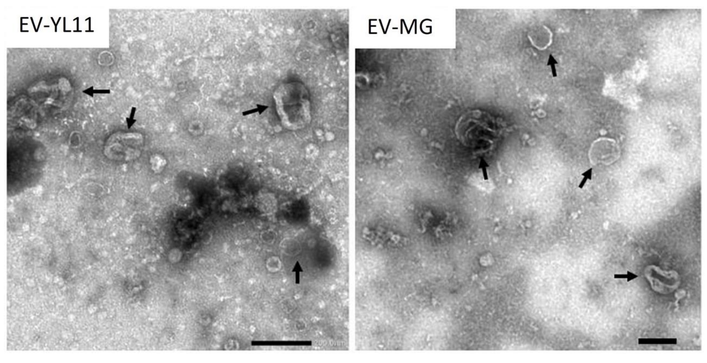


Figure 2. Lactococcal EVs visualized by transmission electron microscopy. Both naturally produced EVs (YL11) and artificially produced EVs (MG) showed typical size and morphology of EVs. Scale bars 200 nm

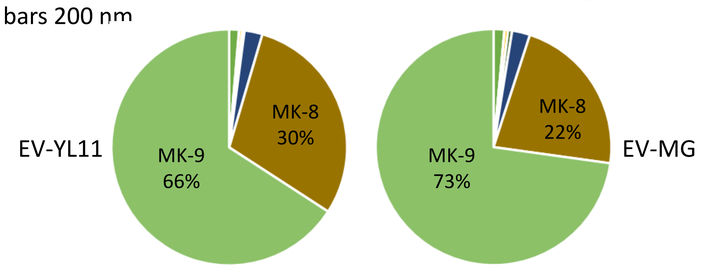


Figure 3. Presence of vitamin K2 in Lactococcal EVs was confirmed by chemical analysis. The major forms (more than 95%) of vitamin K2 in Lactococcal EVs are the long-chain menaquinones MK-9 and MK-8.

Lactococcal EVs deliver VK2 to human cells

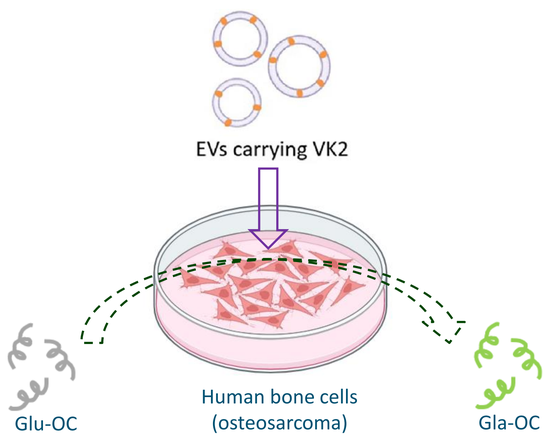


Figure 4. Illustration of the *in vitro* vitamin K2 delivery assay. Lactococcal EVs carrying vitamin K2 were applied to the human osteosarcoma cell line, and the carboxylation status (Glu, undercarboxylated form; Gla, carboxylated form) of bone protein osteocalcin (OC) was the indicator for delivery of vitamin K2.

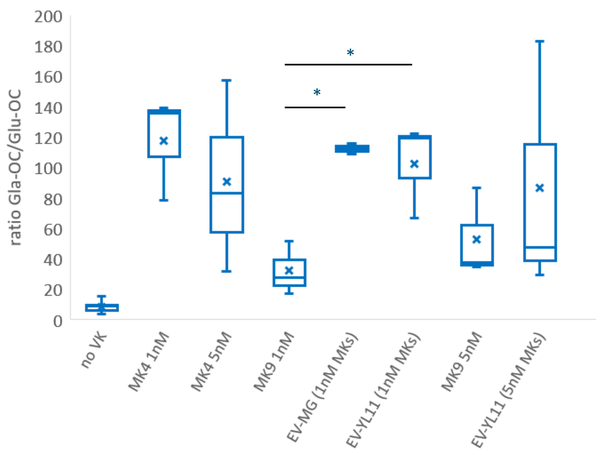
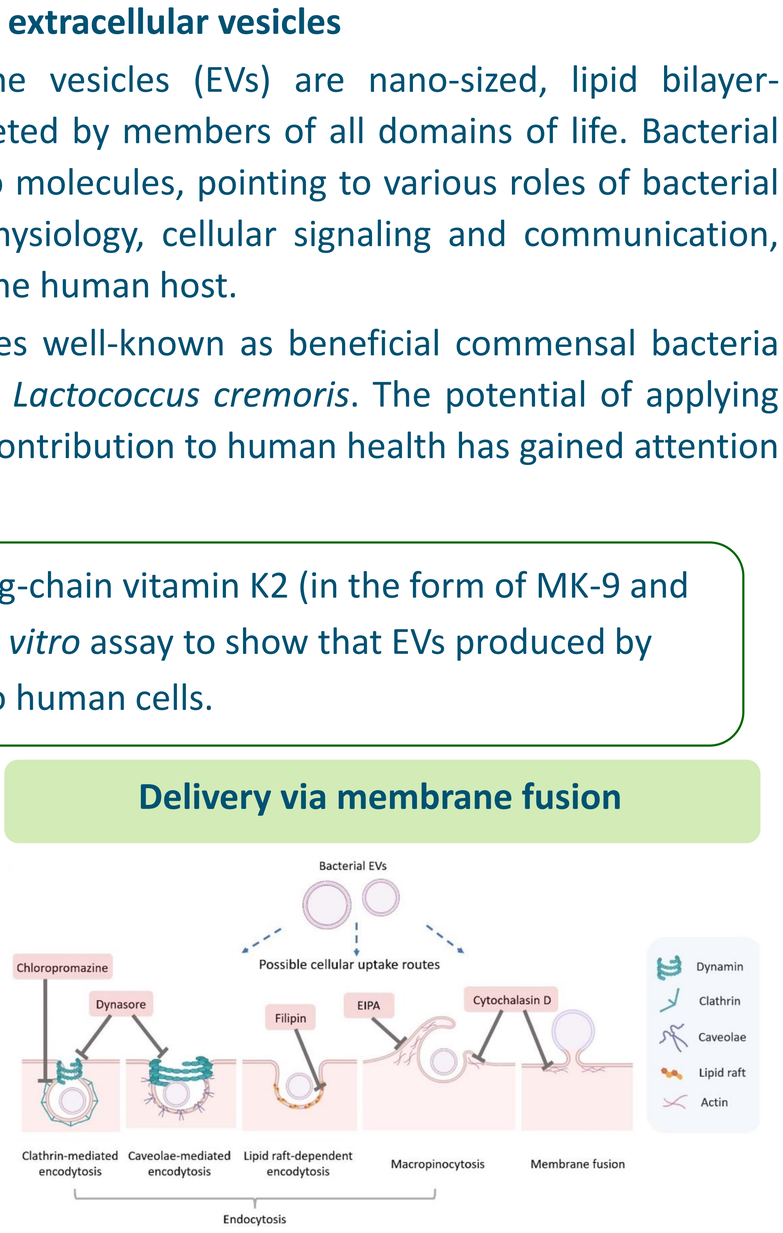


Figure 5. Carboxylation status of OC upon vitamin K2 addition. When vitamin K2 was applied to the osteosarcoma cell line in solvent (short-chain MK-4 and long-chain MK-9, each as pure chemical standard dissolved in ethanol) or carried by EVs (MK-9 and MK-8 as the main forms) in 1 nM and 5 nM, significant ($p < 0.05$) increases in the ratio of Glu-OC and Gla-OC were observed, indicating successful vitamin K2 delivery and functionality. Notably, when long-chain vitamin K2 was applied at 1 nM, the EV-carried form resulted in higher efficiency than the solvent-dissolved form (*, $p < 0.05$).

Delivery via membrane fusion



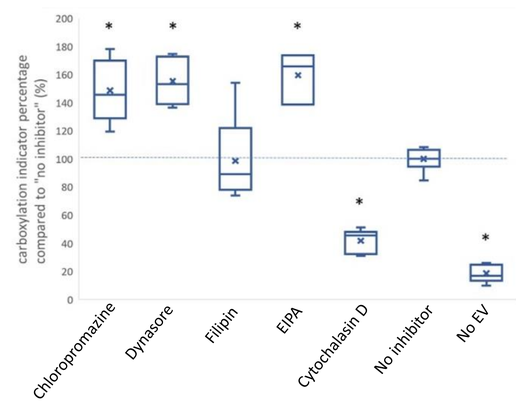


Figure 7. The uptake of bacterial EVs in human cells is blocked by specific inhibitors. The values of Gla-OC/Glu-OC were used as indicators for EV-mediated vitamin K2 delivery, and the indicator levels from samples with EV-MG (10 nM vitamin K2) but without inhibitors (no inhibitor) were set as the reference level (*, $p < 0.05$).

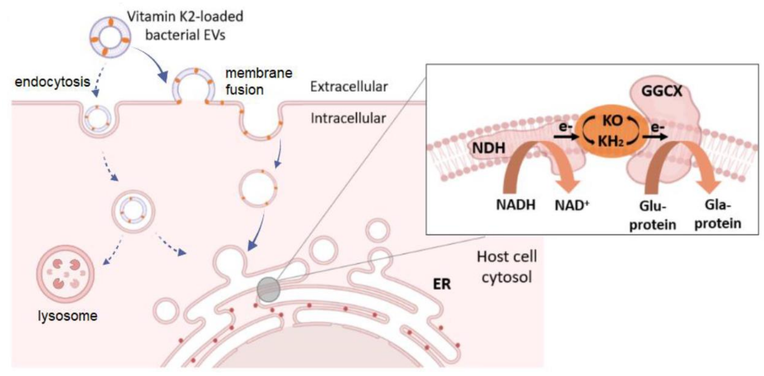


Figure 8. Proposed model for EV-mediated vitamin K2 delivery via membrane fusion to the ER membrane and its action as cofactor in the carboxylation of osteocalcin.

Investigation on EVs produced by bacteria with GRAS status that are key players in food fermentations and insights in their role in delivery of bioactive nutritional compounds to the human host will further contribute to our understanding of their role in human health.

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