Growth Optimisation of *Bacillus subtilis* in medium supplemented with prebiotic gum Arabic

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**Abstract.** *Bacillus subtilis* is a type of gastrointestinal bacteria that build up a healthy gut microbiota. The bacterial species has been well documented to involve in maintaining a healthy gut homeostasis in which disruption in it’s microbial composition or “dysbiosis” has recently been linked to obesity. Probiotic *in vivo* restoration of dysbiosed microbiota have been proved successful in obese CD-1 mice model. However, prebiotic study using Gum Arabic (GA) assessing to optimise an *in vitro* lumen system specifically targeting to enhance the growth of *B. subtilis* are still lacking since this bacteria are depleted in obese individuals. Thus, this study aimed to establish the optimal growth conditions in simulated *in vitro* lumen system and to the best of our knowledge, this is the first *in vitro* study attempted to optimize the growth of *B. subtilis* in medium supplemented with prebiotic GA. Growth screening analysis suggested an optimal dosage of 1.0% and 0.5% glucose and GA, respectively. The highest growth rate was recorded at 0.7995 hours⁻¹ with doubling time of 52.02 minutes with extended period of stationary phase. The optimal GA concentration and fermentation conditions were determined at 0.67%, pH 7.4 and temperature of 37°C. The validated suggested model indicates that the supplementation of GA into an optimal fermentation systems is promising to enhance the growth of gut microflora *Bacillus subtilis*, for restoration of a dysbiosed gut microbiota *in vitro*.

**Introduction**

Obesity has become a worldwide pandemic as its prevalence continue to increase over the last few decades. World Health Organisation (WHO) reported that the worldwide obesity rate has nearly tripled since 1975 [1]. In a survey done in 2016, analysis of the results states that more than 1.9 billion adults aged 18 years and older, were overweight and out of this figure, 650 million adults were obese. The same survey also reported 41 million children under the age of 5 were overweight and over 340 million children and adolescents in the age range of 5 – 18 were either overweight or obese [1]. Among the re-nowned risk factors that cause obesity, dysbiosis in gut microbiota has recently been linked to the disease [2].
Bacteria in the colon capable to regulate the way human body stores fat through modulation of body responds towards carbohydrate metabolism including biologically active fatty acid, bile acids and dietary choline [3]. The human colon has the largest population of bacteria in the body and most of them are anaerobic microorganisms[4]. This microbial community consists of at least 1014 bacterial cells and up to 500–1000 different species of bacteria in which compositions according to The Human Microbiome Project, varies between individuals [5]. Distinct combinations of microbial population followed the health conditions, dietary habits and eating pattern of the host [2]. Despite variations, a type of commensals human gut intestinal probiotic bacteria Bacillus subtilis was shown to consistently involved in maintaining a healthy gut homeostasis. It displaced unfavourable gut bacteria by affecting their ability to colonise the gut [6]. The spores itself was recently been used to normalised a dysbiosoed human microbiota [7].

One of the ways to cultivate a healthy gut ecosystem is to introduce prebiotics into the system by ingestion. Prebiotics are non-digestible food ingredient that selectively stimulates growth of bacteria in the colon as it provides a suitable environment for probiotics to flourish when administered at a suitable dosage [8]. Dried exudate of Acacia Senegal tree, known as Gum Arabic (GA) is a type of heteropolysaccharide declared safe by the the Food and Drug Administration(FDA) since 1970s [8]. The compound is non-digestible as it is not accessible to various digestive enzymes in the small intestine but can be metabolised by selected gut microflora [9]. Fermentation products of GA can selectively stimulates the growth and activity of the good bacteria that produce Short Chain Fatty Acid (SCFA) metabolites without stimulating the bad one [10]. Successful used of prebiotic in obesity has been exempted in mice model CD-1 mice [11]. In human, it was tested in obese children and adult females both resulted in significant reduction of body weight through selective alteration of gut microbiota.[12].

Despite the promising in vivo studies, the optimal condition for effective fermentation of to enhance the growth of B. Subtilis in medium supplemented with prebiotic Gum Arabic shall be investigated further. It was suggested that the wheat-bran derived prebiotics compounds were greatly affected by pH and temperature whereby at pH 2 and pH 3 initiated the hydrolysis of oligosaccharides linkages[13]. Substantial decomposition was also observed when the sample was subjected to temperature higher than body temperature under alkaline condition [14]. Therefore the study intended to establish the optimal fermentation conditions (pH, temperature and prebiotic concentration) in an invitro lumen system, targeting to enhance the growth of B. subtilis.

2. Materials and Methods
2.1 Bacterial strain, growth conditions and preparation treatment
B. subtilis bacteria was bought from ATCC Catalogue number Cohn (ATCC® 6051) (Ehrenberg). The bacteria were grown anaerobically in basal medium broth, pH 6 at temperature 37 °C. AnaeroGen sachet (Oxoid Ltd, Hampshire, UK) was used to maintain the anoxic conditions. Before performing the growth experiment, a colony of B. subtilis was transferred into 10 mL of basal broth and incubated overnight at 37 °C. The culture was then subsequently subcultured (10% v/v) and incubated for 22 h at 37 °C. After incubation, the cultures were concentrated 20 times by centrifugation (10 min, 1500 g). The supernatant was removed and the pellet was suspended in enrich media as the working inoculum [15].

2.2 Fed batch incubation in different concentration of glucose and gum arabic
An inoculum with concentration of 1.0x10⁶ CFU/mL of bacteria suspension was prepared and counted using haemocytometer under microscope. Serial dilution was performed to reduce the inoculum concentration greater than the concentration required by adding 1 mL of the bacteria suspension into 9 mL of sterile distilled water. Then, 10mL of the adjusted inoculum was pipetted into a conical flask with 100 mL of basal medium broth supplemented with either glucose or gum arabic and a combination of both with concentration ranging from 0.5% to 1%. The pH was adjusted to pH7, incubated at 37 °C at 120rpm. The turbidity of the inoculum was measured using a UV
spectrophotometer at 600 nm within an interval of 1 hour until the cell concentration starts to decline. Basal medium was used as baseline to compare the growth efficiency.

2.3 Simulation of lumen and mucus-associated microbiota

Proximal colon conditions was prepared in an airtight 150 mL container. The bottle was filled with 100 mL nutritional medium and mucin agar-covered microcosms in a polyethylene netting that serve as glycoprotein contact surface for close simulation of human microbiota. The nutritional medium contained per litre: 1g peptone (Sigma); 0.25g xylan; 3g yeast extract (Oxoid); 2g mucin (Sigma); 0.5g apple pectin (Sigma); 0.25g arabinogalactan (Sigma); 1g starch (Sigma). The mucin agar consists of 4% (w/v) gastric mucin (Merck) and 0.75% (w/v) agar (Sigma), incubated at 37 °C in the dark and under slow shaking [15].

2.4 Experimental Design and Optimisation: Response Surface Methodology.

Experimental statistic used in this study employed the response surface methodology (RSM) to explain the nature of response surface in the optimum region. Under a broad choice of process variables, the optimal growth conditions of B. subtilis in the simulated mucus lumen could be obtained from analysis of multiple regression of the experimental design that simultaneously solved the multivariable equations. A range of parameters was given in Table 1. In this study, a five levels and three factors CCD requiring 20 experiments was performed (Table 2). The CCD consists of a 2k factorial runs with 2k axil runs and xo number of center points (six replicates). The number of experimental runs was determined from the following equation:

$$N = 2k + 2k + x0$$  \hspace{1cm} (1)

where N is the number of experimental runs required, k is the total number of parameter variables and xo is the total number of central points. Total number of experimental runs were estimated to be 20 (k=3 and xo=6). The relationships of parameter variables were evaluated by fitting a second order polynomial equation to consist of 20 experiments. The quadratic model was established as follows:

$$\hat{Y} = \beta_0 + \sum_{i=1}^{k} \beta_i A_i + \sum_{i=1}^{k} \sum_{j=1}^{i} \beta_{ij} A_i A_j$$  \hspace{1cm} (2)

where $\hat{Y}$ is the predicted response (B subtilis growth), $\beta_0$ is a constant, $\beta_i$ linear terms coefficients, $\beta_{ii}$ quadratic terms coefficient and $\beta_{ij}$ interaction coefficients. The relationship between coded and uncoded form of the variables is given as follows:

$$A_i = ((Z_i - (Z_i)^*) / \Delta Z_i)$$  \hspace{1cm} (3)

where $A_i$ is the coded value of the variable, $Z_i$ is the real value of an independent variable (uncoded), $(Z_i)^*$ is the center point value and $\Delta Z_i$ is the step change between the levels.

| Table 1. Selected range of parameters for optimisation of B. subtilis growth in simulated mucus lumen system |
|-------------------------------------------------------------|
| **Parameters** | **Range** |
| Concentration of GA (%) | 0.2 – 0.7 |
| pH | 5.5 – 7.5 |
| Temperature (°C) | 36 – 38 |
3. Results and Discussion

3.1 Fed batch incubation of B. subtilis in different concentration of glucose and gum arabic

The growth curves of B. subtilis in 0.5% and 1% of glucose and GA were plotted separately in Figure 1(a) and (b). Four phases which are the lag phase, exponential, stationary and death phase were clearly observed. Figure 1(a) suggested that optimal glucose concentration was determined at 1% with the highest growth rate of 1.0024 hours$^{-1}$ and the fastest doubling time at 41.49 minutes (Table 3). Figure (1b) suggested that the optimal dosage for GA was at 0.5% with highest growth rate at 0.9047 hours$^{-1}$ with a shorter doubling time of 45.97 minutes as compared to 0.7745 hours$^{-1}$ and 53.70 minutes for 1.0% of GA. Bacteria shown to favour a nutrient rich environment, our finding suggested otherwise as low GA concentration did not encourage the growth of B. subtilis. This could be explained as high viscosity of GA shown to affect the rate of dissolved oxygen level in the medium leading to formation of micro–anaerobic environment [16]. The dissolved oxygen level could impact the oxygen uptake of the cell which affected the cell growth and its metabolism to some extent [17]. Nonetheless, 1% of glucose is much favoured as glucose is the simplest form of sugar which could easily be metabolised by the microbe and supplementation at 1% concentration did not affect the rheology of the medium. Figure 1(c) confirmed that the growth behaviour of B. Subtilis was consistent when grown in combination of both optimal dosage of glucose and GA as suggested in Figure 1(a) and (b) which were 1% and 0.5%, respectively. The highest growth rate was recorded at 0.7995 hours$^{-1}$ with doubling time of 52.02 minutes with extended period of stationary phase due to catabolite repression which regulates the tiered and progressive use

| Run | A  | B  | C  | Response, R |
|-----|----|----|----|-------------|
| 1   | 0.45 | 8.2 | 37.0 | 1.8435     |
| 2   | 0.45 | 6.5 | 37.0 | 1.8146     |
| 3   | 0.20 | 5.5 | 38.0 | 0.2106     |
| 4   | 0.45 | 6.5 | 37.0 | 1.6231     |
| 5   | 0.45 | 6.5 | 35.3 | 1.5947     |
| 6   | 0.03 | 6.5 | 37.0 | 1.0688     |
| 7   | 0.20 | 7.5 | 36.0 | 1.6206     |
| 8   | 0.70 | 5.5 | 38.0 | 1.4170     |
| 9   | 0.20 | 7.5 | 38.0 | 0.6582     |
| 10  | 0.45 | 4.8 | 37.0 | 0.9682     |
| 11  | 0.70 | 7.5 | 36.0 | 1.6103     |
| 12  | 0.45 | 6.5 | 37.0 | 1.5316     |
| 13  | 0.45 | 6.5 | 38.7 | 0.8917     |
| 14  | 0.45 | 6.5 | 37.0 | 1.5026     |
| 15  | 0.45 | 6.5 | 37.0 | 1.4668     |
| 16  | 0.70 | 5.5 | 36.0 | 1.0353     |
| 17  | 0.87 | 6.5 | 37.0 | 1.7977     |
| 18  | 0.45 | 6.5 | 37.0 | 1.5478     |
| 19  | 0.20 | 5.5 | 36.0 | 0.7818     |
| 20  | 0.70 | 7.5 | 38.0 | 1.7301     |
of carbon sources in a medium by inhibiting the expression of enzymes which catabolise non–preferred carbon sources [18]. Such mechanism allows the bacteria to survive longer in the medium due to availability of carbon source from enzymatic breakdown of GA.

![Figure 1](image1.png)

**Figure 1.** (a) Growth of *B. subtilis* in basal media, 0.5% and 1% glucose (b) Growth of *B. subtilis* in basal media, 0.5% and 1% of GA (c) Growth of *B. subtilis* in basal media, 1% Glucose + 0.5% GA and GA and 1% Glucose + 1% GA.

### 3.2 Experimental Design and Optimisation: Response Surface Methodology.

**Table 3.** Growth rate and doubling time of *B. subtilis* in basal media, and in medium supplemented with 0.5 or 1% of glucose or Gum Arabic or combination of both.

| Medium                  | Growth Rate (hour⁻¹) | Doubling Time (min) |
|-------------------------|----------------------|---------------------|
| Basal                   | 0.9394               | 44.27               |
| **Figure 1(a)**         |                      |                     |
| 0.5% Glucose            | 0.9932               | 41.87               |
| 1.0% Glucose            | 1.0024               | 41.49               |
| **Figure 1(b)**         |                      |                     |
| 0.5% gum Arabic         | 0.9047               | 45.97               |
| 1.0% gum Arabic         | 0.7745               | 53.70               |
| **Figure 1(c)**         |                      |                     |
| 1.0% glucose + 0.5% GA  | 0.7995               | 52.02               |
| 1.0% glucose + 1.0% GA  | 0.7854               | 52.95               |

ANOVA analysis suggested a significant interaction between GA concentration and temperature (p-value 0.0016) as visualised in the 3-D model of interaction (Figure 2(a)). The optimum point recorded at temperature 37°C and 0.7% GA concentration supplemented to the simulated lumen microbiota. 2-D counter plot in Figure 3 suggested cell biomass increased as GA concentration increase but growth deteriorate at lower temperature (36.0°C). The theoretical relationship was that temperature encourage the activation of cellulase enzyme that initiate the breakdown of GA to glucose subunits making it available for *B. Subtilis*. High temperature nonetheless, will deactivate the enzyme making the complex structure of GA inaccessible to be metabolised by *B. Subtilis* [19]. ANOVA analysis (Table 4) suggested no significant interaction for both interaction between pH and GA concentration and temperature and pH (p>0.05). It is of our interest to further investigate the underlying mechanism in our subsequent analysis.
**Figure 2.** (a) 3D Model of interaction between temperature and GA concentration (b) 3D Model interaction between pH and GA concentration (c) 3D Model of interaction between pH and temperature.

**Figure 3.** 2D Contour plot of interaction between temperature and concentration of GA.
Table 4. Analysis of variance (ANOVA)

| Source | Sum of Squares | df | Mean Square | F value | p-value | Remarks |
|--------|----------------|----|-------------|---------|---------|---------|
| Model  | 3.40           | 9  | 0.39        | 13.96   | 0.0001  | Significant |
| A      | 1.03           | 1  | 1.03        | 36.49   | 0.0001  | Significant |
| B      | 0.97           | 1  | 0.97        | 34.56   | 0.0002  | Significant |
| C      | 0.36           | 1  | 0.36        | 12.74   | 0.0051  | Significant |
| AB     | 0.020          | 1  | 0.020       | 0.70    | 0.4211  | Not significant |
| AC     | 0.52           | 1  | 0.52        | 18.37   | 0.0016  | Significant |
| BC     | 0.053          | 1  | 0.053       | 1.89    | 0.1990  | Not significant |
| A²     | 0.14           | 1  | 0.14        | 4.86    | 0.0520  | Not significant |
| B²     | 0.17           | 1  | 0.17        | 5.88    | 0.0358  | Significant |
| C²     | 0.39           | 1  | 0.39        | 13.87   | 0.0039  | Significant |
| Residual | 0.28          | 10 | 0.028       |         |         |         |
| Lack of Fit | 0.20      | 5  | 0.041       | 2.56    | 0.1624  | Not significant |
| Pure Error | 0.079      | 5  | 0.016       |         |         |         |
| Cor Total | 3.82        | 19 |             |         |         |         |

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