Metabolic and Biochemical Responses of Probiotic Bacteria to Oxygen

A. Talwalkar and K. Kailasapathy

Centre for Advanced Food Research, University of Western Sydney,
Locked Bag #1797, SPDC, NSW 1797, Australia

ABSTRACT

The interaction between oxygen and probiotic bacteria was studied by growing *Lactobacillus acidophilus* and *Bifidobacterium* spp. in 0, 5, 10, 15, and 21% oxygen in a hypoxic glove box. The metabolic responses of each probiotic strain in the different oxygen environments were monitored by measuring the levels of lactic acid and determining the lactate-to-acetate ratio. Biochemical changes induced by oxygen were examined by monitoring the specific activities of NADH oxidase, NADH peroxidase, and superoxide dismutase. In addition, the ability to decompose hydrogen peroxide and the sensitivity of each strain to hydrogen peroxide was also determined. With an increase in oxygen percentage, levels of lactic acid in *L. acidophilus* strains decreased, whereas the lactate-to-acetate ratio reduced in all the bifidobacteria tested. At 21% oxygen, the specific activities of NADH oxidase and NADH peroxidase, and the hydrogen peroxide decomposing ability of five probiotic strains was significantly higher than at 0% oxygen. The sensitivity of the probiotic strains to hydrogen peroxide however, remained unaffected in all the different oxygen percentages. Superoxide dismutase levels did not reveal any conclusive trend. In both *L. acidophilus* and *Bifidobacterium* spp., NADH oxidase and NADH peroxidase functioned optimally at pH 5. Growth in the various oxygen environments did not change this optimum pH.

(**Key words:** probiotic bacteria, oxygen, metabolic and biochemical response)

Abbreviation key: H_2O_2 = hydrogen peroxide, NADH = reduced NAD, SOD = superoxide dismutase.

INTRODUCTION

The various therapeutic benefits of probiotic bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium*

spp. (Kailasapathy and Chin, 2000) have led to their increased incorporation in dairy foods such as yogurts (Lourens-Hattingh and Viljoen, 2001). The poor survival of probiotic bacteria in commercial yogurts (Shah et al., 1995; Rybka and Fleet, 1997; Shah et al., 2000) below the recommended 10^6 cfu/g (IDF, 1992), however, can limit their ability to exert positive health benefits in humans (Kurmann and Rasic, 1991). Among the reasons responsible for the loss in probiotic viability, cell death due to oxygen toxicity is considered a significant factor (Brunner et al., 1993; Klaver et al., 1993; Dave and Shah, 1997).

The problem of oxygen toxicity in probiotic bacteria, however, remains poorly understood. Although probiotic bacteria, especially bifidobacteria, are considered as highly susceptible to oxygen, the oxygen tolerance of these organisms has been strain dependent (de Vries and Stouthamer, 1969; Shimamura et al., 1992; Talwalkar et al., 2001). Satisfactory growth of a variety of *Bifidobacterium* spp. in the absence of strict anaerobic conditions was observed by Cheng and Sandine (1989). In another study, *B. lactis*, isolated from fermented milk was found to display good oxygen tolerance (Meile et al., 1997).

It is believed that intracellular levels of H_2O_2 block fructose 6 phosphofructotetolase, a key enzyme in the sugar metabolism of bifidobacteria and therefore scavenging H_2O_2 becomes important for cell survival (de Vries and Stouthamer, 1969). Both *L. acidophilus* and *Bifidobacterium* spp. are devoid of catalase, a key enzyme for the breakdown of H_2O_2 and have to rely on enzymes such as NADH oxidase and NADH peroxidase to scavenge environmental oxygen (Condon, 1987). The activities of NADH oxidases in probiotic bacteria give rise to H_2O_2 , prompting NADH peroxidase to scavenge H_2O_2 and prevent cell death. Shimamura et al. (1992) explored the biochemical mechanisms of oxygen sensitivity of several bifidobacteria and concluded that levels of NADH oxidase and NADH peroxidase play an important role in the prevention of oxygen toxicity. High levels of these enzymes were found in the most aerotolerant *Bifidobacterium* spp.

So far, oxidative studies on probiotic bacteria have mainly focused on bifidobacteria (de Vries and Stou-

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Corresponding author: K. Kailasapathy; e-mail: k.kailasapathy@uws.edu.au.

thamer, 1969; Shimamura et al., 1992; Ahn et al., 2001). Furthermore, in the reported studies on bifidobacteria and *L. acidophilus*, the cells were grown in either aerobic or partially aerobic conditions (Archibald and Fridovich, 1981; Shimamura et al., 1992; Ahn et al., 2001). These undefined concentrations of oxygen may be unsuitable for identifying definitive relationships between the effects of different oxygen concentrations on probiotic bacteria. Understanding the precise metabolic and biochemical changes influenced by known amounts of oxygen is crucial for preventing the problem of oxygen toxicity in probiotic bacteria.

Therefore, in this study, four *Bifidobacterium* spp. and two *L. acidophilus* strains were grown individually in 0, 5, 10, 15, and 21% oxygen in a hypoxic glove box and their metabolic and biochemical responses to oxygen were monitored. Metabolic responses were examined by the measurement of lactic acid and acetic acid levels. The sensitivity of the probiotic strains to different H_2O_2 concentrations in each oxygen percentage was also tested. Furthermore, the ability of the strains to decompose known amounts of H_2O_2 was examined. Specific activities of NADH oxidase, NADH peroxidase, and SOD were regarded as biochemical indices of the probiotic bacterial strains and were measured accordingly. The defined concentrations of oxygen allowed us to study the incremental build up of the oxidative stress response of the probiotic bacteria.

MATERIALS AND METHODS

Organisms and Culture Conditions

Lactobacillus acidophilus 2400, *L. acidophilus* 2409, *Bifidobacterium infantis* 1912, *Bifidobacterium lactis* 1941, *Bifidobacterium pseudolongum* 1944 were supplied by Commonwealth Scientific Industrial Research Organization (CSIRO) stock culture collection, Australia. *Bifidobacterium longum* 55815, an oxygen-resistant strain was obtained from ATCC (Manassas, VA). One hundred microliters of an 18-h-old inoculum of these strains grown anaerobically in MRS broth (Oxoid, Australia) with A_{600nm} of 0.6 was added aseptically to 200 ml of MRS broth in a 500-ml conical flask and stoppered with a cotton plug. Each strain was grown under 0, 5, 10, 15, and 21% oxygen at 37°C for 24 h using a hypoxic glove box (Coy Laboratory Products, Grass Lake, MI). At 0% oxygen, the glove box contained a gaseous atmosphere of 95% N_2 and 5% H_2 . The various oxygen concentrations in the glove box were created by replacing hydrogen with oxygen and adjusting the nitrogen levels accordingly. Each culture was tested in duplicate. The flasks containing the culture broth were agitated using a magnetic stirrer. The culture broth after incubation was centrifuged at $10,000 \times g$ for 20 min at 4°C. The

cell free supernatant was used to estimate lactic acid and acetic acid. The cell pellet was washed three times with 0.1 M phosphate buffer, pH 7.0, and part of it was used for the determination of H_2O_2 decomposing ability and the sensitivity to H_2O_2 . The remaining cell pellet was used for preparing the cell free extract.

Preparation of Cell Free Extract

The washed cell pellet suspended in 0.1 M phosphate buffer (pH 7) was passed through a French 20K pressure mini-cell (Spectronic, USA) at 20,000 psi. Cell debris was removed from the cellular contents by centrifugation at $12,000 \times g$ for 15 min. The cell free extract collected was used for assaying levels of NADH oxidase, NADH peroxidase, and SOD. The protein content of the cell-free extract was determined according to Bradford using bovine serum albumin as the standard.

H_2O_2 Sensitivity Assay

Sensitivity of the cells to H_2O_2 was assayed based on the method reported by Shimamura et al. (1992). Cells were exposed to 10,000 mg/L, 20,000 mg/L, and 30,000 mg/L of H_2O_2 for 1 min. Appropriate dilutions of the cell suspension exposed to H_2O_2 were spread plated on MRS agar (Oxoid, Australia). Plates were incubated under anaerobic conditions at 37°C for 48 h, and the cell counts were enumerated.

H_2O_2 Decomposing Ability

The ability of the cell pellet to decompose H_2O_2 was determined based on method reported by Shimamura et al. (1992). Known amount of cells were incubated anaerobically with 300 nmol H_2O_2 at 37°C for 1 h. The concentration of residual H_2O_2 in the test tube after incubation was estimated by the method described by Marty-Teyssset et al. (2000).

Determination of Acetic Acid and Lactic Acid Levels

The cell-free broth was clarified using Carrez reagents. Five milliliters of Carrez-I-solution [potassium hexacyanoferrate (II), 85 mM] and 5 ml of Carrez-II-solution (Zinc sulfate, 250 mM) were added to 60 ml of distilled water containing 10 ml of the cell free broth. The pH of the solution was adjusted to 8 using 0.1 N NaOH, and the volume was made up to 100 ml with distilled water. The solution was mixed with activated charcoal (1%), agitated and then filtered. The concentrations of lactate and acetate in the clarified broth were determined using commercially available kits

(Boehringer Mannheim) and used for the calculation of the lactate to acetate ratio in the *Bifidobacterium* spp.

Enzyme Assays

Activities of NADH oxidase and NADH peroxidase were assayed spectrophotometrically as described by Talwalkar et al. (2003) by measuring the initial linear slope of oxidation of NADH at 340 nm at 25°C ($\epsilon = 6.22 \text{ M}^{-1} \text{ cm}^{-1}$). The reaction mix contained the cell free extract, NADH (67 μM), FAD (67 μM), H_2O_2 (1 mM) and McIlvaine buffer, pH 4.5 to 6.5 in a total volume of 3 ml. The pH of the McIlvaine buffer was adjusted using an inoLAB pH Level 1 meter (WTW GmbH, Germany). The assay was conducted for 3 min in the presence as well as in absence of oxygen. NADH oxidase activity was derived from the difference in the slopes. The slope of the anaerobic assay provided the NADH peroxidase units. For both these enzymes, 1 U of activity was defined as the amount that oxidized 1 nmol of NADH per minute at 25°C.

SOD was measured based on the method reported by Sun and Zigman (1978). One hundred microliters of epinephrine (0.1 M) was added to 100 μl of cell-free extract in 1.9-ml 50 mM Tris-HCl buffer (pH 7.5) and the inhibition of epinephrine autooxidation was monitored at 320 nm. One unit of SOD was defined as the amount inhibiting the rate of epinephrine autooxidation by 50%.

The specific activities of NADH oxidase, NADH peroxidase and SOD were calculated by dividing the total enzyme units (EU) by the total protein of the cell free extract.

Statistics

The means from six replicates were analyzed using single factor ANOVA ($\alpha = 0.05$) MS Excel software. Significant differences among individual means were determined using Tukey's HSD test.

RESULTS

Levels of Lactic Acid and Lactate-to-Acetate Ratio

Lactobacillus acidophilus 2400 and *L. acidophilus* 2409 demonstrated a significant ($P < 0.05$) reduction in the production of lactate as the oxygen in the hypoxic glove box was increased (Table 1). The decrease in lactate levels correlated strongly ($r^2 = 0.9$) with the increase in the oxygen percentage. From 0 to 21% oxygen, lactate levels in *L. acidophilus* 2400 decreased 71% from 6.9 to 2 mg/ml. These levels were similar to those seen in *L. acidophilus* 2409 in which the lactate produc-

tion decreased by 64%. No acetate was detected in the culture broth of either *L. acidophilus* strains.

Levels of lactate followed a similar correlating trend ($r^2 = 0.9$) in the *Bifidobacterium* spp. (Table 1). Except for *B. lactis* 1941, in all the other bifidobacteria tested, concentrations of lactate at the various oxygen percentages were significantly different ($P < 0.05$) from each other. In *B. lactis* 1941, no significant reduction ($P > 0.05$) was seen in lactate levels when the oxygen was increased from 15 to 21%. The decrease in lactate, however, varied among the strains. The levels of lactate in *B. infantis* 1912 dropped sharply by 85% when the oxygen was increased from 0 to 15%, whereas in *B. pseudolongum* 1944, lactate levels at 0 and 21% oxygen differed by only 24%. Interestingly, under anaerobic conditions, lactate levels in *B. lactis* 1941 and *B. pseudolongum* 1944 were double to that produced by the oxygen tolerant *B. longum* 55815. Except for *B. infantis* 1912, all the other *Bifidobacterium* spp. tested in this study were able to grow in 21% oxygen.

The decrease in lactate levels and increased production of acetate in the *Bifidobacterium* spp. caused significant lowering of the lactate to acetate ratio ($P < 0.05$) (Table 1.). The decrease in the ratio was strain dependent. As the concentration of oxygen was increased to 21%, the ratio decreased differently in *B. pseudolongum* 1944 and *B. longum* 55815 even though both strains had a lactate/acetate ratio of 2.5 at 0% oxygen. While the ratio dropped 36% in *B. pseudolongum* 1944, it decreased steeply by 98% in *B. longum* 55815. Similarly, *B. infantis* 1912 exhibited a sharp decrease of 82% in the lactate-to-acetate ratio when the oxygen concentration was increased from 0% oxygen to 15% oxygen, whereas there was only a 55% decrease in *B. lactis* 1941.

H_2O_2 Decomposing Ability

As the oxygen concentration was increased stepwise from 0 to 21%, except for *B. pseudolongum* 1944, all strains showed a significant ($P < 0.05$) rise in their ability to decompose H_2O_2 (Table 2). In *B. pseudolongum* 1944, no increase in the H_2O_2 decomposition capacity was seen when the oxygen was raised from 15 to 21%. In all the strains, the extent of H_2O_2 decomposed was observed to be strain dependent. At 21% oxygen, while the H_2O_2 decomposing ability of *B. lactis* 1941 was 11 times higher than that observed at 0% oxygen, in *L. acidophilus* 2409 it was found to increase by 73%. When grown in similar concentrations of oxygen, the H_2O_2 decomposing ability of *L. acidophilus* strains was at least twice of that seen in the *Bifidobacterium* spp. At 0% oxygen, the H_2O_2 decomposing ability of *B. longum* 55815 was almost seven times that of *B. pseudolongum*

Table 1. Effect of different oxygen concentrations on the lactic acid produced by *Lactobacillus acidophilus* strains and on the lactate to acetate ratio in *Bifidobacterium* spp.^A.

Strain	% Oxygen	Lactic acid (mg/ml) ^B	Lactate/Acetate ^C
<i>L. acidophilus</i> 2400 ¹	0	6.9	...
	5	5.8	...
	10	4.6	...
	15	2.3	...
	21	2.0	...
<i>L. acidophilus</i> 2409 ²	0	6.5	...
	5	5.8	...
	10	4.3	...
	15	2.8	...
	21	2.3	...
<i>B. infantis</i> 1912 ³	0	11.2	4.1
	5	4.3	1.8
	10	4.0	1.7
	15	1.6	0.7
<i>B. lactis</i> 1941 ⁴	0	13.0	5.9
	5	8.1	4.5
	10	7.6	3.8
	15	5.5 ^a	2.9
	21	5.2 ^a	2.6
<i>B. pseudolongum</i> 1944 ⁵	0	10.5	2.5
	5	9.4	2.3
	10	9.1	2.2
	15	8.2	1.9
	21	7.9	1.5
<i>B. longum</i> 55815 ⁶	0	5.0	2.5
	5	3.1	2.1
	10	2.1	1.7
	15	0.7	0.3
	21	0.1	0.05

^AMean (n = 6).^B1,2,3,4,5,6Standard error of least square means = 0.07, 0.05, 0.007, 0.07, 0.08, and 0.02, respectively; (df = 25)¹, 2, 4, 5, 6, (df = 20)³.^C3,4,5,6Standard error of least square means = 0.007, 0.04, 0.02, and 0.03, respectively; (df = 25)⁴, 5, 6, (df = 20)³.^aMeans in columns with common subscript do not differ significantly ($P > 0.05$).

1944. The H₂O₂ decomposing ability in *L. acidophilus* 2400 and *B. longum* 55815 at 21% oxygen was almost double to that observed when they were grown under 0% oxygen.

Sensitivity to H₂O₂

In all the probiotic strains tested in this study, exposure to 10,000, 20,000, and 30,000 mg/L of H₂O₂ did not cause any significant decrease ($P > 0.05$) in the cell counts (Table 3). Moreover, this trend did not change even when cells were grown in the different oxygen concentrations.

NADH Oxidase and NADH Peroxidase Activities

In both *L. acidophilus* as well as *Bifidobacterium* spp., the pH profiles of NADH oxidase (Figure 1) and NADH peroxidase (Figure 2) revealed maximal activity

at pH 5.0 and it remained unchanged even when cells were grown in different oxygen environments.

Except in *B. infantis* 1912, in all other strains, the specific activities of intracellular NADH oxidase and NADH peroxidase at 21% oxygen were significantly higher ($P < 0.05$) than those observed at 0% oxygen (Table 2). In *B. infantis* 1912, NADH oxidase units increased significantly ($P < 0.05$) when oxygen was increased from 0 to 5%, but no further increase was seen when it was grown in 10 and 15% oxygen. Similarly, the specific activity of NADH peroxidase increased significantly ($P < 0.05$) when the oxygen was increased from 0 to 5%, but no significant change ($P > 0.05$) was seen in its specific activity when oxygen was further increased to 10 and 15% (Table 2). In anaerobic conditions (0% oxygen), the specific activity of NADH oxidase and NADH peroxidase in *L. acidophilus* strains were at least 1.6 times higher than in the *Bifidobacterium* spp. Among the bifidobacteria, *B. longum* 55815 had

Table 2. Effect of different oxygen concentrations on the specific activities of NADH oxidase, NADH peroxidase, and superoxide dismutase (SOD) and on the H₂O₂ decomposing ability of *Lactobacillus acidophilus* strains and *Bifidobacterium* spp.

Strain	% Oxygen	NADH oxidase ^A	NADH peroxidase ^B	S.O.D. ^C	nmol H ₂ O ₂ decomposed ^D
<i>L. acidophilus</i> 2400 ¹	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> 2409 ²	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> 1912 ³	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> 1941 ⁴	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> 1944 ⁵	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 ⁶	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.

^AStandard error (df = 25) = 0.3¹, 0.3², 0.2³ (df = 20), 0.2⁴, 0.1⁵, 0.2⁶.

^BStandard error (df = 25) = 0.3¹, 0.3², 0.1³ (df = 20), 0.1⁴, 0.1⁵, 0.2⁶.

^CStandard error (df = 25) = 0.02¹, 0.03², 0.1³ (df = 20), 0.05⁴, 0.02⁵, 0.05⁶.

^DExpressed as nmol H₂O₂ decomposed per 10⁹ cfu.

^DStandard error (df = 25) = 0.2¹, 0.07², 0.01³ (df = 20), 0.03⁴, 0.07⁵, 0.01⁶.

the highest concentrations of NADH oxidase and NADH peroxidase, although no significant increase ($P > 0.05$) was observed in the enzyme concentrations after increasing the percentage of oxygen from 15 to 21%. In *B. infantis* 1912, *B. lactis* 1941, and *L. acidophilus* 2409, the concentrations of NADH peroxidase were found to correlate strongly ($r^2 = 0.9$) with their H₂O₂ decomposing ability (Table 2).

SOD Activity

Concentrations of SOD failed to demonstrate any conclusive trend with the various oxygen percentages, although it was detected in every strain (Table 2). In contrast to the increasing concentrations of NADH oxidase and NADH peroxidase, the concentrations of SOD were uncorrelated ($r^2 < 0.9$). Among all the strains,

however, *B. longum* 55815 had the highest intracellular concentrations of SOD.

DISCUSSION

Oxygen plays a critical role in the metabolism of bifidobacteria and *L. acidophilus* (Condon, 1987). Although these strains are categorized as anaerobes and microaerophilic, respectively (Hammes and Vogel, 1995; Sgorbati et al., 1995), in our study, except for *B. infantis* 1912, all remaining strains were able to grow well at 21% oxygen. This was unexpected as bifidobacteria are classified as strict anaerobes, suggesting that these particular strains possessed a mechanism to overcome the deleterious effects of oxygen toxicity. Results demonstrated that growth under the various oxygen concentrations clearly altered the metabolic and bio-

Table 3. Effect of exposure to H₂O₂ on the survival (log₁₀ cfu/ml) of *Lactobacillus acidophilus* strains and *Bifidobacterium* spp. grown in different oxygen concentrations.

Strain	% Oxygen	H ₂ O ₂ (mg/kg)			
		0	10,000	20,000	30,000
<i>L. acidophilus</i> 2400	0	9.7 ^a	9.7 ^a	9.7 ^a	9.7 ^a
	5	9.8 ^a	9.8 ^a	9.8 ^a	9.8 ^a
	10	9.7 ^a	9.7 ^a	9.7 ^a	9.7 ^a
	15	9.5 ^a	9.5 ^a	9.5 ^a	9.5 ^a
	21	9.8 ^a	9.8 ^a	9.8 ^a	9.8 ^a
<i>L. acidophilus</i> 2409	0	9.9 ^a	9.9 ^a	9.9 ^a	9.9 ^a
	5	9.6 ^a	9.6 ^a	9.6 ^a	9.6 ^a
	10	9.8 ^a	9.8 ^a	9.8 ^a	9.8 ^a
	15	9.8 ^a	9.8 ^a	9.8 ^a	9.8 ^a
	21	9.9 ^a	9.9 ^a	9.9 ^a	9.9 ^a
<i>B. infantis</i> 1912	0	9.9 ^a	9.9 ^a	9.9 ^a	9.9 ^a
	5	9.7 ^a	9.7 ^a	9.7 ^a	9.7 ^a
	10	9.9 ^a	9.9 ^a	9.9 ^a	9.9 ^a
	15	9.5 ^a	9.5 ^a	9.5 ^a	9.5 ^a
<i>B. lactis</i> 1941	0	9.9 ^a	9.9 ^a	9.9 ^a	9.9 ^a
	5	9.7 ^a	9.7 ^a	9.7 ^a	9.7 ^a
	10	9.8 ^a	9.8 ^a	9.8 ^a	9.8 ^a
	15	9.6 ^a	9.7 ^a	9.7 ^a	9.7 ^a
	21	9.8 ^a	9.8 ^a	9.8 ^a	9.8 ^a
<i>B. pseudolongum</i> 1944	0	9.5 ^a	9.5 ^a	9.5 ^a	9.5 ^a
	5	9.6 ^a	9.6 ^a	9.6 ^a	9.6 ^a
	10	9.7 ^a	9.7 ^a	9.7 ^a	9.7 ^a
	15	9.7 ^a	9.7 ^a	9.7 ^a	9.7 ^a
	21	9.9 ^a	9.9 ^a	9.9 ^a	9.9 ^a
<i>B. longum</i> 55815	0	9.7 ^a	9.7 ^a	9.7 ^a	9.7 ^a
	5	9.9 ^a	9.9 ^a	9.9 ^a	9.9 ^a
	10	9.8 ^a	9.8 ^a	9.8 ^a	9.8 ^a
	15	9.5 ^a	9.5 ^a	9.5 ^a	9.5 ^a
	21	9.8 ^a	9.8 ^a	9.8 ^a	9.8 ^a

^aMeans (n = 6) in rows with common superscripts do not differ significantly ($P > 0.05$).

chemical behavior in both *L. acidophilus* and *Bifidobacterium* spp.

Lactate and acetate are the main end products of fermentation in lactic acid bacteria. While *L. acidophilus* converts glucose to lactic acid via a homolactic fermentation (Hammes and Vogel, 1995), bifidobacteria convert two moles of glucose to form 3 moles of acetate and 2 moles of lactate (Sgorbati et al., 1995). When this molar ratio is expressed in milligrams per milliliter, a theoretical ratio of 1 is obtained and can serve as an index of the metabolic activity of the cell. The absence of acetate in the culture broths of *L. acidophilus* 2400 and *L. acidophilus* 2409 even during aerobic growth confirmed their obligate homolactic fermentation.

Condon (1987) suggested that in anaerobic conditions, lactic acid bacteria convert pyruvate to lactate by the NADH-dependent lactate dehydrogenase, regenerating NAD⁺ needed for the dehydrogenation reactions of sugar metabolism. In the presence of oxygen, however, the pyruvate metabolism can be altered by the competition of NADH oxidases and NADH peroxidases with lactate dehydrogenase for NADH affecting the fermentation end products. This provides clues to the met-

abolic changes observed in *L. acidophilus* 2400 and *L. acidophilus* 2409 when grown under increasing concentrations of oxygen. Smart and Thomas (1987) proposed that in lactic anaerobic streptococci, the regeneration of NAD⁺ by the activities of NADH oxidase: NADH peroxidase system could remove the need for conversion of pyruvate to lactate, resulting in lower lactate production. A mechanism similar to that observed in lactic streptococci may be responsible for the increase in the specific activities of NADH oxidase and NADH peroxidase and the simultaneous decrease in the lactate levels in *L. acidophilus* 2400 and *L. acidophilus* 2409. Apart from the enzyme activities, it is also probable that the decrease in lactate production in these lactobacilli was because of less growth of the cells caused by the increase in oxygen concentration.

In a study conducted on *B. lactis*, Meile et al. (1997) reported a high acetate/lactate ratio when the strain was grown anaerobically, and this ratio was found to decrease in the presence of oxygen. The four *Bifidobacterium* spp. tested in our study, however, gave contrary results. As seen in Table 1, levels of lactic acid in bifidobacteria strains were highest when they were

Figure 1. pH profile of reduced NAD oxidase of (A) *Bifidobacterium infantis* 1912, (B) *Bifidobacterium lactis* 1941, (C) *Bifidobacterium pseudolongum* 1944, (D) *Bifidobacterium longum* 55815, (E) *Lactobacillus acidophilus* 2400, and (F) *L. acidophilus* 2409 under different oxygen concentrations: (—◆—) 0%, (—■—) 5%, (—▲—) 10%, (—x—) 15%, (—●—) 21%.

Figure 2. pH profile of reduced NAD peroxidase of (A) *Bifidobacterium infantis* 1912, (B) *Bifidobacterium lactis* 1941, (C) *Bifidobacterium pseudolongum* 1944, (D) *Bifidobacterium longum* 55815, (E) *Lactobacillus acidophilus* 2400, and (F) *Lactobacillus acidophilus* 2409 under different oxygen concentrations: (—○—) 0%, (—■—) 5%, (—▲—) 10%, (—×—) 15%, (—●—) 21%.

grown anaerobically. With increase in oxygen concentrations, however, the lactate levels dropped, while acetate levels increased, reducing the lactate-to-acetate ratio. Interestingly, this closely resembles the lactate-acetate production patterns seen in other lactic acid bacteria such as lactic streptococci. Smart and Thomas (1987) found that aeration increased the pyruvate dehydrogenase in lactic streptococci, whereas lactate dehydrogenase activities decreased suggesting that aerobically, cells are more suited to produce acetate. This could explain the decrease in lactate levels and the subsequent increase in acetate production in our *Bifidobacterium* spp. when they were grown aerobically.

In earlier studies on bifidobacteria, concentrations of NADH oxidase and NADH peroxidase have been found to correlate with oxygen tolerance (Shimamura et al., 1992; Ahn et al., 2001). The limited knowledge about the pH optima of intracellular NADH oxidases and NADH peroxidases of bifidobacteria as well as the absence of a standard assay may, however, have led to inaccurate estimations of NADH oxidases in *Bifidobacterium* spp. (Talwalkar et al., 2003). The NADH oxidases and NADH peroxidases of bifidobacteria were assayed at neutral pH by de Vries and Stouthamer (1969) and Uesugi and Yajima (1978). Our study, however, revealed that maximum activity of these enzymes was at pH 5.0. The pH optima of the NADH oxidases and NADH peroxidases of our *Bifidobacterium* spp. agreed with the findings of Shimamura et al. (1992). The NADH oxidase: NADH peroxidase system has not been studied in *L. acidophilus* so far and we therefore suggest that in *L. acidophilus* strains, these enzymes be assayed at pH 5 to ensure maximal activity.

Additionally, the consistency of the optimum pH across the different oxygen concentrations in the *Bifidobacterium* spp. and *L. acidophilus* strains suggests that oxygen does not affect the pH profiles of their NADH oxidases and NADH peroxidases. The high specific activity of both NADH oxidase and NADH peroxidase in *L. acidophilus* strains and oxygen tolerant *B. longum* 55815 (Table 2) highlight the role they play in aerotolerance. The inability of *B. infantis* 1912 to increase its NADH oxidase activity seems to have resulted in its failure to grow at 21% oxygen. The strong correlation between these enzymes and the different oxygen concentrations suggests that these enzymes are inducible with oxygen acting as an inducer. These findings are similar to those seen in lactic streptococci (Higuchi, 1984; Smart and Thomas, 1987), in which the activities of NADH oxidase and NADH peroxidase increased when strains were exposed to oxygen.

NADH peroxidase activities correlated well ($r^2 \geq 0.9$) with the H_2O_2 decomposition in all the strains, underpinning this enzyme's relevance in protecting the cell

from the lethal effects of intracellular accumulation of H_2O_2 .

Bifidobacteria have been found to differ in their sensitivities to H_2O_2 and in their ability to decompose H_2O_2 (de Vries and Stouthamer, 1969; Lim et al., 1998). Shimamura et al. (1992) had reported variation in the H_2O_2 sensitivity of four *Bifidobacterium* spp. with *B. infantis* being the least sensitive to H_2O_2 . In our study, however, none of the *L. acidophilus* and *Bifidobacterium* spp. revealed any significant decrease in cell viability, even after exposure to high concentrations of 30,000 mg/L H_2O_2 . This is in contrast to Shimamura et al. (1992), where exposure to 10,000 mg/L H_2O_2 caused significant losses in the viability of three *Bifidobacterium* spp. strains. Lim et al. (1998) used longer H_2O_2 exposure times than those reported in Shimamura et al. (1992), and found a significant decrease in cell viability. It seems likely that the levels of NADH peroxidase in our strains were sufficient to protect them from the short exposure to H_2O_2 . The increase in the intracellular levels of NADH peroxidase may have also contributed to the H_2O_2 sensitivity pattern remaining unchanged over the different oxygen environments.

Anaerobes including lactic acid bacteria usually possess SOD for scavenging toxic oxygen radicals. Previous studies on the SOD of bifidobacteria and *L. acidophilus* strains have found no correlation between its specific activity and the aerobic environment (Shimamura et al., 1992; Soon-Young and Park, 1997; Lin and Yen, 1999). Results obtained in this study were in accordance with these findings. Although all strains possessed SOD, no conclusive trend could be detected with the oxygen concentration suggesting that oxygen did not seem to induce SOD. Jenny et al. (1999) proposed the role of a superoxide reductase that is independent of SOD and catalase, to detoxify oxygen in anaerobes. Superoxide reductase was not tested in this study and therefore its presence and role in the oxygen tolerance in our test strains cannot be ruled out.

Oxygen tolerance in bifidobacteria and *L. acidophilus* remains poorly studied. Oxidative stress protein and changes in cellular morphology have been detected in a *Bifidobacterium* spp., when it was exposed to oxygen (Ahn et al., 2001). The presence of a ferroxidase in bifidobacteria and iron chelation activity in *L. acidophilus* and strains of *Bifidobacterium* spp. (Kot et al., 1994; Kim et al., 2001) may be instrumental in protecting cells from peroxide by an iron sequestering mechanism (Yamamoto et al., 2000). Additionally, it has been suggested that bacteria can exhibit a common stress response offering cross protection against a variety of environmental factors (Kim et al., 2001). Our study indicates that an oxidative response is clearly triggered in these strains. The complexity of the oxida-

tive stress response in bifidobacteria and *L. acidophilus* could therefore involve a concerted action by a number of individual components interacting with each other to bring about a common stress response.

This study hence offers valuable information for understanding the precise details of the oxidative stress response in *L. acidophilus* and *Bifidobacterium* spp. It is hoped that the knowledge gained would be useful for developing techniques to prevent oxygen toxicity in probiotic bacteria. This will ultimately help in the extended survival of probiotic bacteria in dairy foods, thereby ensuring maximum therapeutic benefits to the consumer.

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