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Crystal Ball

Extracellular vesicles in food biotechnology

Pilar Morales, Ana Mencher, Jordi Tronchoni[†] and Ramon Gonzalez[*]
Instituto de Ciencias de la Vid y del Vino (CSIC, Gobierno de la Rioja, Universidad de La Rioja), Finca La Grajera, Carretera de Burgos, km 6, Logroño, La Rioja 26071, Spain.

The purpose of this article is to bring attention to the potential importance of extracellular vesicles (EVs) of microbial origin in the production of fermented foods. We anticipate that understanding the role of the EVs in these processes will contribute to the development of new tools in food biotechnology. The reasons that lead us to make such claim are given as: (i) the production of EVs is a widespread feature in all domains of life; (ii) a growing number of functions are being identified for EVs, the most prominent one being biological communication; (iii) the study of EVs has become important for the understanding of biological processes and as a diagnostic and therapeutic tool in the biomedical sciences; (iv) many fermented foods require the activity of microbial consortia involving multiple species; (v) an increasing number of studies are showing that interactions between microorganisms may be relevant in the development of fermentation processes and would therefore be of biotechnological interest (Curiel *et al*., 2017; Tronchoni *et al*., 2017; Conacher *et al*., 2019).

Extracellular vesicles are structures that have been described in all domains of life. They are surrounded by a lipid bilayer and show a broad range of sizes, from 20 to 500 nm. EVs can have diverse biogenesis routes (Colombo *et al*., 2014) and, most importantly, they can be carriers of molecules with high information capacity, such as proteins, and diverse types of RNAs, including notably miRNAs (Rodrigues and Casadevall, 2018). In humans, EVs have been shown to participate in a wide variety of biological processes, from immunomodulation to cancer development. They are also known to be involved in interactions between pathogenic microorganisms and their host animals or plants. See Bielska *et al*. (2019) for an overview on the most recent advances in the field of EVs. EVs are specifically involved in communication (intra- and interspecific) between living cells in many different contexts (Raposo and Stahl, 2019).

On the other side, with some remarkable exceptions such as beer and white bread, most of the fermented foods consumed around the world are the result of the activity of consortia that incorporate multiple microbial species (and strains), with yeasts and lactic acid bacteria as the main players in most cases. These consortia can be spontaneous, or more or less domesticated, as in the case of kefir or sourdough (Lhomme *et al*., 2015; Walsh *et al*., 2016). Both main microbial players and associated microbiota (including potential spoilage microorganisms) might get involved in microbial interactions during food fermentation. These interactions are classified according to different criteria by different authors. But, for the purpose of this article, we will distinguish only between targeted and untargeted interactions. Untargeted interactions are the consequence of cellular metabolic activities that would take place in the same way in pure cultures. The simplest example is competition for nutrients that are found in limited amounts. Other examples are, the toxic effect on other species of some metabolic end products, such as ethanol or CO_2, or the constitutive production of unspecific toxic substances, unless its production is enhanced in response to other species. In contrast, targeted interactions involve communication between cells. For such interactions to exist there should be a recognition mechanism; that is, a cell perceives the message (presence) of cells from other species (or the same one, as in the case of quorum sensing). This should be followed by integration of the information mediated by, for example, transcriptional regulation cascades. And they will result in a physiological response from the recipient cell. Interspecies cross-feeding might be targeted or untargeted, depending on whether specific signals for the recognition of the other partner are involved. The targeted mechanisms of interaction are intensely studied in parasitism, pathogenesis and some examples of symbiosis, but are much less known in the

Received 7 August, 2020; accepted 9 August, 2020.
*For correspondence. E-mail rgonzalez@icvv.es; Tel. +34 941 89 49 80; Fax +941 89 97 28.
†Present address: Universidad Internacional de Valencia, Valencia, Spain.
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case of weaker interactions between microorganisms sharing the same environments.

There are often methodological difficulties in distinguishing between targeted and untargeted interactions. In the area of food fermentation, untargeted mechanisms of interaction between different microorganisms have often been described, but there are few examples of targeted interactions (Conacher *et al*., 2019). The clearest examples of targeted interactions are those involving physical contact between cells, such as co-aggregation, biofilm building, or contact-dependent killing, that some authors describe as 'direct' interactions (Rossouw *et al*., 2015; Pérez-Torrado *et al*., 2017). However, the molecules or structures that mediate many targeted interactions are often poorly or not characterized. We consider that, in order to fully understand the interactions between the different microorganisms involved in food fermentation, it is necessary to focus on both the targeted and untargeted interactions. And as for the targeted mechanisms of interaction, it is necessary to fill the gaps in knowledge about molecules and cascades mediating intra- and interspecific recognition.

In this context, it seems reasonable to expect that EVs will play an important role in the mechanisms of interaction between different microbial species, as well as between strains of the same species. To confirm this hypothesis, it would be necessary to be able to isolate EVs produced by fermentation microorganisms, characterize their properties and composition and demonstrate an impact of the isolated vesicles on the behaviour of cells of a recipient strain (Fig. 1). In fact, there are already some reports of EVs produced by yeasts and lactic acid bacteria of food interest (Dean *et al*., 2019; Mencher *et al*., 2020). The case of lactic acid bacteria has been more studied, especially in the context of probiotic activities (also a form of biological interaction), which on several occasions were associated with the production of EVs (Liu *et al*., 2020). This includes those released by lactobacilli isolated from kefir (Seo *et al*., 2018), a clear example of a stable consortium of microorganisms involved in a food fermentation process. Yeast EVs have been studied mainly in species pathogenic to humans (Gil-Bona *et al*., 2015; Brown *et al*., 2015). *S. cerevisiae* has provided very interesting results for understanding the mechanisms of EV biogenesis in yeasts (Oliveira *et al*., 2010; Winters *et al*., 2020), although it has been relatively under exploited as a model system in this context. More recently, EVs have been isolated under winemaking-like conditions from six wine yeast species, including *S. cerevisiae* (Mencher *et al*., 2020). In addition, there are a number of studies on yeast-yeast interactions that address the requirement for physical contact of the cells, using double-compartment culture devices separated by semi-permeable membranes. Among them, we can find either works that confirm contact is necessary or works that conclude the opposite (Renault *et al*., 2013; Taillandier *et al*., 2014; Wang *et al*., 2015). These differences can be due to many factors, such as the species and strains of yeast used, or the lack of standardization in experimental conditions and cultivation devices. However, none of these designs take into account the possibility that EVs may be involved in the interaction. It cannot be ruled out that, in some instances, a requirement for transfer of EVs might have been mistaken as a requirement for cell-to-cell contact. Note that even when relatively large pore sizes are used, the diffusion of EVs may be restricted by the membranes being used. Similar experimental designs, but taking into account EVs, and setting appropriate controls, could also shed light on their role in some yeast interactions taking place during fermentation processes.

There is an undeniable academic interest in knowing the role of EVs in the interactions between fermentative microorganisms. For example, the analysis of EV's macromolecular composition (miRNAs, proteins, lipids and other metabolites) and of the physiological responses of yeast cells to EVs might unveil novel intracellular signalling systems, or at least new functions for those already known. This should improve our understanding of the ecology of food fermentations, whether they are spontaneous, use established communities (kefir, yoghurt, sourdough), or use *de novo* assemblies, with selected microbial strains. All this knowledge could contribute to the design of new microbial consortia, optimized for specific applications. Beyond food fermentations, it is likely that these studies will also have an impact on other industrial processes, driven by mixed starter cultures (combinations of microbial strains and species). Certainly, the study of EVs is already becoming relevant in the understanding of the probiotic activity of various microorganisms (Seo *et al*., 2018).

We can venture that the analysis of the EVs isolated from fermentative processes will probably provide relevant information on their status and dynamics. The analogy would be the use of EVs isolated from biological fluids as diagnostic markers (Lianidou and Pantel, 2019). This information would be complementary to others such as metagenomic analysis in its different forms. To be able to interpret and exploit this type of data we still need gaining much more knowledge about the EVs from pure microbial cultures and, in a second step, their binary combinations (Fig. 1). Therefore, the short-term expectation is to increase this body of knowledge by getting a growing number of research groups involved on the study of microbial interactions in fermented food processing and paying

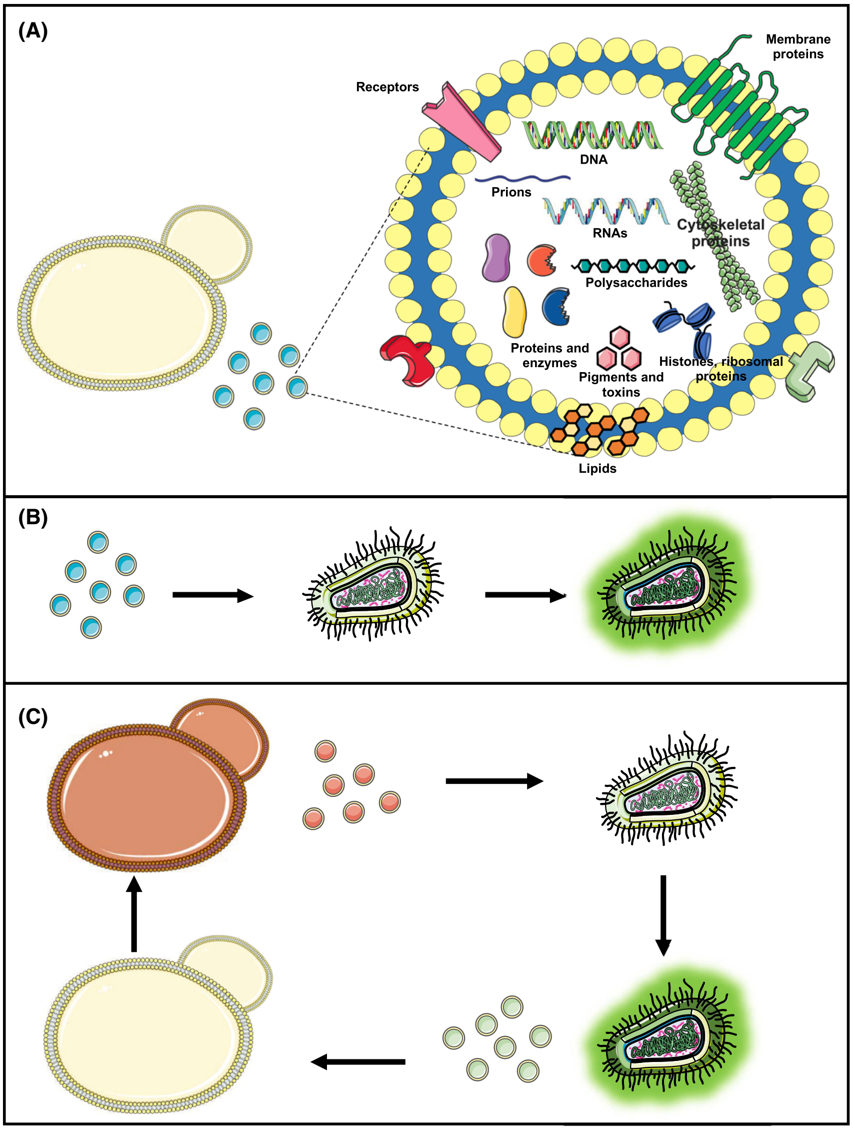

Fig. 1. Different research steps towards understanding the role of EVs in food fermentation.
A. Study of EVs production and composition from pure cultures.
B. Analysis of the biological impact of EVs on other microorganisms found in food fermentation.
C. Study of the reciprocal impact of co-culture (binary combinations) on EVs production and composition by food microorganisms.

attention to the potential impact of EVs on their experimental results.

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