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The Role of Oxygen in the Viability of Probiotic Bacteria with Reference to *L. acidophilus* and *Bifidobacterium* spp.

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Abstract

The various therapeutic benefits of *Lactobacillus acidophilus* and *Bifidobacterium* spp. have resulted in their increased incorporation into dairy foods such as yoghurts. Currently however, the efficacy of these probiotic bacteria is limited by their poor survival during the shelf life of yoghurt. Oxygen toxicity is widely considered to be responsible for the cell deaths of these bacteria. The intestinal origins and the microaerophilic and anaerobic characteristics of *L. acidophilus* and *Bifidobacterium* spp. respectively, can render them susceptible to oxygen contained in the food products. This review discusses the influence of the dissolved oxygen in yogurt on the viability of these bacteria. Suggested techniques to protect these probiotic bacteria from oxygen toxicity are evaluated. Although the problem of oxygen toxicity in probiotic bacteria is regarded as significant, little is known however about the cellular interaction of these bacteria with oxygen. This review summarizes what is known about the biochemistry of oxygen toxicity in these bacteria. The various metabolic and biochemical responses of *L. acidophilus* and *Bifidobacterium* to oxygen are examined. Additionally, the importance of NADH oxidase and NADH peroxidase in the oxygen tolerance of these bacteria is evaluated and assays used to measure their cellular concentrations are discussed.

Introduction

The previous century witnessed an extensive dominance of antibiotics in the treatment of various diseases. Antibiotic therapies however cause unpleasant side effects as they alter the intestinal balance, which is critical for maintaining good human health. Moreover, there has been a rapid development of antibiotic resistance in pathogenic microorganisms (Ney, 1994). Consequently a climate has been created wherein both doctors and patients are searching for preventive rather than curative approaches to diseases, in which the intestinal microflora is not adversely affected.

One such approach is the concept of probiotics, currently defined as 'live microbes which transit the gastrointestinal tract and in doing so benefit the health of the consumer' (Tannock *et al.*, 2000). Bacteria that fulfill this definition are therefore termed as probiotic bacteria.

In this regard, intestinal bacteria such as lactobacilli, particularly *Lactobacillus acidophilus* and *Bifidobacterium* spp. have gained recognition as probiotic bacteria due to their various therapeutic health benefits such as prevention of diarrhea in children, modulation of immunity, alleviating lactose intolerance, preventing some forms of cancers and lowering of serum cholesterol (Kailasapathy and Chin, 2000). Moreover, both *L. acidophilus* and *Bifidobacterium* spp. belong to the Lactic Acid Bacteria (LAB), a group of bacteria that are generally considered safe for human administration (Salminen *et al.*, 1998).

The health benefits of *L. acidophilus* and *Bifidobacterium* spp. have led to their increased incorporation in dairy foods, particularly in yoghurts. This has created a new generation of health foods. The growing health awareness among consumers has helped to spark a boom in the sale of probiotic yoghurts, especially in Europe, Japan and Australia (Anon., 1998; Sanders, 1998, Stanton *et al.*, 2001). Presently over 70 products all over the world including sour cream, buttermilk, yoghurt, powdered milk and frozen desserts contain bifidobacteria and lactobacilli (Shah, 2001).

For probiotic bacteria to be effective however, it is essential that they reach the small intestine in sufficiently high numbers. This involves surviving through the various manufacturing processes of the food product and its storage. Additionally, the probiotic strain must endure the hostile conditions of the gastrointestinal tract such as high acidity in the stomach and bile in the small intestine. For probiotic bacteria to deliver their therapeutic effects, it has been suggested that the daily intake should be at least 10^8 cfu (Lourens-Hattingh and Viljoen, 2001). It is therefore recommended that the minimum counts of probiotic bacteria in probiotic yoghurts be 10^6 cfu/g at the time of sale (Kurmann and Rasic, 1991). These high numbers have been suggested to compensate for the possible reduction in the numbers of probiotic organisms during passage through the stomach and intestine. The expanding popularity of probiotic dairy products has led to several food organizations introducing standards which require a minimum of 10^6 - 10^7 cfu g⁻¹ of *L. acidophilus* and/or bifidobacteria in fermented milk products (IDF, 1992; Shah, 2000; Bibiloni *et al.*, 2001).

Market surveys on the viability of *L. acidophilus* and bifidobacteria in probiotic yoghurts vary in their findings with some studies reporting low counts of these bacteria (Iwana *et al.*, 1993; Shah *et al.*, 1995; Rybka and Fleet,

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1997; Anon, 1999; Shah *et al.*, 2000) while others citing satisfactory viability (Lourens *et al.*, 2000; Shin *et al.*, 2000). Variable counts have been reported elsewhere (Pacher and Kneifel, 1996; Dave and Shah, 1997c; Micanel *et al.*, 1997; Vinderola and Reinheimer, 1999; Vinderola *et al.*, 2000).

Several factors are reported to influence the survival of *L. acidophilus* and bifidobacteria in yoghurts. These include acid and hydrogen peroxide produced by yoghurt bacteria, type of strain, culture conditions, concentrations of lactic and acetic acids, whey proteins and interaction of the probiotic species with the yoghurt starters (Kailaspathy and Supraidi, 1996; Godward *et al.*, 2000; Shah, 2000; Vinderola *et al.*, 2000). Among these, the oxygen content in the product and oxygen permeation through the package is considered most significant in reducing the viability of *L. acidophilus* and bifidobacteria in fermented milk products (Klaver *et al.*, 1993; Dave and Shah, 1997c).

Oxygen toxicity of probiotic bacteria in fermented milks, particularly yoghurts

Organisms inhabiting the human gut are generally anaerobic or microaerophilic and therefore lack effective oxygen scavenging cellular mechanisms such as catalases. Hence, exposure to oxygen in these bacteria causes toxic oxygenic metabolites to accumulate in the cell leading to cell death from oxidative damage. This lethal effect of oxygen is termed as oxygen toxicity.

Both *L. acidophilus* and *Bifidobacterium* spp. are derived from the intestine and are classified as microaerophilic and strictly anaerobic respectively. Dairy foods such as yoghurt contain high levels of oxygen, which is incorporated during the various homogenization, mixing and agitation steps of yoghurt manufacture. Additionally, oxygen diffuses through the packaging material during the shelf life storage (Ishibashi and Shimamura, 1993; Miller *et al.*, 2002). The resulting oxygen environment in yoghurts or fermented milks is thought to induce cell death and lead to poor viability of *L. acidophilus* and *Bifidobacterium* spp. (Brunner *et al.*, 1993a; Brunner *et al.*, 1993b; Klaver *et al.*, 1993).

Although bifidobacteria are considered more susceptible to oxygen than *L. acidophilus* due to their anaerobic nature, the oxygen susceptibility of bifidobacteria could however be strain dependent. Dave and Shah (1997b) found that bifidobacteria survived well over a 35 day period in yoghurt, regardless of the oxygen content and redox potential of the yoghurt. Miller *et al.* (2002) too found better survival of bifidobacteria compared to *L. acidophilus* even as the dissolved oxygen of the yoghurt was seen to rise steadily over the shelf life. While counts of bifidobacteria remained above the recommended 10^6 cfu/g throughout the shelf life of the yoghurt, *L. acidophilus* counts were found to decrease below 10^3 cfu/g by the third week of storage. The strain dependent phenomenon of oxygen sensitivity was further demonstrated by Meile *et al.* (1997) who were able to isolate a moderately oxygen tolerant species of *Bifidobacterium*, *B. lactis* sp. nov. from fermented milk.

Techniques to protect *L. acidophilus* and *Bifidobacterium* spp. from oxygen toxicity in yoghurts

1. Use of ascorbate and L-cysteine as oxygen scavengers in yoghurts

The property of ascorbic acid and L-cysteine to act as oxygen scavengers and maintain low redox potential was evaluated by Collins and Hall (1984) and by Dave and Shah (1997a; 1997b) for maintaining conditions suitable for the extended viability of probiotic bacteria in yoghurts. Although there was a reduction in the oxygen content and redox potential of yoghurt, together with an improvement in the counts of *L. acidophilus* and *Bifidobacterium* spp., the incorporation of ascorbic acid in yoghurts can however reduce the amount of oxygen required for the activities of *S. thermophilus*, an aerobic organism used as a starter culture in the manufacture of yoghurt. Cysteine too was found to suppress the growth of the yoghurt starter cultures, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. This can have a detrimental effect on the textural and nutritional qualities of yoghurt and this technique may hence be undesirable in the industrial manufacture of yoghurt.

2. Use of special high-oxygen consuming strains

To protect probiotic bacteria, especially bifidobacteria from oxygen in yoghurt, the incorporation of a high-oxygen consuming strain of *Streptococcus thermophilus* has been suggested (Lourens-Hattingh and Viljoen, 2001). *S. thermophilus*, which relies heavily on oxygen for its metabolic activities can act as an effective oxygen scavenger by its consumption of the dissolved oxygen in the yoghurt. This can therefore help to reduce oxygen exposure to bifidobacteria (Ishibashi and Shimamura, 1993). This technique however suffers from the drawback that fast acidifying strains of *S. thermophilus* used commercially, can lead to a rapid accumulation of acid in the growth medium. As both *L. acidophilus* and bifidobacteria are sensitive to high acidity, this can have a negative impact on the viability of probiotic bacteria. Moreover, this method is useful in providing protection against oxygen toxicity only during the stages of yoghurt manufacture. It does little to protect the probiotic bacteria from subsequent oxygen ingress into yoghurt through the packaging material.

3. Microencapsulation

Micro-encapsulation of probiotic bacteria is a process in which cells are retained within an encapsulating membrane to reduce cell injury or cell loss (Shah, 2000; Kailaspathy, 2002). The physical retention of cells in the encapsulating matrix can facilitate the separation of cells from direct exposure to the adverse factors such as oxygen while at the same time allow the diffusion of nutrients in and out of the matrix to support the viability of the cells. Microencapsulation has also been applied to increase the survival of probiotic bacteria in yoghurt and other dairy products by protecting probiotic bacteria from lyophilization and rehydration, and acidity in the product (Shue *et al.*, 1993; Dinakar and Mistry, 1994; Kim *et al.*, 1996; Kebary *et al.*, 1998; Adhikari *et al.*, 2000; Shah and Ravula, 2000; Sultana *et al.*, 2000). Little is known about the protective

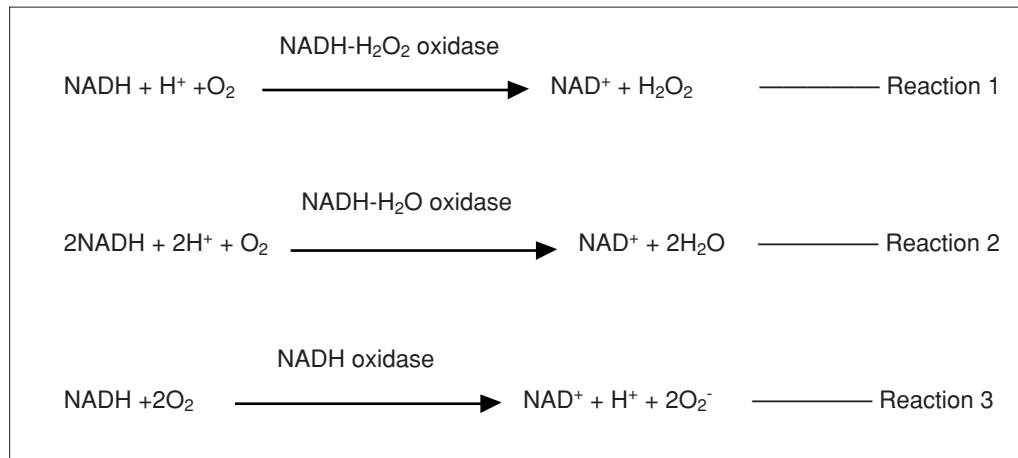


Figure 1. Reactions of NADH oxidase with oxygen in LAB (Condon, 1987).

effect of microencapsulation from oxygen toxicity. Talwalkar and Kailaspathy (2003a) evaluated the protective effect of microencapsulation against oxygen toxicity in *L. acidophilus* and *Bifidobacterium* spp. in both culture broth as well as in prepared yoghurt. Cells were encapsulated in calcium alginate and grown in the presence of oxygen. When tested in optimum conditions of culture broth and temperature (37°C), encapsulated cell counts were one log higher than their free cell counterparts. Mixed results were however observed when microencapsulation was tested in yoghurt stored at 6°C. Microencapsulation was found to significantly increase viability in six strains while no significant difference was observed between encapsulated cell counts and free cells counts in the other six strains. Preliminary evidence therefore suggests that although microencapsulation can offer protection to probiotic bacteria against oxygen toxicity in broth culture, further optimization studies are needed before its application in yoghurt.

4. Packaging material

The oxygen permeability of the packaging material used currently for probiotic yoghurts is considered a key factor in the high levels of oxygen present in yoghurt. It is well known that packaging materials such as polyethylene and polystyrene are gas permeable and allow the diffusion of oxygen into yoghurt during storage (Ishibashi and Shimamura, 1993).

The exclusion of oxygen during the manufacturing process can be costly. Few current packaging techniques are capable of preventing oxygen permeation.

Dave and Shah (1997c) found improved survival of *L. acidophilus* over a 35-day period in yoghurts that were packaged in glass bottles as compared to when the yoghurt was packaged in plastic cups. The oxygen content in yoghurts stored in plastic cups increased due to the permeation of oxygen whereas the yoghurts contained in the glass bottles retained a low oxygen environment. This led to the suggestion that to prevent oxygen toxicity in probiotic bacteria, yoghurts be packed in glass containers. Although effective, glass jars are however neither convenient nor practical owing to their high cost and handling hazards. On the other hand, polyethylene and

polystyrene do not have sufficient oxygen barrier properties and are therefore unsuitable to prevent oxygen ingress into yoghurt during storage.

In this regard, Miller *et al.* (2002) suggested a relatively cheaper packaging option using specific oxygen impermeable packaging materials. Yoghurts when packaged in polystyrene based packaging containing an added gas-barrier layer (Nupak™) were found to demonstrate no increase in their dissolved oxygen levels. In comparison, the dissolved oxygen in yoghurts packaged with conventional polystyrene tubs was seen to rise steadily over the shelf life. Such packaging technologies thus seem promising and their application needs to be explored further.

Biochemistry of the oxidative response

Aerobic bacteria derive their energy primarily through oxidative phosphorylation, involving the electron transport chain, which is composed of a chain of carriers capable of undergoing reversible oxidation and reduction. Anaerobic bacteria including lactic acid bacteria such as *L. acidophilus* and *Bifidobacterium* spp. cannot synthesize cytochromes and other heme containing enzymes vital to the electron transport chain. They are thus unable to synthesize ATP by respiratory means and have to depend strictly on a fermentative mode of metabolism. Due to the lack of participation of an external electron acceptor (oxygen in aerobic bacteria) in anaerobes, the organic substrate undergoes a balanced series of oxidative and reductive reactions mediated by pyridine nucleotides such as NADH. As the energy in anaerobes is derived mainly through substrate level phosphorylation, the regeneration of NAD⁺ from NADH assumes critical importance.

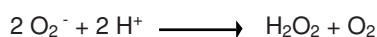
The simplest way to oxidize NADH is by the reduction of molecular oxygen (O₂) via the activity of NADH oxidase. Coincidentally, possession of a NADH oxidase appears to be a universal property of lactic acid bacteria (Condon, 1987).

Generally, the NADH oxidizing reactions in lactic acid bacteria including *L. acidophilus* and *Bifidobacterium* spp. catalyze the transfer of one, two or four electrons to the dioxygen molecule (Figure 1).

Mainly, two types of NADH oxidases: NADH-H₂O₂ oxidase and NADH-H₂O oxidase have been reported in lactic acid bacteria. While the NADH-H₂O₂ oxidase catalyzes the reduction of O₂ to H₂O₂ (Reaction 1, Figure 1) (Condon, 1987; Smart and Thomas, 1987), the NADH-H₂O oxidase carries out the four-electron reduction of oxygen to water (Reaction 2, Figure 1) (Condon, 1987; Higuchi *et al.*, 2000).

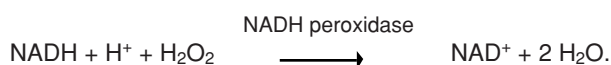
The activities of NADH oxidase can also result in the incomplete reduction of oxygen, generating reactive oxygen species such as the superoxide anion (O₂⁻) (Reaction 3, Figure 1). O₂⁻ can easily dismutate to hydrogen peroxide (H₂O₂) or the hydroxyl radical

(HO[•]) either spontaneously or by the activity of superoxide dismutase (SOD) as shown below (Fridovich, 1975; Sanders *et al.*, 1995):



In addition, O₂⁻ can also be dismutated by high intracellular Mn₂⁺ to the more stable H₂O₂ (Condon, 1987). These compounds can readily diffuse across cellular membranes and oxidatively damage a number of vital cellular components including membrane lipids, enzymes and DNA (Miller and Britigan, 1997; Hassen and Fridovich, 1979; Higuchi *et al.*, 2000). It is well known that the hydroxyl radical is highly reactive with biological molecules. Although H₂O₂ is a weak oxidant, it can generate hydroxyl radicals in the presence of transition metals (Lin and Yen, 1999). Accumulation of H₂O₂ during aerobic growth has been shown to inhibit the growth of several lactobacilli (Condon, 1987). Additionally, excess intracellular H₂O₂ may also produce further oxidation products (O₂SCN⁻ and O₃SCN⁻) that could be bacteriostatic in lactic acid bacteria (Reiter, 1985). Hydrogen peroxide is also claimed to inactivate fructose-6-phosphate phosphoketolase, the major enzyme responsible for sugar metabolism in bifidobacteria (Shah, 1997). Thus to live in the presence of oxygen, lactobacilli and bifidobacteria have to convert these reactive oxygen species to nontoxic molecules, failing which, these compounds can accumulate in the cell and cause cell death from oxidative damage.

Accordingly, some lactic acid bacteria possess NADH peroxidase that reduce H₂O₂ to H₂O as shown below (Mizushima and Kitahara, 1962; Anders *et al.*, 1970; Thomas and Pera, 1983):



Studies on the oxygen tolerance of *L. acidophilus* and *Bifidobacterium* spp.

Only a few studies have been conducted on the oxygen tolerance of probiotic bacteria. Moreover, most of these studies have focussed mainly on *Bifidobacterium* spp. Little is known about the effect of oxygen on the physiology of *L. acidophilus*.

Research into the oxygen tolerance of bifidobacteria has been conducted from as early as 1969 when de Vries and Stouthamer (1969) examined the sensitivity of twenty

Bifidobacterium strains to oxygen by measuring the size of the inhibition zones obtained when the bacteria were grown in deep agar cultures under air. The different strains were then classified into three categories based on their degree of oxygen tolerance and formation of H₂O₂ during aerobic growth. Uesugi and Yajima (1978) also classified seven *Bifidobacterium* spp. strains based on their oxygen sensitivity. Shimamura *et al.* (1992) and Shin and Park (1997) also found bifidobacteria to differ in the sensitivity to oxygen.

In all of the above studies however, the oxygen tolerance of bifidobacteria was evaluated qualitatively, such as growth on agars or constant or partial shaking of the culture broth. A quantitative estimate of the oxygen tolerance of several strains of *L. acidophilus* and bifidobacteria was first reported by Talwalkar *et al.* (2001) using a methodology called as Relative Bacterial Growth Ratio (RBGR). RBGR is the ratio of aerobic growth of the strain to its anaerobic growth, thus providing a numerical index for oxygen tolerance for each strain. In this study, strains were found to vary in the sensitivity to oxygen. Overall however, *L. acidophilus* strains demonstrated higher RBGRs than *Bifidobacterium* spp. indicating that they could tolerate oxygen better.

Ahn *et al.* (2001) studied the interaction of oxygen with bifidobacteria at a cellular level and found that *B. longum*, when exposed to oxygen underwent changes in its cellular fatty acid profiles. Besides an extension of the lag phase, cells became elongated and developed a rough surface due to abnormal or incomplete cell division. Elsewhere, a correlation between intracellular polysaccharide accumulation *Bifidobacterium* spp. and its oxygen uptake was also reported (Shimamura *et al.*, 1990). As the oxygen uptake was observed only in the presence of NADH, it was suggested that NADH oxidase operated as the terminal oxygen oxidoreductase in *Bifidobacterium* spp. A similar involvement of NADH oxidase in oxygen uptake in bifidobacteria has been suggested by Cox and Marling (1992).

Many studies on the aerotolerance of lactic acid bacteria suggest that the ratio and specific activities of the NADH oxidase and NADH peroxidase determine the elimination of oxygen from the cell (Higuchi, 1984; Lucey and Condon, 1986; Smart and Thomas, 1987; Soon-Young and Park, 1997).

Shimamura *et al.* (1992) explored the enzymatic machinery behind the oxygen sensitivity of *Bifidobacterium* spp and found a good correlation between the levels of NADH oxidase and NADH peroxidase and the oxygen tolerance of the strains, with the oxygen sensitive strain displaying low activities of these enzymes. All strains accumulated H₂O₂ under aerobic conditions but no notable correlation was observed between the growth inhibition by oxygen and sensitivity to H₂O₂. Similarly, Shin and Park (1997) found that the activities of NADH oxidase and NADH peroxidase were low in the most aerosensitive strains of bifidobacteria whereas maximum activities were observed in the most aerotolerant strain.

The measured activities of NADH oxidase and NADH peroxidase in the above- mentioned studies could however be unreliable. The NADH oxidase-NADH oxidase enzyme

Table 1. Effect of different oxygen concentrations on the specific activities of NADH oxidase, NADH peroxidase, and SOD and on the H₂O₂ decomposing ability of *L. acidophilus* strains and *Bifidobacterium* spp.

Strain	% Oxygen	NADH oxidase ^A	NADH peroxidase ^B	S.O.D. ^C	nmol H ₂ O ₂ decomposed
<i>L. acidophilus</i> CSCC 2400	0	20.62	18.37	1.10 ^a	17.4
	5	25.38 ^a	22.86 ^a	1.06 ^a	18.7
	10	25.64 ^a	23.87 ^{a,b}	1.02 ^{a,b}	28.4
	15	26.44 ^{a,b}	25.18 ^b	1.00 ^{a,b}	32.4
	21	27.26 ^b	25.28	0.94 ^b	38.4
<i>L. acidophilus</i> CSCC 2409	0	21.09 ^a	20.20 ^a	1.64 ^a	20.3
	5	21.85 ^{a,b}	21.06 ^{a,b}	1.58 ^a	22.3
	10	21.94 ^{a,b}	22.29 ^b	1.35 ^b	28.5
	15	23.05 ^b	23.87	1.30 ^b	33.7
	21	25.21	25.65	1.36 ^b	35.2
<i>B. infantis</i> CSCC 1912	0	2.10	5.35	0.86 ^a	2.69
	5	4.67 ^a	6.46	1.02 ^a	4.72
	10	4.38 ^a	7.67 ^a	1.58	5.42
	15	4.66 ^a	7.56 ^a	1.30 ^a	5.48
<i>B. lactis</i> CSCC 1941	0	4.97 ^a	5.32	1.36	0.78
	5	5.37 ^a	6.35	2.03 ^a	1.16
	10	6.50 ^b	7.68	2.10 ^a	4.27
	15	7.05 ^b	8.65	1.90 ^a	7.99
	21	7.32 ^b	10.53	1.19	8.87
<i>B. pseudolongum</i> CSCC 1944	0	1.99 ^a	3.47	0.86 ^a	1.05
	5	2.2 ^a	4.08 ^a	0.65 ^b	1.34
	10	3.2	4.19 ^a	0.81 ^a	3.29
	15	5.2	4.30 ^a	0.70 ^b	3.71 ^a
	21	6.2	6.07	0.57 ^b	3.71 ^a
<i>B. longum</i> 55815	0	12.74	10.37	2.71 ^a	6.97
	5	14.57	12.38	2.89 ^a	7.87
	10	15.97	16.40 ^a	2.66 ^a	8.28
	15	18.11 ^a	16.86 ^a	2.36 ^b	10.07
	21	18.91 ^a	16.86 ^a	2.52 ^{a,b}	13.32

a, b, c Means in columns with like superscripts do not differ significantly ($P > 0.05$)
Means in columns with no superscripts differ significantly ($P < 0.05$)
A, B, C Expressed as Enzyme Units/ per mg of total protein of the cell free extract.

system in lactic acid bacteria is interdependent with the product of NADH oxidase (H₂O₂) being a substrate for NADH peroxidase. Additionally, both enzymes require NADH for their activity. NADH oxidase is generally assayed as the slope of NADH oxidation at 340nm. in the presence of cell free extract. However, when the cell free extract also contains NADH peroxidase, the slope of NADH oxidation is actually the sum of the total NADH oxidised by the activities of both oxidase and peroxidase. Most of the reported studies on NADH oxidase and NADH peroxidase of lactic acid bacteria, particularly bifidobacteria have failed to take this into account and therefore estimates of NADH oxidase activities in those studies may be erroneous. Additionally, considerable variation exists in the assays reported to measure NADH peroxidase. While Shimamura *et al.* (1992) estimated activities of NADH peroxidase by measuring the consumption of H₂O₂ under anaerobic conditions, Shin and Park (1997) assayed NADH peroxidase activity by measuring the slope of NADH oxidation under anaerobic conditions. On the other hand, Uesugi and Yajima (1978) and de Vries and Stouthamer (1969) estimated NADH peroxidase as the slope difference in the presence and absence of H₂O₂ under aerobic conditions.

In this regard, an improved spectrophotometric assay developed by Talwalkar *et al.* (2003) was found to address the complexity of the NADH oxidase-NADH peroxidase enzyme system of lactic acid bacteria and provide accurate estimations of NADH oxidase even in the presence of NADH peroxidase. This assay was thus recommended as a standard assay for the measurement of NADH oxidase and NADH peroxidase in lactic acid bacteria, particularly *L. acidophilus* and *Bifidobacterium* spp.

Among the oxidative studies conducted on *L. acidophilus*, Archibald and Fridovich (1981) found that *L. acidophilus* lacked high intracellular levels of Mn (II). Lin and Yen (1999) investigated the antioxidative ability of *L. acidophilus* and *B. longum* and found that both bacteria were capable of chelating metal ions, scavenge reactive oxygen species or possess reducing activity. Similarly, iron chelation activity in *L. acidophilus* and strains of *Bifidobacterium* spp. as well as the presence of a ferroxidase in bifidobacteria has been reported (Kot *et al.*, 1994; Kim *et al.*, 2001).

Perhaps the most comprehensive study on the various metabolic and biochemical responses of both *L. acidophilus* and *Bifidobacterium* spp. oxygen was conducted by Talwalkar and Kailasapathy (2003b). Strains of these probiotic bacteria were grown in either 0%, 5%, 10%, 15%

or 21% oxygen and its effect on various metabolic and biochemical parameters such as level of lactic acid produced, lactate to acetate ratio, sensitivity to H₂O₂, decomposition of H₂O₂, specific activities of NADH oxidase, NADH peroxidase and superoxide dismutase were monitored. The stepwise increase in the concentrations of oxygen provided a better inspection of the development of the oxidative response in both *L. acidophilus* and bifidobacteria. Overall, as the oxygen concentration increased, the levels of lactic acid in *L. acidophilus* decreased while the lactate to acetate ratio in bifidobacteria reduced. Interestingly, specific activities of NADH oxidase and NADH peroxidase were found to increase as the oxygen concentration increased, highlighting their inducibility and participation in the oxidative response in these bacteria. The increase in the activities of NADH peroxidase was found to correlate with the increase in H₂O₂ decomposition. Although superoxide dismutase is considered significant in detoxifying molecular oxygen, no correlation was observed between the increase in oxygen concentration and its levels (Table 1). Similar observations of superoxide dismutase levels in *Bifidobacterium* spp. and *L. acidophilus* strains being independent of the oxygen sensitivity have been reported (Shimamura *et al.*, 1992; Shin and Park, 1997; Lin and Yen, 1999; Talwalkar and Kailasapathy, 2003b).

This study therefore provided a clear evidence that probiotic bacteria such as *L. acidophilus* and *Bifidobacterium* spp. are capable of mounting a cellular response against oxygen and that NADH oxidase and NADH peroxidase seem to play an important role in the oxygen scavenging mechanism in these bacteria.

Conclusions

The problem of oxygen toxicity in probiotic bacteria, particularly *L. acidophilus* and *Bifidobacterium* spp. is still an unresolved issue. Although both these microorganisms are considered highly susceptible to oxygen, studies have revealed that considerable differences exist in their oxygen tolerance. The biochemistry of the oxidative response in these bacteria suggests a series of complex mechanisms that act in unison to protect the cell from oxygen toxicity. Activities of NADH oxidase and NADH peroxidase seem critical in the oxidative responses in these bacteria. Knowledge about the various biochemical pathways and their regulation in these bacteria is however limited. Consequently, the research available on the oxygen tolerance of these bacteria offers only a glimpse into the influence of oxygen at a cellular level. It would be interesting to elucidate the effect of oxygen at a molecular level in these bacteria and the possibility of stress proteins developed in response to oxidative stress. Certain proteins that reverse oxidative damage have been found in *B. longum* (Schell *et al.*, 2002) while genes encoding various oxidative stress-related proteins have been reported in the genome of *L. plantarum* (Kleerebezem *et al.*, 2003). Research in this direction would enable a thorough documentation of the biochemical pathways of these bacteria and promote better understanding of their responses to stresses such as oxygen.

The inducible oxidative response in both *L. acidophilus* and bifidobacteria offers avenues for stress adapting these strains to oxygen. It is possible that passaging these bacteria through gradually increasing concentrations of oxygen will allow cells to adapt to elevated levels of oxygen.

Caution should however be exercised in extrapolating the findings of the oxidative studies to food systems like yoghurts. The effect of oxygen on the viability and cellular physiology of probiotic cells can be more pronounced when the strains were tested at optimum temperature (37°C) and in a suitable culture broth. Food products such as yoghurts are stored at sub optimum temperatures such as 4-8°C. As observed by Talwalkar and Kailasapathy (2003b), it is likely that oxygen can affect cells differently at these temperatures. The activities of NADH oxidase and NADH peroxidase can also be influenced by low temperatures altering the oxidative response. It is therefore vital that the oxidative studies be also conducted in conditions that simulate the product conditions. This would allow a better estimate of the oxygen susceptibility. In this regard, it would be worthwhile to evaluate the effect of various oxygen impermeable or oxygen scavenging packaging materials on the viability of *L. acidophilus* and *Bifidobacterium* spp. in food products such as yoghurts.

The insufficient research on oxygen toxicity in probiotic bacteria makes it difficult to draw any conclusive trend about the influence of oxygen on cell viability. Considering the importance of high cell numbers in foods such as yoghurts, it is imperative that the influence of oxygen on cell numbers be elucidated in detail. This would require a thorough investigation of the precise biochemical pathways in both *L. acidophilus* and *Bifidobacterium* spp. and their susceptibility to oxygen. Furthermore, the low storage temperatures of food makes it essential that oxygen toxicity be evaluated in both optimal conditions as well as those encountered in the product in which probiotic bacteria would be incorporated. Together this would allow the development of effective techniques to protect probiotic bacteria from oxygen and ensure the maintenance of adequate cell numbers in food products such as yoghurts. The consequent delivery of therapeutic benefits to consumers would further serve to expand the highly dynamic probiotic food market.

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