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Current Opinion in Food Science

Molecular and technological insights into the aerotolerance of anaerobic probiotics: examples from bifidobacteria

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Probiotics are live microorganisms that confer a health benefit on the host when administered in adequate amounts. Toxicity induced by molecular oxygen significantly affects the viability of probiotic bacteria during biomass generation, product manufacturing and storage also constitute a major challenge for their inclusion in dairy products. Oxygen toxicity is particularly critical in the case of bifidobacteria, which are strict anaerobes. This mini-review aims at compiling the knowledge on the response mechanisms, mainly in this bacterial group, focused on counteracting the deleterious effects of oxygen on probiotic cells. The effect of other technological and microbiological ways to attenuate oxygen toxicity, such as milk deaeration, microencapsulation or co-culture is reviewed. Potential uses of the isolation of better adapted strains of probiotic *Bifidobacterium* strains, as well as the next generation probiotics such as *Faecalibacterium prausnitzii*, are discussed.

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Current Opinion in Food Science 2017, **14**:110–115

This review comes from a themed issue on **Functional foods and nutrition**

Edited by **Cristina Delgado-Andrade**

For a complete overview see the Issue and the Editorial

Available online 27th March 2017

http://dx.doi.org/10.1016/j.cofs.2017.03.002

Significance of maintaining probiotic viability

According to the last expert consensus of the International Scientific Association for Probiotics and Prebiotics, probiotics are "live microorganisms" which when administered in adequate amounts confer a health benefit on the host [1•]. According to this definition, probiotics must be alive in the moment of administration, highlighting the importance of the technological (and mostly abiotic) factors impacting on probiotic viability during industrial handling [2].

Many probiotic strains, notably those belonging to genus *Bifidobacterium* spp. and *Lactobacillus* spp., have been a target of food industry due to their long history of safe use and attributed health benefits, and they have been mostly included in dairy products. In fact, more than 75% of new probiotic products launched in the market are included in dairy products, mostly fermented milks [3]. Inclusion of probiotics in foods has evidenced many technological problems for adapting these anaerobic bacteria to different production processes, among which oxygen toxicity is noteworthy. In fact, in the food industry the selection of probiotic bacteria was based more on their technological performance rather than in other properties, as in the case of the selection of bifidobacteria a species with intrinsic aerotolerance which is dominant in dairy food products [4,5]. The possibility of using an aerotolerant species such as *Bifidobacterium animalis* subsp. *lactis*, has led to a reduction in the research effort in this subject and thus knowledge on oxygen resistant mechanisms is scarce.

This is critical, as probiotic viability is seriously affected after oxygen exposure, notably in anaerobic microorganisms, will compromise the beneficial health effects that are claimed for, since they are generally linked to an ingestion dose effect. The aim of this minireview is to discuss the main technological advances in this field, as well as to address the challenges to be overcome in order to include the so-called emerging probiotics as part of functional foods.

Oxygen toxicity and ROS generation

Oxygen can reach probiotic foods in many steps during the elaboration process. In the case of fermented milks, this concerns mainly the oxygen that is dissolved during milk homogenization, cooling after pasteurization, and mixing after addition of starters. In addition some fermented products are again mixed and stirred during product packaging to maximize the concentration of dissolved oxygen, as many dairy starters require such conditions to develop their optimal metabolic abilities [6]. The physical composition of packages also offers a source of oxygen diffusion into final products. Plastics used in food industry present different porosity and permeability leading to continuous diffusion of oxygen in the food matrix, which is also dependent on container thickness. However, Talwalkar and Kailasapathy showed that viability of oxygen and non-oxygen adapted strains of the genus *Lactobacillus* and *Bifidobacterium*, was not affected regardless the oxygen permeability of the packaging material, mainly oxygen permeable high-impact polystyrene, a specific oxygen-barrier material (Nupak^{TM}) and Nupak^{TM} combined with a commercial oxygen scavenging film (Zero2^{TM}) [7].

Presence of oxygen in fermented matrices is of special relevance since dairy bacteria and many probiotics are unable to reduce oxygen as aerobic bacteria do and, in addition, they often lack oxygen-scavenging mechanisms such as catalase-coding genes. This ends in formation and accumulation of the so-called reactive oxygen species (ROS), among which hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) are noteworthy. ROS accumulation is highly toxic for living cells, notably at DNA and phospholipid level, and at the end induces cell death [8]. ROS act mainly damaging cell membrane phospholipids, DNA and proteins but oxidation at the level of membrane fatty acids is supposed to be the most deleterious mechanism on living cells [9].

Oxygen has different effects regarding the taxonomy of the probiotic strain considered. The relative bacterial growth ratio (RBGR) has been proposed as an index that classifies bacteria from obligate aerobes (value close to infinite) to obligate anaerobes (values close to zero) [10]. In this sense, simple methodologies such as milk deaeration impacts positively in the survival of bifidobacteria included in fermented milks, mainly by lowering dissolved oxygen concentrations and therefore lowering the redox potential [11]. Moreover, molecular mechanisms underlying bacteria aerotolerance may improve human health by decreasing ROS formation and limiting gut inflammation, as it is known that metabolic pathways involved in antioxidant compound production are decreased in gut metagenomes from Inflammatory Bowel Disease patients [12].

Mechanisms of response to molecular oxygen

In anaerobic bacteria energy is obtained through substrate level phosphorylation rather than the oxidative phosphorylation pathway of aerobic microorganisms. This is mainly due to the absence of cytochrome-coding genes and other components of the electron respiratory chain in the genomes of the former. In consequence, and due to the inability of using O_2 as external electron acceptor, redox reactions in anaerobic bacteria are controlled by pyridine nucleotides such as NADH [8].

Indeed aerotolerance in anaerobic bacteria is tightly related to their ability to regenerate NAD^+ from NADH. For instance, many lactic acid bacteria and bifidobacteria possess NADH oxidases that can transfer up to four electrons to molecular oxygen. Depending on the number of transferred electrons, oxygen can be reduced to H_2O or to H_2O_2 [13]. In addition, an incomplete NADH oxidase activity can lead to formation of other ROS, such as the O_2^-. Finally, another type of NADH peroxidase can reduce H_2O_2 to H_2O [14].

Other mechanism of response in anaerobic bacteria includes superoxide dismutase, which converts O_2^- into H_2O_2, which can be further destroyed by other enzymes such as catalase or peroxidase. Anaerobic bacteria present also many non-enzymatic response mechanisms to oxygen, including accumulation of water- or lipid-soluble antioxidant compounds such as glutathione, ascorbate, alpha-tocopherol or beta-carotene [15]. Accumulation of certain flavonoids such as catechins with phospholipids has also been proposed as a protection mechanism against membrane oxidation [16].

Mechanisms of response to oxygen in bifidobacteria

Bifidobacteria are commonly described as strictly anaerobic bacteria as they are not able to form colonies on agar-plates in the presence of air [17]. This poor tolerance to abiotic stresses such as oxygen has an impact in their claimed probiotic properties. In fact, oxygen exposure has a direct effect on the fatty acid profiles in *Bifidobacterium longum* subsp. *longum*, inducing morphological changes notably in cell-shape [18]. Because of their poor survival in presence of oxygen (and the formation of other not-desirable fermentation products such as acetic acid), bifidobacteria have no proper technological aptitudes to be used as starters in food fermentation or to even survive the process of food elaboration after its addition to milk. One exception is *Bifidobacterium animalis* subsp. *lactis* [19], whose intrinsic aerotolerance has allowed for its massive use in fermented milks as a probiotic supplement [20]. Other aerotolerant bacteria are *Bifidobacterium thermophilum*, *Bifidobacterium boum* LMG 10736^T, *Bifidobacterium minimum* DSMZ 10105^T and *Bifidobacterium pyschraerophilum* [21]. As a general rule, potential probiotic strains such as *Bifidobacterium bifidum* displays usually low tolerance to oxygen [22].

Some detoxification mechanisms have been identified in some bifidobacteria species (Figure 1). In the absence of other detoxification mechanisms, oxygen tolerance in bifidobacteria appears to be dependent on the presence of a set of oxygen-scavenging NADH oxidases. Indeed, increasing oxygen concentrations correlate positively with increases in the NADH oxidase activity and negatively with lactate production in *Bifidobacterium* spp. [23]. Maximum activities of these enzymes are found in the most aerotolerant strains [2]. Examples of NADH oxidases identified in bifidobacteria is the b-type dihydroorotate dehydrogenase, (H_2O_2-forming) from *B. bifidum* JCM 1255^T, catalysing the oxidation of dihydroorotate to orotate in pyrimidine biosynthesis [24]. Concomitantly,