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Autoaggregation, Coaggregation and Hydrophobicity -A Mechanism to Explain Oral Probiotic Function by Isolates from Dairy Source

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KEYWORDS

Probiotics, oral health, pathogen, autoaggregation, coaggregation, hydrophobicity

ABSTRACT:

Probiotics have been shown to improve health in a number of bodily systems in order to effectively use the potential of probiotic bacteria, strains had to be examined for the collective traits that support host well-being. The aim of this study was to assess probiotic attributes such as adhesion and aggregation, this study evaluated the aggregation properties and hydrophobicity of *Bacillus valenzensis* DGM7 against oral pathogen *Enterobacter cloacae* OP1. These properties are crucial for therapeutic manipulation of oral microbiota and are desirable for probiotics in health-promoting foods. In the present study the hydrophobicity was examined against xylene, 60.34% hydrophobicity was seen at 2 hrs. of incubation and 87.12% after four hours. The probiotic isolate showed autoaggregation rates of 21.22%, 38.97%, and 55.62% at 20°C and 23.05%, 39.8%, and 57.85% at 37°C, while the pathogen isolate showed lesser autoaggregation rates of 5.69%, 19.3%, and 22.61% at 20°C and 5.79%, 20.68%, 21.75% at 37°C at different time intervals. At 20°C, the probiotic and pathogen coaggregated at rates of 6.71%, 15.21%, and 23.8% after 2,4, 24 hrs. of incubation respectively. In contrast, the coaggregation observed at 37°C was 5.94%, 13.92%, and 24.93% at 2, 4, and 24 hours. Higher the value of autoaggregation, coaggregation, and hydrophobicity properties of the isolates suggest its potential as effective probiotic for maintaining oral health and preventing pathogenic microbe colonization.

1. Introduction

Probiotics are described as live bacteria that are given in sufficient quantity to remain viable in the gut environment and they need to benefit the host showing positive effect. Probiotic organisms are increasingly used in food as dietary supplements or non-food preparations, with most commercialized probiotics containing *Lactobacillus* and *Bifidobacterium* bacteria. Other strains of *Bacillus*, *Pediococcus*, and yeasts are also suitable.

They protect the host against harmful microorganisms and strengthen immune systems. Research is also focusing on other organisms for their beneficial effects (Soccol *et al.,* 2010; Tomičić *et al.,* 2022). Bacterial colonization of the gut starts at birth and evolves over a lifetime, influenced by the host's diet, genome, lifestyle, and antibiotic use. Age-specific compositional shifts in the gut microbiota include a decrease in the Bacteroidetes/Firmicutes ratio and a significant decrease in *Bifidobacteria* in individuals over 60 years of age, during the decline of the immune system (Kerry *et al.,* 2018).

Probiotic species exhibit multicellular aggregates, either autoaggregative or coaggregative. Surface hydrophobicity and autoaggregation are closely related, with bacteria with higher hydrophobicity exhibiting stronger autoaggregation due to their cell surfaces' ability to form interactions with similar cells. Coaggregation and hydrophobicity influence bacteria's adhesion ability and interaction, with a positive correlation between adhesion ability and autoaggregation (Aslim *et al.,* 2007; Rahman *et al.,* 2008).

Aggregation and adhesion assays are crucial for identifying effective probiotic strains for various applications. These properties, promote gut/oral health and prevent pathogenic infections like dental caries,

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gingivitis, periodontitis etc. are essential for probiotics to survive harsh gut environments, effectively colonize, and offer health benefits. Understanding these properties can help select suitable probiotic candidates. Probiotic adherence is associated with stability, including autoaggregation, hydrophobicity, and the capacity of strains to survive stimulation in the gut (Lee *et al.,* 2015). Autoaggregation and coaggregation are important properties exhibited by commensal bacteria in the oral cavity.

These phenomena play a crucial role in maintaining a healthy oral microbiome and preventing the colonization of pathogenic microorganisms. The mechanism of auto-aggregation and coaggregation is quite common as this process is a resultant of the association between the group of similar (related genus) or with different (unrelated genus) microorganisms respectively (Caggia *et al.,* 2015). Although adhesion capacity doesn't guarantee health benefits, it can protect against harmful bacteria by competing for host cell binding sites (Krausova *et al.,* 2019).

The initial phase of bacterial adhesion is based on non-specific physical contacts between two surfaces, which allow adhesins-typically proteins and complementary receptors to interact specifically (Kos *et al.,* 2003). Adhesion to epithelial cells is crucial for selection, as it allows bacterial cells to have long-lasting effects, extrude pathogens, and immunomodulate the host organism, while probiotics' ability to adhere to intestinal mucus increases their survival chances (Rahman *et al.,* 2008; Grigoryan *et al.,* 2018). Human oral microbiome comprises of quite unique, having second most diverse microflora after the gut. In addition to being the initiation point of digestion, the oral microbiome is crucial in maintaining oral as well as systemic health. Because of the ease of sample collection, it has become the most well-studied microbiome till date.

Inspite of all these, about 88% of the oral microbes are not properly understood, of which 53% are not nomenclatured appropriately or well documented and 35% remain still uncultured (Chen *et al.,* 2010; Deo and Deshmukh, 2019). Oral cavity or the buccal cavity is the site of both kinds of microorganisms (beneficial and harmful), and likely also harbor the commensals. Commensals of the oral cavity keep a close watch on the growth of species which may pose harm to human health. The diversity of microorganisms in oral cavity can be from *Streptococcus, Eubacteria, Fusobacterium, Capnocytophaga, Eubacteria, Staphylococcus, Eikenella, Porphyromona, Leptotrichia, Prevotella, Peptostreptococcus, Treponema, Actinomyces* genera. Among these diverse florae, *Lactobacillus,* the spore formers, include those from the genus *Bacillus*, have been studied for potential probiotic functionalities in both *in-vitro* and *in-vivo* models. *Bacillus* strains have shown higher acid tolerance, stability during heat processing and low temperature storage, pathogen exclusion, anti-oxidant, antimicrobial, immuno-modulatory, and food fermentation abilities (Elshaghabee *et al.,* 2017). Probiotics have emerged as a promising adjunctive treatment for various oral diseases, leveraging their ability to modulate the oral microbiome and enhance oral health.

These live microorganisms, when administered in adequate amounts, can effectively prevent and treat conditions such as dental caries, periodontal disease, halitosis, and oral candidiasis (Doron and Gorbach, 2006). It has been suggested that the mode of action involves both systemic and local interactions; probiotic strains may produce antimicrobial compounds, modulate the immune system through cytokine modulation, and compete with pathogens for resources and binding sites (Keller *et al.,* 2011).

The current study was thus conducted to investigate the surface hydrophobicity, autoaggregation and coaggregation potential of isolated probiotic *Bacillus valenzensis* DGM7 (accession number-PP832844). The probiotic isolate was tested against oral pathogen *Enterobacter cloacae* OP1 (accession number- PP886733) isolated from oral diseased variables/patients (dental caries, periodontal disease) for its coaggregation capacity/ability and both the isolates were assessed for their autoaggregation abilities.

2. Materials and Methods

2.1 Selection of isolates/samples:

Isolates used in this study are an oral pathogen and a probiotic strain. The probiotic strain, *Bacillus valenzensis* DGM7 (accession number-PP832844) was isolated from dairy source and the oral pathogen *Enterobacter cloacae* OP1 (accession

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number- PP886733) was isolated from diseased variables (dental caries, periodontal disease) from SDM Dental college Dharwad in association with Dr. Roseline Meshramkar, Professor, SDM Dental College & Hospital, Dharwad.

2.2 BATH (bacterial adhesion to hydrocarbons) assay:

The bacteria (probiotic cells) were grown in luria broth for 24 hours to ascertain their surface hydrophobicity. The probiotic cells were extracted by centrifugation at 14,000 g for 5 minutes, and they were washed twice using phosphate-buffered saline (PBS; 130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2), the cells were resuspended in the same buffer until the bacterial suspension had an absorbance of around 0.5 OD at 660 nm.

An equivalent amount of xylene was then added to the cell suspension. Xylene was chosen as a non-polar solvent because it exhibits both hydrophilicity and hydrophobicity on the surface of the cell. The mixture was vortexed for five minutes, to mix the two-phase system TO homogeneity. After being incubated for one hour at room temperature, the aqueous phase was removed, and its absorbance at 600 nm was measured (Collado *et al.,* 2008; Rahman *et al.,* 2008). Surface hydrophobicity was expressed as surface hydrophobicity percentage (SHb %) and calculated using

$$\mathbf{SHb\ \% = [(A_0\text{-}A)/A_0] \times 100}$$

Where, $\mathbf{A_0}$ and **A** are the absorbances before and after extraction with xylene.

2.3 Auto aggregation assay

2.3.1 Visual autoaggregation:

Select strains were cultured overnight and then rinsed with distilled water at least three times. They were then resuspended in the same initial volume of 1x PBS solution and allowed to incubate at room temperature with at least 1% (v/v) of freshly made sterile culture supernatant.

When the aggregated cells produced visible sand-like particles and settled to the tubes' bottoms, leaving a clear supernatant, within two hours, the aggregate was considered to have adequately aggregated (Reniero *et al.*, 1992).

2.3.2 Spectophotometry autoaggregation assay:

The selected isolates were incubated in luria broth medium for 18-24 hours at 37°C to determine auto-aggregation ability of the isolate. After incubation, the cultures were centrifuged, washed with PBS (pH 7.2), and resuspended to 8 log cfu/mL.

The bacterial suspension was vortexed for 10 seconds, and its absorbance was measured at 600 nm using a UV spectrophotometer. The suspension was incubated at 37°C for 5 hours, and the absorbance was measured (Hojjati *et al.,* 2020; Topçu *et al.,* 2020). Auto-aggregation was determined in percentages with the following formula:

$$\text{Autoaggregation \%} = \left[\frac{\mathbf{OD1-OD2}}{\mathbf{OD1}}\right] \times \mathbf{100}$$

OD1 is the initial optical density at time 0hrs. and OD2 is the optical density after 5hrs. of incubation.

2.4 Coaggregation:

Spectrophotometric analysis was used to determine co-aggregation between the two isolates. After adjusting the absorbance of both the isolates to OD 0.5 at 600 nm (~10^8 cells/ml), equal quantities (3ml each) of the pathogenic and probiotic strains were mixed together and incubated at 37°C without stirring.

After one, two, and four hours of incubation, the mixed suspensions were observed by, comparing the pathogen suspension's absorbance with the mixed suspensions' absorbance, the co-aggregation ratio was calculated using the following formula (Keller *et al.,* 2011; Twetman *et al.,* 2009):

$$\text{Coaggregation \%} = \left[\frac{\mathbf{(OD1+OD2)-(OD3)}}{\mathbf{(OD1+OD2)}}\right] \times \mathbf{100}$$

OD1 is the optical density of probiotic culture, OD2 is the optical density of pathogen culture and OD3 is the optical density of mixture of probiotic and pathogen culture.

3. Results

3.1 Selected isolates:

The pure cultures and microscopic images of the selected isolates for this study are shown below in the figure 1.

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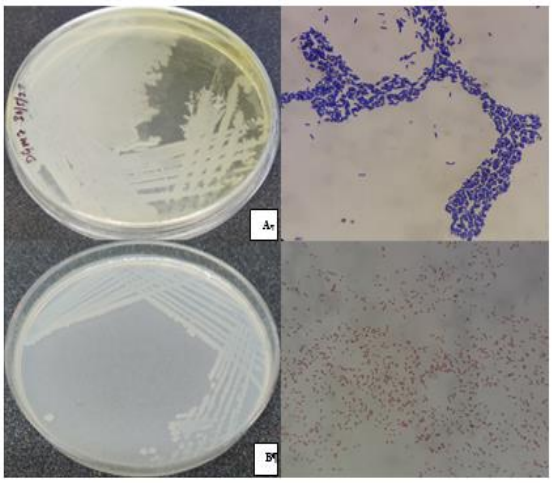

Figure 1. Pure culture and microscopic images of A-*Bacillus valenzensis* DGM7, B-*Enterobacter cloacae* OP1.

3.2 BATH assay:

The surface hydrophobicity/BATH assay was performed by using xylene as a hydrocarbon. The hydrophobicity of the isolate *Bacillus valenzensis* DGM 7 against the xylene was found to be 60.34% at 2 hrs. of incubation however, the attachment of the cells to the hydrocarbon increased with the increase in the time of incubation i.e. 4hrs. of incubation where hydrophobicity was observed to be 87.12%. figure 2 shows the percentage hydrophobicity against the hydrocarbon at different time intervals.

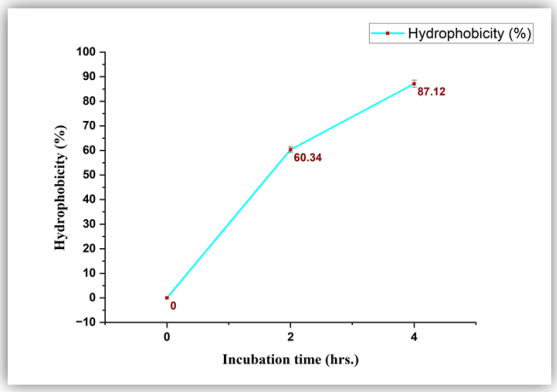

Figure 2: Hydrophobicity of *Bacillus valenzensis* DGM7.

3.3.1 Visual autoaggregation:

Probiotic strains exhibited greater autoaggregation capacities than pathogenic strains. After being incubated for 24 hours, probiotic strain tested in this study exhibited increased percentages of aggregation than the pathogenic strain. The isolate with high autoaggregation capacity showed cell aggregates formation leaving behind a clear supernatant at the end of the incubation which was further confirmed quantitatively by the spectrophotometric analysis. Figure 3 shows the cell aggregation/ aggregates in the PBS buffer.

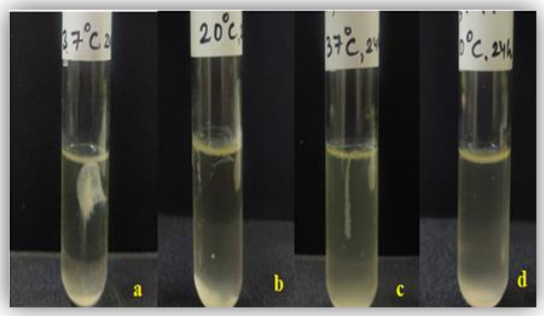

Figure 3: Visual auto aggregation of probiotic and pathogenic strains. a, b shows the autoaggregation of probiotic strain at 37℃ and 20 ℃ whereas, c, d shows auto aggregation of pathogenic strain at 37℃ and 20 ℃.

3.3.2 Spectophotometry autoaggregation assay:

Autoaggregation ability of the probiotic isolate *Bacillus valenzensis* DGM7 and the pathogenic bacterial isolate *Enterobacter cloacae* OP1. was determined by subjecting them to two different temperatures 37℃ and 20℃ at different time intervals. The following table 1 shows the % autoaggregation of both the strains.

Both the strains showed higher autoaggregation at 37℃ than 20℃. The probiotic isolate showed 21.22%, 38.97%, 55.62% autoaggregation at 2hrs, 4hrs, 24hrs respectively at 20℃ and 23.05%, 39.8%, 57.85% at 2hrs, 4hrs, 24hrs at 37℃. The pathogen isolate showed comparatively lesser autoaggregation, 5.69%, 19.3%, 22.61% at 2hrs, 4hrs, 24hrs respectively at 20℃ and 5.79%, 20.68%, 21.75% at 2hrs, 4hrs, 24hrs at 37℃ (figure 4a and 4b).

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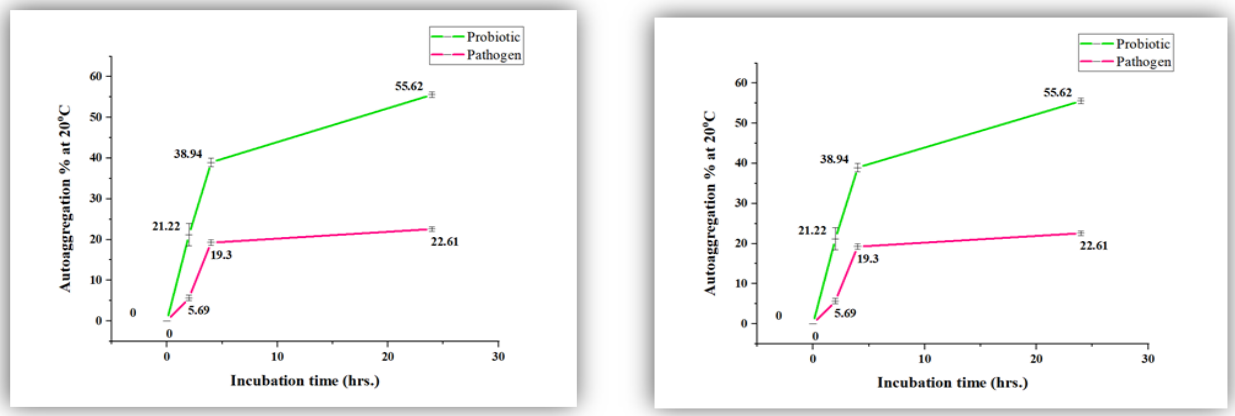
Table 1: The standard deviation (n=3) mentioned with average values of parameters autoaggregation % of probiotic and pathogenic strain at 37℃ and 20℃ at different time intervals. Tukey's test was used to differentiate the mean values at a significance level of $p<0.05$(mean±SD). a, b and c demotion are Tukey's test showing that there is significant difference at $p<0.05$. statistical analysis was carried out using Origin -9 software (Origin Lab Corporation, Massachusetts, USA) and by one way analysis of variance (Anova).

	Time	Autoaggregation % (in triplicates)			Mean %	(n≥3) ± s. d.
Probiotic isolate (20^0C)	0hrs	0.0	0.0	0.0	0.0	0.0
	2hrs	18.08	22.56	23.04	21.22667	21.22 ± 2.73^c
	4hrs	38.08	38.7	40.13	38.97	38.97 ± 1.05^b
	24hrs	55.55	55.01	56.3	55.62	55.62 ± 0.64^a
Probiotic isolate (37^0C)	0hrs	0.0	0.0	0.0	0.0	0.0
	2hrs	18.96	23.4	26.8	23.05	23.05 ± 3.93^c
	4hrs	38.98	39.86	40.56	39.8	39.8 ± 0.79^a
	24hrs	57.62	57.01	58.93	57.85333	57.85 ± 0.98^b
Pathogen isolate (20^0C)	0hrs	0.0	0.0	0.0	0.0	0.0
	2hrs	5.16	6.5	5.41	5.69	5.69 ± 0.7^b
	4hrs	19.33	20	18.57	19.3	19.3 ± 0.7^b
	24hrs	22.2	23.29	22.36	22.61667	22.61 ± 0.58^a
Pathogen isolate (37^0C)	0hrs	0.0	0.0	0.0	0.0	0.0
	2hrs	5.25	5.89	6.23	5.79	5.79 ± 0.49^b
	4hrs	20.03	20.98	21.05	20.68667	20.68 ± 0.56^c
	24hrs	21.33	21.89	22.04	21.75333	21.75 ± 0.37^a



a **b**

Figure 4 a and b. Fig. 4a shows comparative autoaggregation % of probiotic and pathogen at 37 ℃ and fig. 4b shows autoaggregation % of probiotic and pathogen at 20℃ at different time intervals.

3.3. Coaggregation:

Coaggregation was determined by spectrophotometry, where the optical densities of the probiotic, pathogen and the mixture of both the isolates was measured and calculated. The coaggregation of the probiotic and pathogen at 20℃ was found to be 6.71%, 15.21%, 23.8% at 2hrs., 4hrs. and 24hrs. of incubation respectively (table

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2). Whereas the coaggregation observed at 37℃ was 5.94%, 13.92%, 24.93% at 2hrs., 4hrs. and 24hrs. Coaggregation % of probiotic and pathogen at different time intervals and temperatures is shown in the figure 5.

Table 2: The standard deviation (n=3) mentioned with average values of parameters coaggregation % of probiotic and pathogen at different time intervals and temperatures. Tukey's test was used to differentiate the mean values at a significance level of $p<0.05$(mean±SD). a, b and c demotion are Tukey's test showing that there is significant difference at $p<0.05$. statistical analysis was carried out using Origin -9 software (Origin Lab Corporation, Massachusetts, USA) and by one way analysis of variance (Anova).

	Time	Coaggregation % (in triplicates)			mean %	(n≥3) ± s.d
Incubation at 20^0C	0hrs	0.0	0.0	0.0	0.0	0.0
	2hrs	8.05	5.6	6.5	6.716666667	6.71 ± 1.23[a]
	4hrs	16.66	13.57	15.4	15.21	15.21 ± 1.55[b]
	24hrs	22.4	21.3	27.7	23.8	23.8 ± 3.42[c]
Incubation at 37^0C	0hrs	0.0	0.0	0.0	0.0	0.0
	2hrs	6.13	6.01	5.69	5.943333	5.94 ± 0.22 [a]
	4hrs	15.05	12.55	14.17	13.92333	13.92 ± 1.26 [b]
	24hrs	23.4	25.7	25.71	24.93667	24.93 ± 1.33 [c]

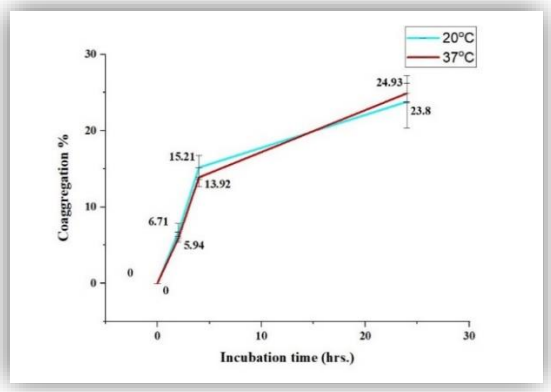

Figure 5. Coaggregation % of probiotic and pathogen at different time intervals and temperatures.

4. Discussion

Cell adhesion is an important characteristic of probiotics, involving contact between bacterial cell membranes and interacting surfaces. Many strains used as probiotics have the ability to adhere to epithelial cells and mucosal surfaces. Aggregation ability is often related to cell adherence properties, especially in the human gut, where probiotics are active (Collado *et al.,* 2008). Stimulating the beneficial bacteria and enhancing the variety of oral biofilms can serve as a supplementary approach to avoid oral illnesses. So, probiotic candidates with potential to combat oral disease can be screened using aggregation and growth inhibition tests, which are helpful methods to analyse the capabilities of probiotics (Keller *et al.,* 2011).

The capacity of probiotics to adhere is often assessed by combining surface hydrophobicity and bacterial auto-aggregation (Liu *et al.,* 2020). The benefit of probiotic bacteria lies in their adhesion to epithelial cells, enabling colonization of the oral cavity or the gastrointestinal tract. The BATH assay is commonly used to measure the hydrophobicity of probiotic's cell surface, evaluating their affinity to solvents like hexane, xylene, or toluene. In this study xylene was used to which the hydrophobicity of the *Bacillus valenzensis* DGM7 was found to be 76.34 % at 2 hrs and 87.12 % at 4hrs of incubation. BATH values ranged from 2.2% to 56.4% adherence in *Bacillus subtilis* (Ritter *et al.,* 2018) also, in a study conducted by Mohkam et al. (2016) adherence to hydrocarbon in *Bacillus sp.* ranged from 10-60%. Sarojini et al., (2020) in their study stated that hydrophobicity of *B. subtilis* subsp. *inaquosorum* was 44.81% against xylene, whereas it was 52.67% against toluene. In our present study the autoaggregation of probiotic strain was comparatively higher to pathogen strain the probiotic isolate showed 21.22%, 38.97%, 55.62% autoaggregation at 2hrs, 4hrs, 24hrs respectively

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at 20℃ and 23.05%, 39.8%, 57.85% at 2hrs, 4hrs, 24hrs at 37℃. The pathogen isolate showed 5.69%, 19.3%, 22.61% autoaggregation at 2hrs, 4hrs, 24hrs respectively at 20℃ and 5.79%, 20.68%, 21.75% at 2hrs, 4hrs, 24hrs at 37℃. Based on a significant correlation between auto-aggregation and cell attachment to the digestive system, the three isolates namely, *Bacillus velezensis* TPS3N, *Bacillus subtilis* TPS4, and *Bacillus amyloliquefaciens* TPS17 showed less than 30% autoaggregation ability during the first three hours of incubation. Autoaggregation, however, rose to 92.97%, 84.83%, and 89.13% following a 24-hour period (Kuebutornye *et al.*, 2020). The autoaggregation ability of *B. subtilis* P223 was high. Following an incubation period of 4 hours, the autoaggregation abilities of *B. subtilis* P223 and *B. clausii* ATCC 700160 were found to be 88.13 ± 3.55% and 85.10 ± 1.56%. When *B. subtilis* P223 was incubated for 24 hours, its value was found to be the same, whereas that of *B. clausii* ATCC 700160 rose (Jeon *et al.*, 2017). In a study conducted by AlGburi et al., (2020) among the examined bacteria, *B. amyloliquefaciens* B-1895 had the greatest auto-aggregation rate of 89.5 percent over a 24-hour period.

The probiotic strains' capacity to co-aggregate is essential for the development of biofilms and their rivalry with pathogens for binding sites. Compared to more complex approaches, the screening procedures employed in this work have been shown to be simple and dependable techniques (Keller *et al.*, 2011). In this investigation the coaggregation % observed by the pathogen and probiotic strain was found to be 6.71%, 15.21%, 23.8% at 2hrs., 4hrs. and 24hrs at 20℃ incubation. Whereas the coaggregation observed at 37℃ was 5.94%, 13.92%, 24.93% at 2hrs., 4hrs. and 24hrs respectively. Similarly in a study conducted by AlGburi et al., (2016) *B. amyloliquefaciens* B-1895 strain co-aggregated poorly (29.9%) with *S. aureus*, it co-aggregated effectively with *E. coli* (47.1%), *P. aeruginosa* (46.9%), S. enterica (43.9%), and *L. monocytogenes* (41.9%). With respect to *B. subtilis* KATMIRA1933, the coaggregation percentage with *E. coli* and *P. aeruginosa* was highest at 50.3%, followed by that of *L. monocytogenes* at 48.2%, *S. enterica* at 47.4%, and *S. aureus* at 34% and *S. mutans* at 31.8%. Also, City et al., (2021) stated that Co-aggregation between *B. subtilis* SM10.1 and *E. coli* was slightly higher than *B. subtilis* SM10.1 and *S. aureus*, though the percentages of both coaggregation were close to 40%. Jeon et al., (2018) The co-aggregation of *B. polyfermenticus* SCD with pathogens was higher than that of *B. subtilis* P229 after 4 h of incubation, and found to be similar after 24 h. *B. polyfermenticus* SCD and *B. subtilis* P229 showed the highest levels of coaggregation with *S. aureus* ATCC 6538 after 4 h i.e. 54.04% and 30.72%, respectively.

5. Conclusion

The probiotic *Bacillus valenzensis* DGM7 was isolated from dairy source. The pathogen strain *Enterobacter cloacae* OP1 was isolated from oral diseased variables (dental caries, periodontal disease) at SDM Dental College, Dharwad. The probiotic was assayed for its hydrophobicity it was found that the adhesion was high at 4hrs of incubation that 2 hrs. The autoaggregation assay suggest that the probiotic aggregation levels at different temperatures and time intervals was found to be higher as compared to the pathogen strain this suggest the competitive exclusion of the pathogen by probiotic can be considered. The coaggregation was not significantly differing in both the strains. As the oral diseases have effect on systemic diseases such information may be useful for the eradication of this bacterium, *Enterobacter cloacae* from the oral cavity, thus leading to the successful prevention of transmission and recolonization of the same leading to serious oral health conditions.

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