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A NOVEL, RAPID ADHERENCE ASSAY FOR SCREENING FOR ORAL PROBIOTICS

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Abstract: Probiotics are emerging as one of the novel oral health care management tools in the recent years. Assessing bacterial candidates as potential oral probiotics using various criteria forms an indispensable part in their development. Adherence to tooth surface being an essential quality for probiotic cells, has been evaluated earlier by several time consuming, expensive and tedious quantitative methods. Here a rapid, reliable and cost effective qualitative assay for screening of microbial cultures as candidates as oral probiotics, is described. The proposed assay is considered to provide a preliminary assessment for probable probiotics with ability to adhere to tooth surfaces. This approach is based on the novel utilization of extracted human tooth to mimic the actual oral cavity conditions. Sensitivity of this method is attributed to the incorporation of a redox indicator dye resazurin in the growth medium. Selection of the bacteria showing desired characteristic was made by the visual detection of color change of dye from blue to pink. Twenty probable lactic acid bacteria from a total of 54 were found to have ability to strongly bind to extracted tooth. This method is proposed to be suitable for rapid primary qualitative determination prior to quantitative evaluation which is a crucial part in developing oral probiotics.

Keywords: Oral probiotics, adherence, human tooth, resazurin, qualitative screening.

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INTRODUCTION

In the recent years oral health has become a major concern all over the world as these problems are rapidly increasing due partly to changes in lifestyle and food habits [1].There is a well-established link between oral health and overall wellbeing due to which oral health care systems are offering improved ways to manage associated problems [2]. Use of antimicrobial and anti-inflammatory agents and mechanical removal of dental plaques form the major core of conventional means for treating oral problems like dental caries, periodontitis, gingivitis, halitosis, *etc*. Emergence of antibiotic resistance by pathogens and some side effects of aforementioned treatments have generated a strong requirement for alternative ways to prevent or treat oral health issues[3].In this context, using probiotics to balance oral microflora has proven to be very effective way to control oral conditions. Oral probiotics are claimed to show their benefits by competing for adhesion sites and nutrients with pathogens, production of antimicrobial compounds against them, enhancing host immune response, etc. [4,5].

Screening of microbial cultures for potential use as oral probiotics can be performed by assessing certain attributes. Adherence to oral tissues helps the probiotic bacteria to establish themselves in the oral cavity. Such adherence ability significantly benefits the bacteria that form the oral microbial flora. Therefore it is one of the important selection criteria to be applied for screening of oral probiotics [6].Microbial adhesion to hydrocarbons or non-polar solvents like n-Hexane, Xylene, etc to measure hydrophobicity of bacterial cell surfaces has been very commonly used means for such adherence studies [7].Prior art also reports various methods like measurement of bacterial attachment to saliva coated hydroxyapatite beads or oral epithelium for assessing this criterion [8].Development of in vitro models to evaluate adhesive properties with respect to glass, human enamel, different dental materials have also been considered[9,10].

Such assays, however, rely on indirect evaluation of measurement of bacterial adhesion and may be misleading as they do not necessarily mimic the real oral cavity conditions. Few assays which are based on minimizing these disconformities, by keeping experimental conditions as close as possible to in vivo oral conditions, are too laborious and time consuming for screening purpose. Hence, there is a need to develop some direct method which rapidly evaluates bacteria for their ability to adhere to real human tooth surface. The present work has been focused on development of a rapid, adherence screening method to select potential probiotics for oral health in humans. This assay system employs resazurin dye to qualitatively assess the ability of bacteria to adhere to extracted healthy human tooth.

Extracted healthy human teeth were procured from dental clinic of Juhu Church Trust, Mumbai, India. Primary cleaning procedure involved initial sonication pulses in ethanol for 20 min at

36+/- 3 KHz, followed by distilled water to remove any debris attached to teeth surfaces[11].Additionally, teeth individually suspended in 5ml D/W in 15 mL screw cap glass vial were sterilized by autoclaving for 20 min and then used in the assay. Probable lactic acid bacteria (LAB) isolated from various fermented foods by procedure previously described by us [12], were subjected to the screening assay. Each isolate was initially grown in3 mL Brain Heart Infusion Broth (BHIB) at 37 °C under microaerophilic conditions for 16 h using an anaerobic jar. The culture broth was centrifuged at 11500 g for 5 min to obtain a pellet which was resuspended in 3 ml normal saline. Optical density of this culture suspension was adjusted to 0.11 +/-0.01 at 620nm. The extracted sterilized healthy human tooth was incubated in 5 ml of culture suspension at 37 °C. After 90 min the treated tooth was aseptically transferred from culture suspension to fresh saline. It was then subjected to a washing step that involved sonication for 1 min at 36+/- 3 KHz followed by 2 washes in sterile saline. Washed tooth was then incubated in 5 mL BHIB containing resazurin dye at a concentration of 10 ppm at 37 °C up to 4 hours under microaerophilic conditions using an anaerobic jar. Reduction of blue color of resazurin to pink color due to growth of cells adhering to the tooth indicated the ability of LAB isolates to adhere to teeth surfaces [13]. Tooth suspended in sterile saline served as negative control. Figure 1 shows the results for representative LAB isolates under study and indicates the change in color of resazurin dye post final incubation. Phase contrast microscopy was used to ascertain the morphology and purity of the cultures screened.

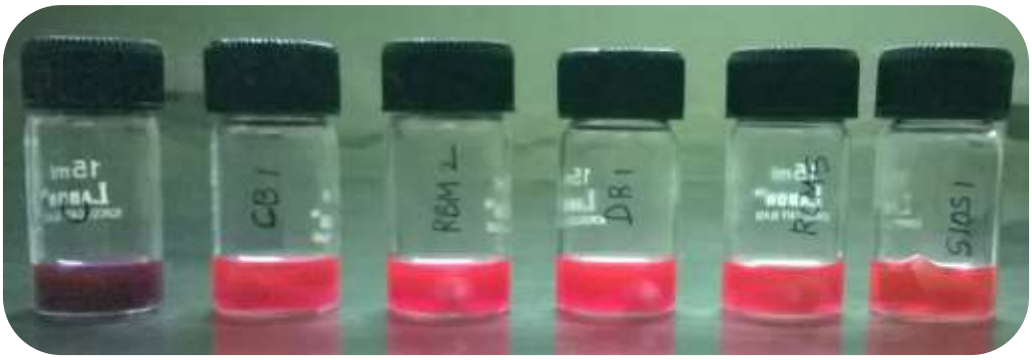

Figure 1: Visual qualitative color change in resazurin due to actively growing LAB adhering to tooth surface.

Total of 54 LAB were subjected to the aforementioned screening procedure and qualitative results were recorded as positive (pink color) or negative (no change in the blue color). Twenty out of 54 LAB isolates showed ability to adhere to tooth surface. Figure 2 graphically summarizes the results obtained. A score +1 (pink bars) indicates ability to adhere whereas score of -1 (blue bars) indicates lack of adhesion. As can be noted from the figure, 34 isolates failed to show the adhesion potential.

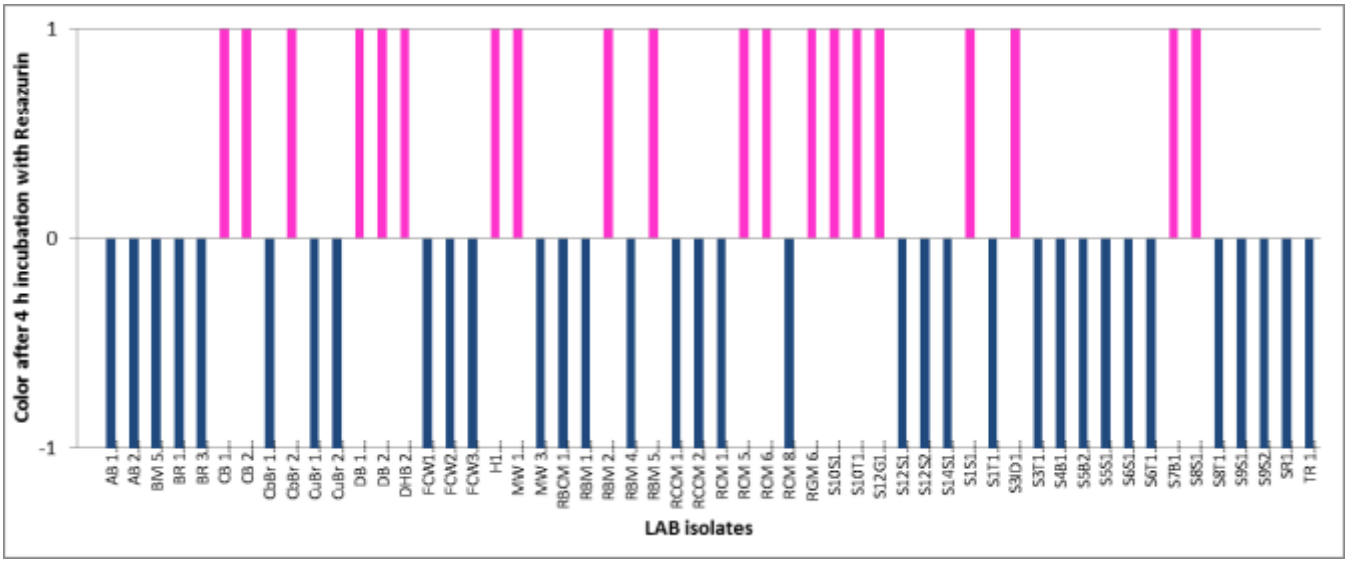

Figure 2: Graphical representation of adhering ability shown by LAB Isolates

The objective for developing this assay was to be able to select only the LAB isolates demonstrating ability to adhere to authentic human teeth. Initial incubation of LAB culture suspension with human tooth gives enough time for the bacteria with adhering ability to attach themselves to tooth surface with sufficient strength and hence can withstand the subsequent washing and sonication steps. Bacteria loosely bound due to poor ability to attach to the surface get easily dislodged during the subsequent washing step involving brief sonication. Only strongly bound isolates start growing when provided with the growth medium in the next step of assay. During this growth phase, they reduce resazurin dye to resorufin indicated by color change from blue to pink by the end of 4 h incubation indicating ability of bacteria to adhere to tooth surface.

Type of culture medium, culture conditions and growth phase of the bacteria, temperature and duration of exposure, initial number of bacteria may have a significant impact on early bacterial adhesion when tested in vitro [14,15]. Hence to minimize variation in results arising due to these factors, all the isolates were grown under identical conditions for all the 3 trials. Use of extracted human tooth is proposed as a superior alternative to commonly used and relatively expensive hydroxyapatite beads, as extracted healthy teeth are readily available and more closely reproduce human oral cavity conditions [16,17]. The assessment of bacterial adhesion to the selected surface has been performed by others through quantitative measurement of adhering cells by microscopic cell counts, viable cell count by plate assay, absorbance measurement, enumeration by radiolabelling or microcalorimetry, *etc* [10,18,19]. Such procedures are rather gruelling, time consuming and expensive and hence could be more suitable and befitting if employed subsequent to a rapid yet simple, qualitative screening

method like the one described here. Resazurin, a redox indicator dye is easy to obtain and is able to permeate easily inside the cells [20]. Further this dye can be advantageously used in measurement of cell growth, as it is more sensitive but less expensive than tetrazolium dyes and significantly reduces the time required compared to conventional methods[21].

Thus, an attempt has been made to develop a unique, rapid and inexpensive assay to screen bacterial candidates suitable as oral probiotics by treating an extracted healthy human tooth with them followed by growth in BHIB containing resazurin. We believe that we are first one to propose use of human tooth as a substrate for rapid and more realistic screening procedure for initial assessment of adherence potential of potentially useful probiotic bacterial isolates.

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