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## The Effect of Environmental Stress on the Growth of Plaque Bacteria

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**Abstract:** In this study the effect of environmental stress on the growth of *Strep. sanguinis* was investigated based on the efficiency of the bacterium to produce lactate while growing under the limitation of nutrients and environmental pH conditions. For the former, four different nutrients which include glucose, sucrose, mannitol and xylitol were used and for the later, three different environmental pH of 5.0, 5.5 and 7.0 were selected. Results showed that *Strep. sanguinis* metabolizes glucose to lactate more efficiently than sucrose and grows best at the pH 7.0. Growth in the presence of mannitol and xylitol, as well as at the pH 5.0 and 5.5, was greatly suppressed with no lactate produced. In perspective of the oral cavity, these results provide some insight to the effect faced by plaque streptococci, more specifically *Strep. sanguinis*, when the availability of nutrients and the stability of pH fluctuate in the oral environment.

**Key words:** Plaque bacteria, *Strep. sanguinis*, environmental stress, intracellular pH, glycolytic activity

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### INTRODUCTION

The residents of the oral cavity including those residing in the plaque are subjected to environmental pressure that may affect or influence their normal growth cycle. Changes in the oral environment such as those brought about by the ingestion of foods, may incur physiological and biochemical stresses on the growth and functions of these cells. Two of the most important factors that may affect the growth and functions of oral bacteria are changes in the pH and the availability of nutrients in the oral cavity (Dashper and Reynolds, 2000; Marsh, 1992).

Fluctuation of pH in the mouth from neutral to acidic may pose crucial implication to bacteria cells. Under low environmental pH, there will be influx of protons into the cells which may lead to the lowering of its intracellular pH. In addition to that, when nutrients especially carbohydrates are fermented for ATP by acidogenic plaque streptococci such as *Strep. sanguinis*, organic acid end products such as lactate are produced alongside, which may further drop the intracellular pH (Dashper and Reynolds, 2000; Carlsson, 1997; Marsh, 1991). Glycolytic activity under such condition is largely dependent on the ability of the bacteria to maintain its intracellular pH above that of the environment. In a normal functioning cell, intracellular pH is regulated by a biochemical mechanism which removes the acid from the bacteria cell, thus preventing the acidification of the cytoplasm. This way, the integrity of the bacteria cell is maintained and the process of glycolysis runs normal. In bacteria that lack the mechanism however, the drop in intracellular pH would lead to the acidification of its cytoplasm and eventually, the inhibition of its glycolytic activities (Carlsson and Hamilton, 1996). *Strep. mutans* for example, have been shown to efficiently remove lactate end products in an energy-dependent carrier mediated electroneutral process (Dashper and Reynolds, 2000). The ability of

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streptococci in removing the protons out of its cell however, varies among bacterial species. Cariogenic bacteria like *Strep. mutans* has a very efficient mechanism of doing so and that accounts for it being able to tolerate drastic pH changes in the oral environment (Dashper and Reynolds, 1996).

In contrast to the cariogenic *Strep. mutans*, not much work has been done on non-cariogenic plaque streptococci such as *Strep. sanguinis*, with respect to its responses to environmental stresses. Therefore, the aim of the study was to identify the nutritional and physical growth requirements of *Strep. sanguinis*. Four nutrients which include glucose, sucrose, mannitol and xylitol and three ranges of environmental pHs of 5.0, 5.5 and 7.0, were studied for the effect they incurred on the growth of *Strep. sanguinis*. The effect of these environmental variations on the growth of the bacteria were indicated and determined by the amount of lactate produced by the cells.

## MATERIALS AND METHODS

### Preparation of *Strep. sanguinis* Suspension

*Strep. sanguinis* used in the study was isolated from dental plaque and had been stored at -70°C under 20% glycerol as pure culture. The bacteria stock was revived in Brain Heart Infusion, BHI (Oxoid, England) broth medium. The growing bacteria were then harvested at the exponential phase (t<sub>i</sub>) by centrifugation (Centrifuge Sigma 302K, Germany) at 10,000 rpm for 20 min at 4°C. The pellet was resuspended in saline and the concentration of bacteria cells was standardized spectrophotometrically (Shimadzu UV 160A, Japan) to an optical density of 0.144 at 550 nm for used in the study.

### Bioassay for Lactate Determination

One milliliter of *Strep. sanguinis* suspension was inoculated into culture flasks containing growth media with the various growth conditions. The culture flasks were placed in a shaking water bath at a temperature of 37°C and the *Strep. sanguinis* were let to grow and propagate. To determine the efficiency of *Strep. sanguinis* in producing lactate, 500 µL of the growth suspension was aseptically pipetted out over a period of 30 min at specific intervals of 0, 2, 4, 6, 8, 10, 15, 20, 25 and 30 min. Colorimetric assay of Barker and Summerson which was later modified by Taylor (1996) was employed for the determination of lactate. Quantification of lactate was based on a standard curve obtained from readings of known lactate concentrations measured using the spectrophotometer.

### Standard Curve for Lactate Determination

Lactate (0 to 20 µg) was dispensed into test tubes at an increment of 5 µg. The volume of each tube was made to 500 µL with the addition of deionized water. Concentrated sulphuric acid (3 mL) was slowly added to the tubes and the content was thoroughly mixed with the use of a vortex mixer. The test tubes were then placed in a water bath at a temperature of 95-100°C for 10 min, following which they were taken out and put in a water bath to cool. Fifty microliter of 4% copper sulphate reagent was added to the test tubes followed by 100 µL of 1.5% p-phenylphenol in 95% ethanol. The mixture was then thoroughly mixed using a vortex mixer and left aside to cool for 30 min. The Optical Density (OD) of each tube was read at 570 nm and a standard curve of the OD readings against lactate concentration was plotted.

### Determining the Effect of Nutrients on the Production of Lactate by *Strep. sanguinis*

A 1.0 mL volume of *Strep. sanguinis* suspension was aseptically inoculated into culture flasks containing 50 mL of basic nutrient medium (NM) which have been enriched with 5% of, (i) glucose, (ii) sucrose, (iii) mannitol and (iv) xylitol. The culture flasks were placed in a shaking water bath at a temperature of 37°C to allow the cells to grow and propagate. At specific intervals, 500 µL aliquots of the growth medium were taken for lactate determination.

**Determining the Effect of Environmental pH on the Production of Lactate by *Strep. sanguinis***

A 1.0 mL volume of *Strep. sanguinis* suspension was inoculated into culture flasks containing 50 mL of basic nutrient medium (NM) at pH which have been adjusted with H<sub>2</sub>SO<sub>4</sub> or NaOH to, (a) pH 7.0, (b) pH 5.5 and © 5.0. The cells were then allowed to grow and propagate in a shaking water bath at 37°C. Likewise the above procedure, 500 µL aliquots of the growth medium in (a), (b) and © were then pipetted out at specific intervals for the determination of lactate production. Throughout the study a basic nutrient medium, NM (Oxoid, England) was used as a control growth medium for *Strep. sanguinis*.

**RESULTS**

A normal sigmoidal growth curve was obtained when the cells were cultured in BHI broth medium, the pattern was not obtained when the cells were grown in nutrient medium (NM) (Fig. 1). The mean exponential growth phase (t<sub>1</sub>) in BHI was determined at 415 min. t<sub>1</sub> for growth in NM was not determinable as much of the curve was spend under the lag phase.

**The Effect of Nutrients on the Production of Lactate by *Strep. sanguinis***

Figure 2 shows that sugars, glucose and sucrose were more favored by *Strep. sanguinis* as substrates in glycolysis compared to the derivatives mannitol and xylitol. In the first 2 min glucose

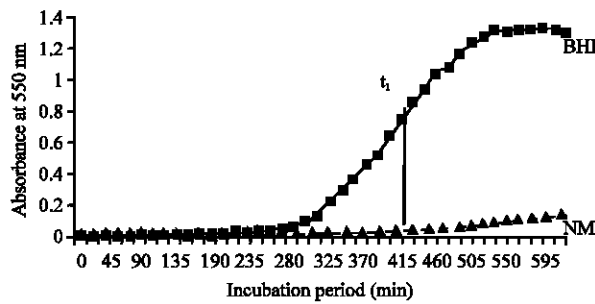


Fig. 1: Normal growth profiles of *Strep. sanguinis* when grown in a rich Brain Heart Infusion (BHI) and basic Nutrient Media (NM) t<sub>1</sub> is the time taken by the cells to reach an active exponential growth phase

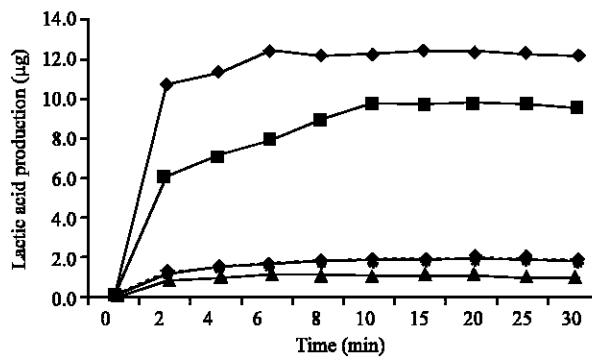


Fig. 2: Lactate produced by *Strep. sanguinis* when cultured in Basic Nutrient (NB) enriched with 5% of glucose, sucrose, mannitol and xylitol. *Strep. sanguinis* was cultured in NM for control (♦) glucose; (■) Sucrose; (x) control; (\*) mannitol and (▲) xylitol

was more efficiently utilized and metabolized to lactate compared to sucrose at a rate of  $5.290 \pm 0.016$  and  $2.990 \pm 0.013 \mu\text{g min}^{-1}$ , respectively. In contrast, lactate was produced at a lower rate of  $0.610 \pm 0.004 \mu\text{g min}^{-1}$  (mannitol) and  $0.380 \pm 0.001 \mu\text{g min}^{-1}$  (xylitol) (Table 1). Lactate was produced at a similar rate as mannitol in basic nutrient medium (control).

### The Effect of Environmental pH on the Production of Lactate by *Strep. sanguinis*

Environmental pH has a direct influence on the ability of *Strep. sanguinis* to produce lactate. The production of lactate was only observed when *Strep. sanguinis* was grown in a medium of pH 7.0. No lactate was produced when the pH of growth medium was altered to pH 5.5 or 5.0 (Fig. 3). At neutral growth environment, the rate of lactate production in the first 2 min was  $0.607 \pm 0.004 \mu\text{g min}^{-1}$ . The rate however decreases from the first 2 min onwards to  $0.091 \pm 0.043 \mu\text{g min}^{-1}$  (Table 2).

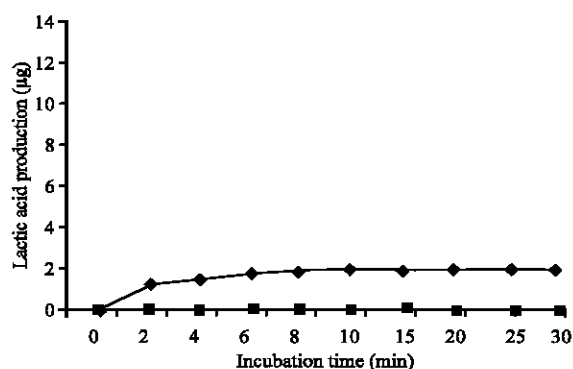


Fig. 3: Lactate produced by *Strep. sanguinis* when cultured in basic nutrient (NM) with pH adjusted to pH 7.0, 5.5 and 5.0. (◆) pH 7.0; (●) pH 5.5 and (■) pH 5.0

Table 1: The rate of lactate produced when *Strep. sanguinis* was cultured in the presence of favorable (glucose and sucrose) and stressed (mannitol and xylitol) nutrient requirement

Growth media enriched with substrate (pH 7.0)	Lactate production		
	Rate of production within the first 2 min ( $\mu\text{g min}^{-1}$ )	Time taken for lactate production to stabilize (min)	Rate of production at stabilized condition ( $\mu\text{g min}^{-1}$ )
Control	$0.59 \pm 0.004$	6	$0.30 \pm 0.050$
Glucose	$5.29 \pm 0.016$	6	$2.03 \pm 0.126$
Sucrose	$2.99 \pm 0.013$	10	$0.96 \pm 0.123$
Mannitol	$0.61 \pm 0.004$	6	$0.30 \pm 0.050$
Xylitol	$0.38 \pm 0.001$	6	$0.17 \pm 0.063$

Table 2: The rate of lactate produced when *Strep. sanguinis* was cultured in basic nutrient medium but under the influence of different environmental pHs. Minimal production of lactate was observed at neutral pH. The growth at pH 5.5 and 5.0 was greatly suppressed as there was no production of lactate observed (ND represents a non-determined reading)

Growth media with adjusted (pH)	Lactate production		
	Rate of production within the first 2 min ( $\mu\text{g min}^{-1}$ )	Time taken for lactate production to stabilize ( $\mu\text{g min}^{-1}$ )	Rate of production at stabilized condition ( $\mu\text{g min}^{-1}$ )
7.0	$0.607 \pm 0.004$	20	$0.091 \pm 0.043$
5.5	ND	ND	ND
5.0	ND	ND	ND

## DISCUSSION

The microbial component of the dental plaque lives in harmony with the host without causing adverse effect to the oral environment. *Strep. sanguinis* is one of the resident microflora which benefits the host by contributing to the normal development of the host's physiology and defense system. Like all bacteria, the growth of *Strep. sanguinis* follows a normal sigmoidal curve when all the requirements needed, both nutritional as well as physical, is provided adequately. Oral streptococci are saccharolytic bacteria that prefer carbohydrates as their main energy contributor. Within the mouth, shortage of these nutrients does not happen as the saliva is loaded with carbohydrates such as glycoproteins and mucins (Frandsen, 1994; Marsh, 1992; van der Hoeven *et al.*, 1990). In *in vitro* experiment, the effect of a growth condition on a bacterium can be seen on the growth curve it produced. BHI is a suitable growth medium for *Strep. sanguinis* as it provided both nutritional and physical requirements to produce a sigmoidal growth curve. Basic nutrient medium (NM) only contained the extract of yeast and peptone as nutrient provider and thus the effect on the growth curve was the reversed of the BHI.

When ATP is produced from the catabolism of the carbohydrates, acids like lactate are generated as well (Dashper and Reynolds, 2000). Results obtained showed that comparative to sucrose, more lactate was produced when glucose was provided as nutrient provider. The generation rate was also higher with glucose than sucrose. Being a monomer, glucose is readily transported into the cell to be metabolized compared to sucrose, a disaccharide which needs to be hydrolyzed prior to transportation into the cell for glycolysis. The production of lactate which eventually stabilized after the first few minutes show that there exist a natural mechanism in the cells to rid of excess protons. The presence of such mechanism has been shown to be a proton-translocating ATPase in *Strep. mutans* (Dashper and Reynolds, 2000). Results obtained suggested the presence of a similar mechanism for the *Strep. sanguinis*. The efflux of protons ensures that intracellular pH is maintained at pH that the cell can tolerate. In the oral cavity, production of acidic by-products would not impose any changes to the plaque ecology if the plaque were thin because the flow of saliva, with its buffering properties continuously neutralizes the acids.

Sugar alcohols like mannitol and xylitol are not favorably utilized by *Strep. sanguinis* which was in accordance with earlier reports (Edgar, 1998; Tapitsoglou *et al.*, 1983). Very low or no lactate was produced under these nutrient-stressed conditions. Xylitol interferes with sugar metabolism by consuming PEP and NAD<sup>+</sup> during futile cycle and competitively inhibit glycolysis by forming either xylitol-5-phosphate or xylulose-5-phosphate (Trahan, 1995; Waaler, 1992). There has been much interest to study the effect of these sugar alcohols on oral bacteria because of their great potential as sucrose substitutes. *Strep. mutans* and *Lactobacillus* has been reported to have the ability to ferment some sugar alcohols like mannitol and sorbitol, although the rate of utilization was very slow (Edgar, 1998; Yamada *et al.*, 1980; Tanzer, 1989).

When plaque gets thicker and supports the accumulation of plaque acids, the pH extracellular to the bacteria cells becomes very acidic and creates a stressed condition for its growth. In the study, when the growth requirement was minimally provided (growth in NM, at pH 7.0) some production of lactate (<2 µg) was observed, meaning that some glycolytic activity has taken place and that the growth of *Strep. sanguinis* has been minimally supported by NM. At the low pHs of 5.5 and 5.0 however, the production of lactate was not observed. Under the stressed conditions caused by the very low environmental pH, the glycolytic activity might have been inhibited or severely suppressed so much so that lactate production was very minimal or negligible.

Therefore it can be concluded from the study that the growth of plaque bacteria such as *Strep. sanguinis* can be greatly affected by factors in its growth environment. The availability of nutrients such as glucose and sucrose supports the growth of *Strep. sanguinis*, while mannitol and xylitol did

not. However, when the production of acid gets too much, the glycolytic activity of the bacteria may be inhibited. The growth of *Strep. sanguinis* was optimal at neutral pH. Acidic pH of pH 5.5 and 5.0 inhibited its growth. In perspective of the oral cavity, results of this study provide some insight to the effects faced by plaque bacteria, more specifically *Strep. sanguinis*, when there are nutritional and environmental fluctuations in the oral environment.

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