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Delayed differential equations as a *Salmonella* biofilm model

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Abstract. *Salmonella* is a widespread bacterial pathogen that is the primary cause of many food-borne illnesses worldwide. It gains significant strength against antibacterial treatments when it forms the biofilm structure, which can be considered a multicellular-like form where the pathogen is compartmentalized based on function in which each part communicates, further adding to the capabilities of resistance. To overcome this problem, it is important for practitioners to know how *Salmonella* biofilms will evolve through time under the presence of various carbon resources which are mostly present in food products. In this work, a mathematical model of *Salmonella* biofilm trajectories was made using Delayed Logistic Differential Equations after an experimental procedure which comprised treatments with seven carbon sources of six different concentrations. This model proved to be efficient for modeling *Salmonella* biofilm formation even without significant amount of data.

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Keywords. *Salmonella*, biofilm, mathematical modeling, carbon source.

1. Introduction

Biofilms are defined as microbial communities surrounded by a self-produced extracellular polymeric matrix (EPS) containing polysaccharides, extracellular DNA (eDNA), proteins and other chemical components. These communities are formed by the attachment of planktonic cells to each other or to biotic and abiotic surfaces [6]. Bacterial biofilm forms are more resistant to numerous adverse environmental circumstances than planktonic forms, such as inadequate nutrition sources, high pressure, temperature, or the presence of different antimicrobials [5]. The reason for this is that the cells in the biofilm structure are embedded in the matrix and thus, they can protect themselves against external stimuli and survive longer. Although biofilm production is crucial for bacterial life, it presents significant problems in the food, water, energy, and notably healthcare industries due to their resistant structure. Biofilm formation is a multi-stage process. The first step begins with the attachment of planktonic cells to a suitable surface which can be organic or inorganic. Once the cells have attached to the appropriate environment, they move on to the second stage called the colonisation stage. These stages are followed by exopolysaccharide matrix (EPS) production, maturation and dispersal processes. Once the biofilm formation process is complete, due to various environmental conditions (depletion of environmental resources, lack of oxygen,

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etc.), they can move on to the dispersal stage. In this process, bacteria in the biofilm structure switch to planktonic form and search for a more suitable environment where they can perform invasion and initiate the formation of a new biofilm [10]. These processes occur simultaneously and the biofilm is continuously being formed and deformed; displaying oscillatory behaviour. Therefore, biofilm production is not linear, but rather a cyclical process that can thus be modeled with sinusoidal functions.

Salmonella is one of the bacteria that has the ability to form biofilms and is mostly transmitted through contaminated foods [14]. *Salmonella* biofilm forms pose a problem in terms of economy and health, particularly in the food industry and medical fields, as they acquire resistance to classical treatment and eradication methods such as antibiotics and antimicrobials. For this reason, alternative strategies need to be developed for the control and eradication of biofilm structures [4]. In order to combat these structures, it is necessary to have a good understanding of the mechanisms that play a role in biofilm formation and regulation. Studies on this subject have shown that genes such as csgD, fimH, fimF, fliZ, rmbA are involved in the formation and regulation of biofilms. In addition to genetic factors, environmental factors such as temperature, salt concentration, pH variation and carbon sources in the environment have also been found to play a role in biofilm regulation [13,16,19]. Glucose is one of the carbon sources used most effectively by *Salmonella* and many other bacteria in the infection of the host cell [2,12]. If glucose is depleted in the environment, bacteria tend to switch to alternative carbon sources available in the environment [9]. As an alternative to glucose, *Salmonella* can use sugars such as fucose, rhamnose and metabolites such as malic acid, citric acid, succinic acid and acetic acid in the tricarboxylic acid cycle (TCA) as carbon sources [8]. The effect of these metabolites on the biofilm formation of *Salmonella* has been examined in previous studies and it has been determined that there are changes in the amount of biofilm depending on the concentration of the carbon source in the environment [11]. Sugars such as fucose and rhamnose have been shown to be catabolized by biofilm forms of *Salmonella* [8].

In this study, the effects of the aforementioned carbon sources and various concentrations of these sources in the environment on the biofilm formation of *Salmonella* were investigated over time using mathematical modeling. Differential equations used in mathematical modeling are described as mathematical tools used to predict many physical phenomena and to gain more information about these phenomena [3]. It is anticipated that modeling the relationship between carbon sources and *Salmonella* biofilm through equations will shed light on studies on the mechanisms involved in the biofilm formation and regulation of *Salmonella*. Furthermore, modeling the relationship between biofilm and sugars and similar organic compounds used in the production of packaged foods, particularly in the food industry, will enable the determination of carbon sources and amounts to be used in these products in a way that does not allow the development of *Salmonella* and other contaminants. A question of why would mathematical models of *Salmonella* would be required while statistical modeling have proven to be successful for the same task as indicated above might be asked. This preference is due to the fact that in most biofilm experiments the sampling frequency is not frequent enough for statistical methods to be applicable. Deterministic modeling as done in this work introduces domain information and compensates for this possibility of data acquisition.

2. Methods

As this study required intricate wet laboratory work, exact laboratory methodologies will be given to retain reproducibility.

2.1. **Bacterial Strains and Growth Conditions.** The *S.* Typhimurium 14028 strain used in this study was obtained from the culture collection of Ankara University Prokaryotic Genetics Laboratory. *Salmonella* cultures were grown in Luria-Bertani (LB) broth medium at 37 °C and 200 rpm.

2.2. **Determination of the Amount of Biofilm Formation on Polystyrene Surface.** Biofilm experiments were conducted to determine the time-dependent effects of different carbon sources on the amount of biofilm formation in *S.* Typhimurium. Seven different carbon sources including sugars such as glucose, fucose, rhamnose and metabolites such as citric acid, acetic acid, succinic acid, malic acid were used for these experiments. M9 medium was used to test the effects of different concentrations of the mentioned carbon sources on biofilm formation. Each carbon source was added to M9 medium at final concentrations of 0.1% (w/v), 0.2% (w/v), 0.3% (w/v), 0.4% (w/v), 0.5% (w/v) and 0.6% (w/v) after sterilisation by filtration. After this stage, separate 96-well polystyrene surface microtiter plates

were set for each carbon source, and 100 µL of M9 medium containing different concentrations of carbon sources was transferred to the wells. Then, 30 µL of *Salmonella* cultures grown in LB broth (OD_{600} = 0.2) were added to the wells containing M9 medium. Following this process, incubation was carried out for 72 hours at 20 °C under static conditions. During the incubation period, the amount of biofilm was measured every 12 hours. When the incubation period ended, the wells were washed three times with sterile physiological serum (0.85%) (Merck, Germany) to remove unattached cells in order to determine the amount of biofilm, and then they were left to dry for 5 minutes at room temperature. After drying process, the biofilm matrix was fixed for 15 minutes by adding 130 µL of 95% methanol to the wells. These fixed structures were stained by adding 130 µL crystal violet (1%) and kept at room temperature for 30 minutes. The wells were washed three times with distilled water to remove unattached dyes. After washing, 130 µL glacial acetic acid (33%) was added to the wells and the plates were kept at room temperature for 45 minutes. At the end of this period, the amount of dye adhering to the biofilm was measured at OD_{595} nm with an Elisa reader (Biorad, California, USA) and the amount of biofilm formed was determined [15]. Biofilm assays were repeated two times to test the fitness of the models that are constructed according to the data obtained from the first biofilm experiments.

3. Modeling Time Trajectories of Bacterial Biofilms

In this study, a combination of theory and observation-based modelling approaches were adopted. That is, in addition to biological knowledge that described biofilm growth, data observed from initial laboratory work as given in earlier sections were used in conjunction. In this regard, it was aimed to use a growth model that mathematically best expresses the experimental data obtained for *S.* Typhimurium examined in this study. It was assumed that biofilms are a population of their own, rather than being composed of individual bacterial cells, as the structure has behavioral properties that do not necessarily apply to individual bacteria. Therefore, ecological modeling approaches were adopted and initial considerations for the model were based on logistic growth models. There are many mathematical models for population growth models in the literature, one of the most well-known being the logistic equation given by the mathematician Pierre François Verhulst. This equation, $P(t)$ corresponds to the size of the population at time t, is expressed as:

$$\frac{dP}{dt} = rP(t)(1 - \frac{P(t)}{K}) \tag{1}$$

Here, the rate of the biofilm formation process is described as being proportional to the size of the population at time t, $P(t)$, by a factor of r, which represents the growth rate. However, biofilm formation process does not continue indefinitely. Due to molecular signalling and interactions of bacterial cells with each other as well as their environment, the biofilm is being formed and degraded simultaneously. Therefore, the third component of the model, $(1 - \frac{P(t)}{K})$ is added. This component implies that when the population reaches a certain threshold,K, it reduces the growth rate to zero, implying no growth. At population sizes larger than K, this component outweighs the growth component, $rP(t)$, which in turn implies reduction in growth (negative derivative). If the logistics equation (1) is considered with the delay constant a ($a > 0$), the lagged logistic equation also known as the Hutchinson-Wright equation, is obtained [7,18].

$$\frac{dP}{dt} = rP(t)(1 - \frac{P(t-a)}{K}) \tag{2}$$

Adding the lag constant to the equation here means that earlier populations can influence later populations. For example, although the impact of population size on food resources may not be felt immediately, it may be felt after a certain period of time a as a consequence of biological adaptions, e.g response to starvation [1]. For *S.* Typhimurium considered in this study, Hutchinson-Wright equation was modified with a parameter $b \geq 1$ to add a degree of elasticity so that the model can describe observed data more

effectively, resulting in the model of the form

$$\frac{dP}{dt} = rP(t)(b - \frac{P(t-a)}{K}) \tag{3}$$

The parameter b here does not have an inherent biological significance. It is used here to give more degree of freedom to the equation, i.e. to increase the number of possible parameters, so that more curves can be fit to the data. If this parameter is not added, the equation represents a negative rate of growth at $\frac{P(t-a)}{K} > 1$, however after its addition, negative rate of growth occurs at $\frac{P(t-a)}{K} > b$. The lagged logistic equation was taken into consideration and the amount of biofilm formation under different concentrations of glucose, fucose, rhamnose, citric acid, succinic acid, acetic acid and malic acid was examined separately to check whether this explanation of time trajectories of biofilms apply under different environmental conditions, as these carbon sources affect metabolism differently. The model (3) was fit to the data using the Maple software, and the fitness of the model was measured through Pearson correlation coefficient.

Examining the structure of this model would lead to the expectation that the process would possess oscillatory characteristics, since the lag term implies that reduction in biofilm amount would be followed by its growth, and its growth would be followed by reduction, a process which will go on consistently.

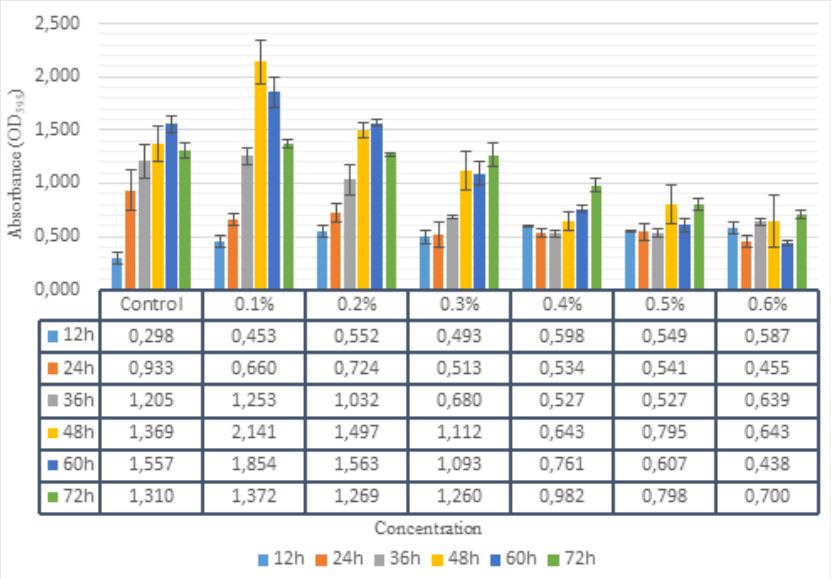
4. Results

4.1. **Effects of Various Concentrations of Carbon Sources on Biofilm Formation.**

4.1.1. *Glucose.* In these experiments, the capacity of *S.* Typhimurium 14028 strain to produce biofilm in the presence of different concentrations of glucose was determined. The control group includes the *S.* Typhimurium 14028 strain grown in M9 medium without any carbon source. When the amount of biofilm change in the control group is examined within 72 hours, it is discovered that the amount of biofilm increases until the 60th hour and then decreases. When 0.1% glucose was added to the M9 medium in which *Salmonella* strains were grown, it was discovered that the biofilm reached its maximum value after 48 hours and then decreased. In the presence of 0.2% glucose, the amount of biofilm reached the highest value at 60 hours and then decreased, similar to the control group. After 72 hours, the amount of biofilm peaked when the medium's glucose content was raised to 0.3%. Examining the biofilm measurements in the medium containing 0.4% and 0.5% glucose concentration, it is found that the amount reached its maximum value after 72 hours, and variations in the biofilm amount up to this point were seen to fluctuate between increases and decreases. When the glucose concentration in the medium was increased to 0.6%, the amount of *Salmonella* biofilm increased until 48th hour and then decreased. At 72 hours, an increase in the amount of biofilm recurred (Fig. 1).

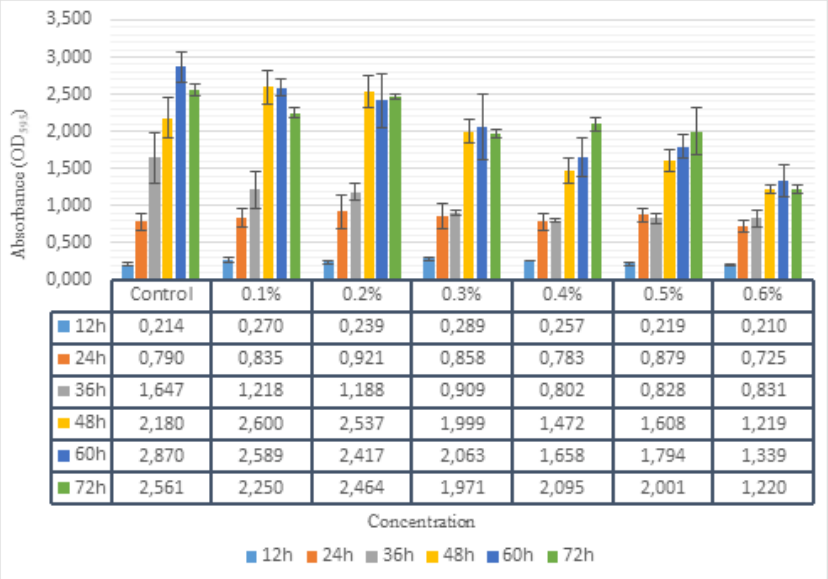
4.1.2. *Fucose.* In the investigation of the effect of various fucose concentrations in the medium on the biofilm formation capacity of *Salmonella*, it was observed that the amount of biofilm in the control strains grown in the medium without a carbon source reached its maximum at 60 hours and then decreased. The highest biofilm amount was observed at 48 hours when the fucose ratio in M9 medium was 0.1 and 0.2%. The maximum amount of biofilm was observed at the 60th hour when the fucose ratio was 0.3%. In the presence of 0.4 and 0.5% fucose, the amount of biofilm tended to increase until the 72nd hour, when it reached its peak. When the fucose content of the medium was increased to 0.6%, the highest value in the amount of biofilm was observed at the 60th hour, after which it began to decrease (Fig. 2).

4.1.3. *Rhamnose.* When the biofilm formation capacities of *S.* Typhimurium were examined in the presence of rhamnose, another sugar that *Salmonella* uses as a carbon source, it was discovered that the amount of biofilm in the control group reached a maximum at the 60th hour and then tended to decrease. When the values of 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% rhamnose in the medium are examined, it can be seen that the biofilm amount of *S.* Typhimurium increases and decreases over the course of 72 hours, peaking at 72nd hour. In the presence of 0.6% rhamnose, it was observed that there was an increase in



	Control	0.1%	0.2%	0.3%	0.4%	0.5%	0.6%
12h	0,298	0,453	0,552	0,493	0,598	0,549	0,587
24h	0,933	0,660	0,724	0,513	0,534	0,541	0,455
36h	1,205	1,253	1,032	0,680	0,527	0,527	0,639
48h	1,369	2,141	1,497	1,112	0,643	0,795	0,643
60h	1,557	1,854	1,563	1,093	0,761	0,607	0,438
72h	1,310	1,372	1,269	1,260	0,982	0,798	0,700

FIGURE 1. Observed effects of glucose treatment (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%) on *S.* Typhimurium biofilm formation

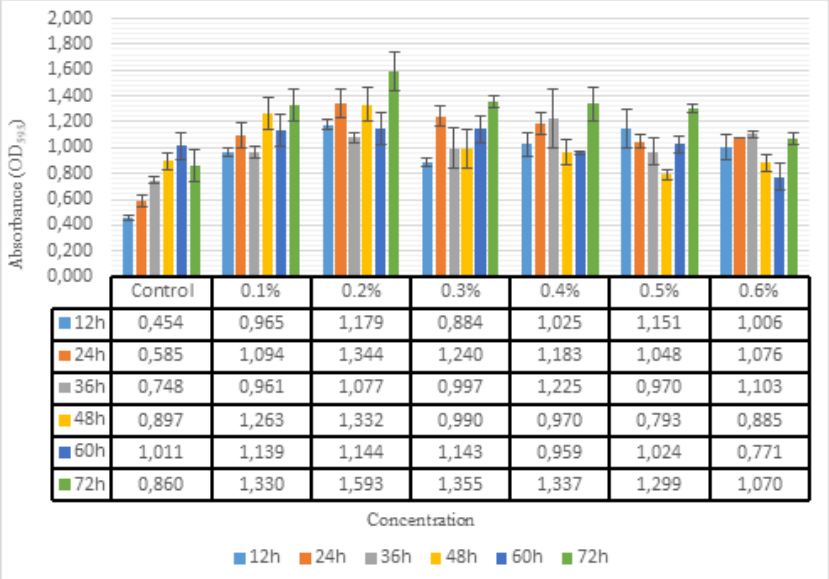


	Control	0.1%	0.2%	0.3%	0.4%	0.5%	0.6%
12h	0,214	0,270	0,239	0,289	0,257	0,219	0,210
24h	0,790	0,835	0,921	0,858	0,783	0,879	0,725
36h	1,647	1,218	1,188	0,909	0,802	0,828	0,831
48h	2,180	2,600	2,537	1,999	1,472	1,608	1,219
60h	2,870	2,589	2,417	2,063	1,658	1,794	1,339
72h	2,561	2,250	2,464	1,971	2,095	2,001	1,220

FIGURE 2. Observed effects of fucose treatment (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%) on *S.* Typhimurium biofilm formation

the amount of biofilm until the 36th hour, and a decrease in the period from 36th hour to the 60th hour. It was determined that this amount began to increase at the 72nd hour (Fig. 3).

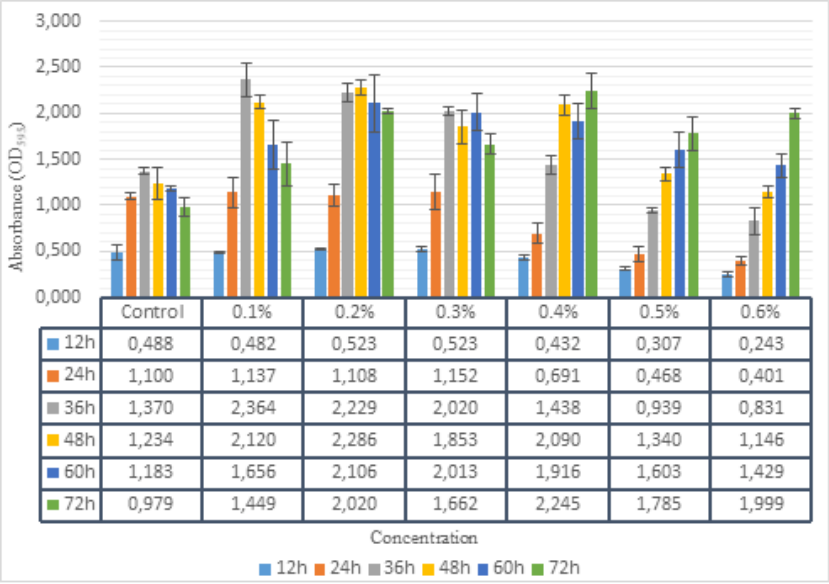
4.1.4. *Citric Acid.* When the effects of citric acid, a metabolite in TCA that *Salmonella* utilizes as a carbon source, on the amount of biofilm were investigated, it was discovered that the amount of biofilm in the control group developed in the medium without any carbon source reached its peak at 36 hours and then decreased. The amount of biofilm formed by *Salmonella* in the presence of 0.1% citric acid in the medium reached a maximum at 36 hours and then decreased, similar to the control group. The amount of biofilm reached its peak after 48 hours in the presence of 0.2% citric acid. The amount of biofilm reached a maximum in the presence of 0.3% citric acid in the medium. The behavior of the biofilm at 0.4% citric



	Control	0.1%	0.2%	0.3%	0.4%	0.5%	0.6%
12h	0,454	0,965	1,179	0,884	1,025	1,151	1,006
24h	0,585	1,094	1,344	1,240	1,183	1,048	1,076
36h	0,748	0,961	1,077	0,997	1,225	0,970	1,103
48h	0,897	1,263	1,332	0,990	0,970	0,793	0,885
60h	1,011	1,139	1,144	1,143	0,959	1,024	0,771
72h	0,860	1,330	1,593	1,355	1,337	1,299	1,070

FIGURE 3. Observed effects of rhamnose treatment (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%) on *S.* Typhimurium biofilm formation

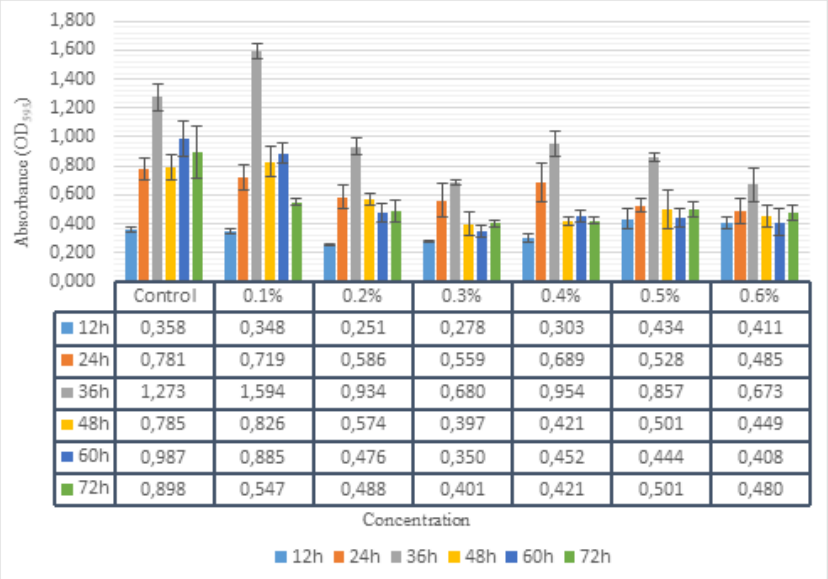
acid content was similar to that at 0.3% citric acid content, increasing until the 48th hour, decreasing at the 60th hour, and increasing again at the 72nd hour. When the citric acid content was between 0.5 and 0.6%, the amount of biofilm increased until the 72nd hour, when it reached its peak (Fig. 4).



	Control	0.1%	0.2%	0.3%	0.4%	0.5%	0.6%
12h	0,488	0,482	0,523	0,523	0,432	0,307	0,243
24h	1,100	1,137	1,108	1,152	0,691	0,468	0,401
36h	1,370	2,364	2,229	2,020	1,438	0,939	0,831
48h	1,234	2,120	2,286	1,853	2,090	1,340	1,146
60h	1,183	1,656	2,106	2,013	1,916	1,603	1,429
72h	0,979	1,449	2,020	1,662	2,245	1,785	1,999

FIGURE 4. Observed effects of citric acid treatment (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%) on *S.* Typhimurium biofilm formation

4.1.5. *Succinic Acid.* The investigation into the effects of succinic acid on *Salmonella* biofilm formation revealed that the biofilm amount of the control group reached its peak at 36 hours and subsequently decreased. When the concentration of this metabolite in the medium was increased to 0.1%, the biofilm amount reached its peak at 36 hours. Similarly, at other ratios of succinic acid in the medium (0.2%, 0.3%, 0.4%, 0.5%, 0.5%, and 0.6%), the amount of biofilm reached its peak at 36 hours (Fig. 5).



	Control	0.1%	0.2%	0.3%	0.4%	0.5%	0.6%
12h	0,358	0,348	0,251	0,278	0,303	0,434	0,411
24h	0,781	0,719	0,586	0,559	0,689	0,528	0,485
36h	1,273	1,594	0,934	0,680	0,954	0,857	0,673
48h	0,785	0,826	0,574	0,397	0,421	0,501	0,449
60h	0,987	0,885	0,476	0,350	0,452	0,444	0,408
72h	0,898	0,547	0,488	0,401	0,421	0,501	0,480

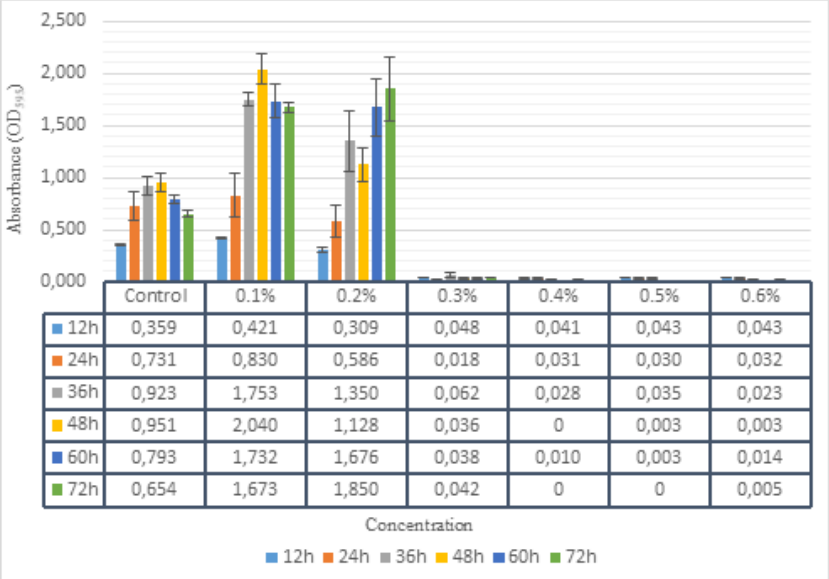
FIGURE 5. Observed effects of succinic acid treatment (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%) on *S.* Typhimurium biofilm formation

4.1.6. *Acetic Acid.* The amount of biofilm in the control group reached the maximum value at 48 hours in the experiments to investigate the effects of acetic acid on the biofilm formation of *S.* Typhimurium. When the acetic acid concentration in the medium was 0.1%, the amount of biofilm formed peaked at 48 hours and then decreased. When the concentration was increased to 0.2%, the amount of biofilm reached its maximum after 72 hours, but there were intermittent increases and decreases in the amount of biofilm during this time. Similarly, at other acetic acid concentrations in the medium, fluctuating increases and decreases in biofilm amount can be observed within 72 hours. In addition to these findings, it is possible to see that increasing the concentration of acetic acid in the medium to 0.3% and higher reduces the amount of biofilm formed by *Salmonella* (Fig. 6).

4.1.7. *Malic Acid.* When the effects of malic acid on the amount of biofilm formed by *Salmonella* were investigated, it was determined that the highest biofilm amount was reached at 36 hours in the control group. When the malic acid concentration in the medium was 0.1%, the biofilm amount peaked at the 36th hour and then began to decline. The highest value of biofilm amount was observed at the 36th hour in the presence of 0.2% malic acid and at 48th hours at malic acid concentrations of 0.3% and 0.4%. The biofilm amount reached its maximum value at 60 hours at malic acid concentrations of 0.5% and 0.6% (Fig. 7).

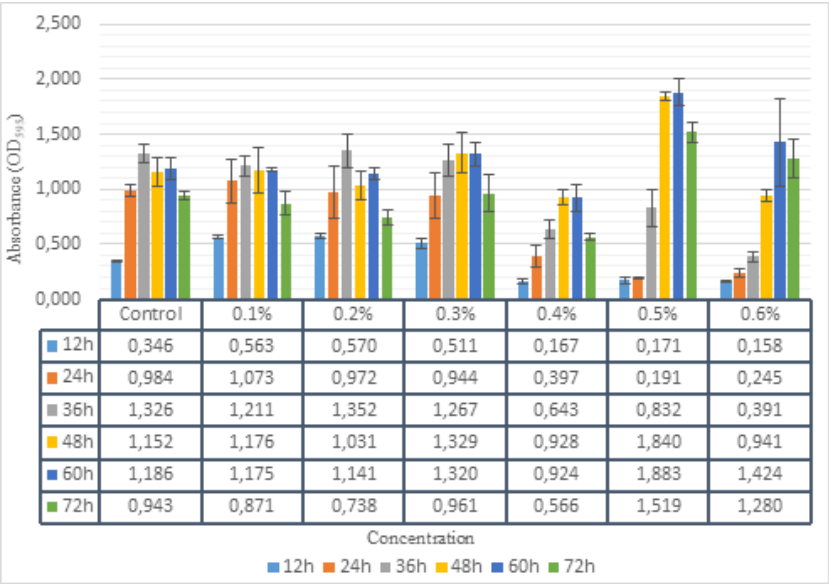
4.2. **Application of Delayed Logistic Differential Equation on *Salmonella* Biofilm Time Trajectories.** The mathematical models are formed in accordance with the data obtained from biofilm assays to determine the amount of biofilm formed by *Salmonella* depending on various carbon sources and concentrations. According to the biofilm assay results, parameter values obtained for equation (3) at different concentrations of the carbon sources are given in Table(s) 1-7.

Figure(s) 8-14 displays fitted model lines in addition to the data obtained from experiments. It is obvious that with the correct choices of parameters the model offers a solution to all of the empirical data. It can be seen that the distance from model lines to data points are considerably small, i.e. the model has a significant explanatory power. Even the abnormal anti-biofilm properties of acetic acid can be explained by the model. A plausible continuation to this model would be finding the distribution of estimated parameters [17], which is to be discussed further in the discussion section.



	Control	0.1%	0.2%	0.3%	0.4%	0.5%	0.6%
12h	0,359	0,421	0,309	0,048	0,041	0,043	0,043
24h	0,731	0,830	0,586	0,018	0,031	0,030	0,032
36h	0,923	1,753	1,350	0,062	0,028	0,035	0,023
48h	0,951	2,040	1,128	0,036	0	0,003	0,003
60h	0,793	1,732	1,676	0,038	0,010	0,003	0,014
72h	0,654	1,673	1,850	0,042	0	0	0,005

FIGURE 6. Observed effects of acetic acid treatment (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%) on *S.* Typhimurium biofilm formation



	Control	0.1%	0.2%	0.3%	0.4%	0.5%	0.6%
12h	0,346	0,563	0,570	0,511	0,167	0,171	0,158
24h	0,984	1,073	0,972	0,944	0,397	0,191	0,245
36h	1,326	1,211	1,352	1,267	0,643	0,832	0,391
48h	1,152	1,176	1,031	1,329	0,928	1,840	0,941
60h	1,186	1,175	1,141	1,320	0,924	1,883	1,424
72h	0,943	0,871	0,738	0,961	0,566	1,519	1,280

FIGURE 7. Observed effects of malic acid treatment (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%) on *S.* Typhimurium biofilm formation

TABLE 1. Parameter values obtained for equation (3) at different concentrations of glucose

	Control	**0.1%**	**0.2%**	**0.3%**	**0.4%**	**0.5%**	**0.6%**
r	1.2669	0.8175	0.9837	0.5163	0.0375	0.0875	1.8097
K	1.0043	1.2694	0.8929	0.9122	2.5304	1.9841	0.3441
a	0.0009	1.5521	2.5249	2.6280	6.6782	6.9945	0.7897
b	1.4050	1.0517	0.9524	0.9834	2.0788	1.0000	1.6091

TABLE 2. Parameter values obtained for equation (3) at different concentrations of fucose

	Control	**0.1%**	**0.2%**	**0.3%**	**0.4%**	**0.5%**	**0.6%**
r	1.3257	0.6129	1.0371	0.7403	0.6579	0.8449	0.9938
K	2.5626	1.3710	2.2875	1.5829	1.7676	1.6526	0.9529
a	0.0012	0.8965	0.6181	0.9445	0.0015	0.0016	0.0010
b	1.0656	1.6508	1.0559	1.1781	1.2773	1.2496	1.3551

TABLE 3. Parameter values obtained for equation (3) at different concentrations of rhamnose

	Control	**0.1%**	**0.2%**	**0.3%**	**0.4%**	**0.5%**	**0.6%**
r	1.3123	0.1953	0.0483	2.1371	1.9611	1.7468	14.3207
K	0.7412	1.2377	1.3295	1.0436	1.0710	0.9756	0.9982
a	2.8242	5.4901	5.4979	0.4329	1.0654	2.4287	4.0274
b	0.7914	1.0815	1.5976	1.0820	1.0344	1.1194	1.0052

TABLE 4. Parameter values obtained for equation (3) at different concentrations of citric acid

	Control	**0.1%**	**0.2%**	**0.3%**	**0.4%**	**0.5%**	**0.6%**
r	1.1529	1.1428	0.9132	1.3122	1.4153	0.6283	0.4994
K	1.1755	1.6873	1.6966	1.9615	3.4677	1.6025	1.9761
a	0.8253	0.9121	0.7163	0.5633	0.5640	0.6399	0.0010
b	0.9907	1.0683	1.2415	0.9439	0.6128	1.1250	1.3272

TABLE 5. Parameter values obtained for equation (3) at different concentrations of succinic acid

	Control	**0.1%**	**0.2%**	**0.3%**	**0.4%**	**0.5%**	**0.6%**
r	1.4036	1.0281	1.3699	1.4592	3.5809	3.5902	1.5121
K	0.8996	0.6972	0.6041	0.4687	1.3597	1.4038	0.5060
a	0.6880	1.0155	0.9747	1.0346	1.0538	1.1358	1.113
b	1.0498	1.3208	1.0018	1.0000	0.4249	0.4145	1.0000

TABLE 6. Parameter values obtained for equation (3) at different concentrations of acetic acid

	Control	**0.1%**	**0.2%**	**0.3%**	**0.4%**	**0.5%**	**0.6%**
r	1.1540	0.7207	0.7827	0.1825	1.1965	1.2004	0.9413
K	0.9075	1.1912	1.4936	0.0756	0.0823	0.0789	0.0965
a	1.0459	0.8169	0.0014	0.1975	0.0014	0.0014	0.0014
b	0.8779	1.4697	1.2237	0.3208	0.0515	0.0224	0.1535

TABLE 7. Parameter values obtained for equation (3) at different concentrations of malic acid

	Control	**0.1%**	**0.2%**	**0.3%**	**0.4%**	**0.5%**	**0.6%**
r	2.1466	1.0327	1.0369	1.0596	1.0034	0.8977	1.3376
K	1.4987	1.1588	1.0218	1.3709	0.6863	1.3363	0.3284
a	0.5111	1.0398	1.0953	1.1918	1.4118	1.0404	3.2706
b	0.7555	0.9269	1.0000	0.8256	0.9257	1.1231	0.9134

5. Discussion

The food pathogen *Salmonella* and the biofilm structures formed by *Salmonella* are seen as a threat to public health and many areas, especially the food industry, and therefore cause worldwide concerns. In order to combat biofilms, it is important to understand these structures well. In this study, in order to explain the metabolic activity of *Salmonella* biofilms, the time-dependent responses of these structures

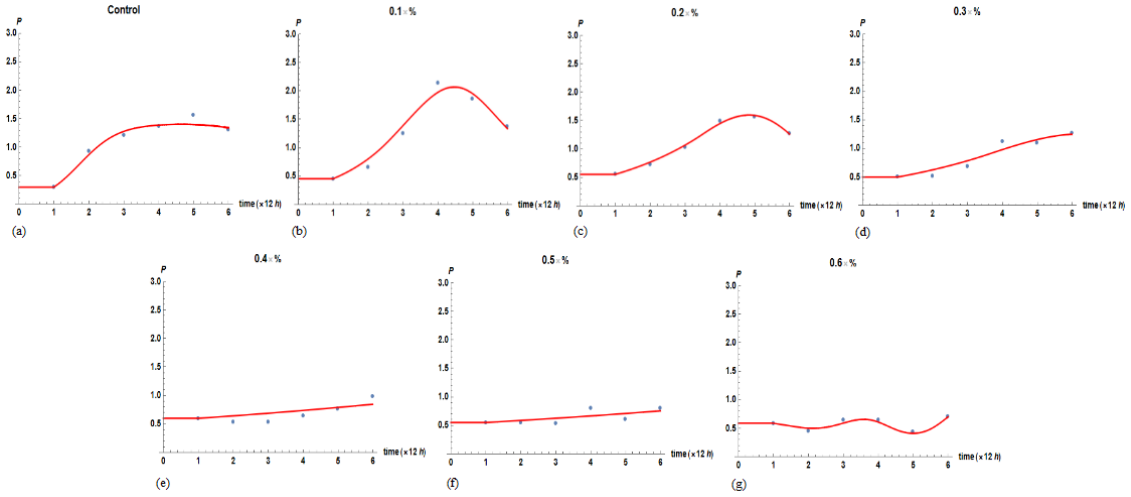


FIGURE 8. (a): Trajectory of *Salmonella* biofilm without any carbon source (b): Trajectory of *Salmonella* biofilm under 0.1% glucose (c): Trajectory of *Salmonella* biofilm under 0.2% glucose (d): Trajectory of *Salmonella* biofilm under 0.3% glucose (e): Trajectory of *Salmonella* biofilm under 0.4% glucose (f): Trajectory of *Salmonella* biofilm under 0.5% glucose (g): Trajectory of *Salmonella* biofilm under 0.6% glucose

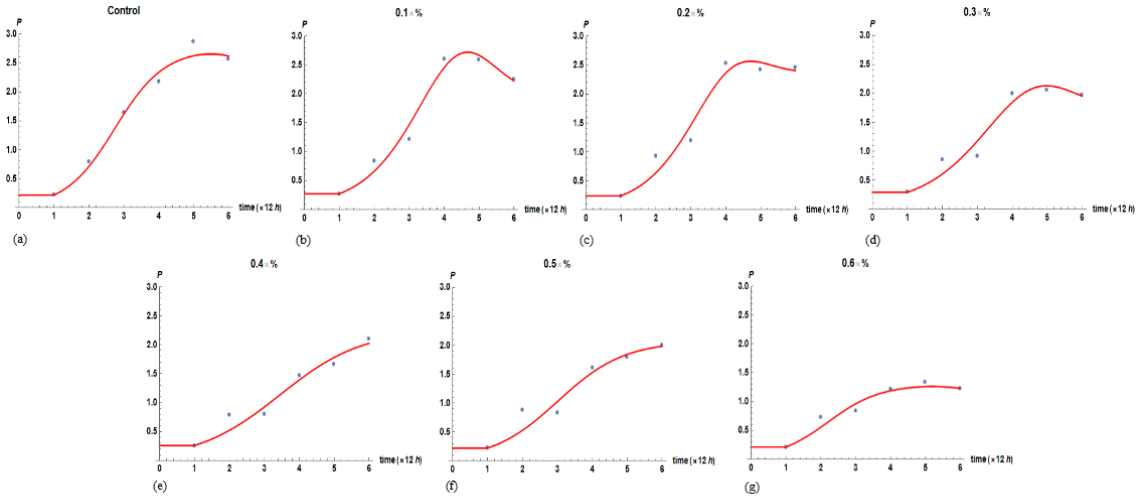


FIGURE 9. (a): Trajectory of *Salmonella* biofilm without any carbon source (b): Trajectory of *Salmonella* biofilm under 0.1% fucose (c): Trajectory of *Salmonella* biofilm under 0.2% fucose (d): Trajectory of *Salmonella* biofilm under 0.3% fucose (e): Trajectory of *Salmonella* biofilm under 0.4% fucose (f): Trajectory of *Salmonella* biofilm under 0.5% fucose (g): Trajectory of *Salmonella* biofilm under 0.6% fucose

to different carbon sources and their different concentration levels were investigated. It was aimed to determine the trajectory of the amount of *Salmonella* biofilm in the time dimension depending on various carbon sources and different concentrations. In order to define the trajectory, the hour (speed) at which the biofilm amount reached its highest value was considered. According to the results obtained from

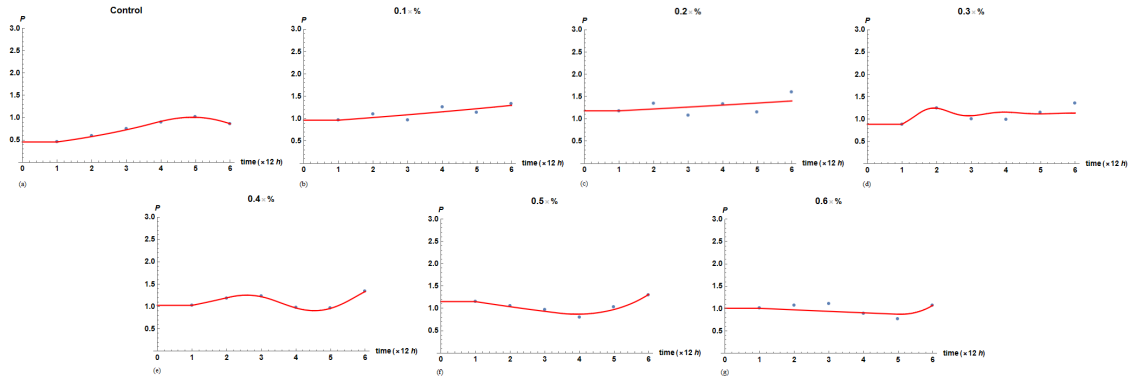


FIGURE 10. (a): Trajectory of *Salmonella* biofilm without any carbon source (b): Trajectory of *Salmonella* biofilm under 0.1% rhamnose (c): Trajectory of *Salmonella* biofilm under 0.2% rhamnose (d): Trajectory of *Salmonella* biofilm under 0.3% rhamnose (e): Trajectory of *Salmonella* biofilm under 0.4% rhamnose (f): Trajectory of *Salmonella* biofilm under 0.5% rhamnose (g): Trajectory of *Salmonella* biofilm under 0.6% rhamnose

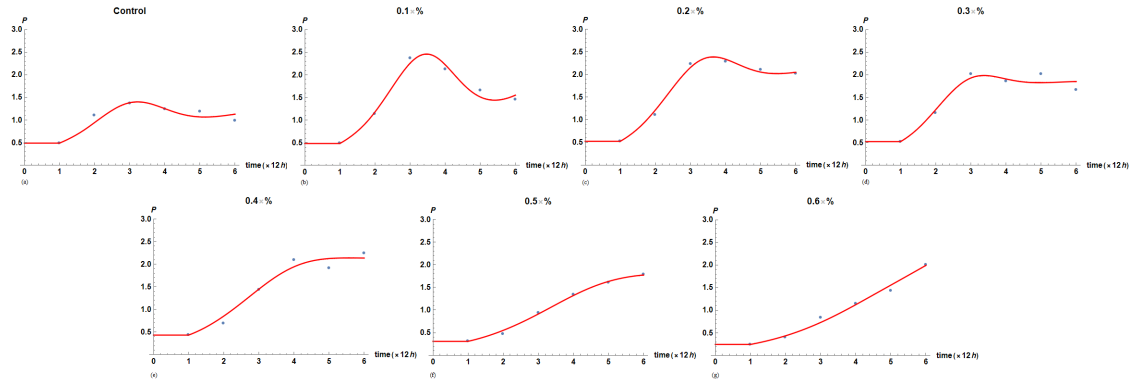


FIGURE 11. (a): Trajectory of *Salmonella* biofilm without any carbon source (b): Trajectory of *Salmonella* biofilm under 0.1% citric acid (c): Trajectory of *Salmonella* biofilm under 0.2% citric acid (d): Trajectory of *Salmonella* biofilm under 0.3% citric acid (e): Trajectory of *Salmonella* biofilm under 0.4% citric acid (f): Trajectory of *Salmonella* biofilm under 0.5% citric acid (g): Trajectory of *Salmonella* biofilm under 0.6% citric acid

the biofilm experiments, it was observed that the speed slowed down as the concentration of fucose in the medium increased (Fig. 2). When the relationship between malic acid and biofilm amount was analyzed, it was observed that similar to fucose, the rate slowed down as the concentration increased, but the process was faster than fucose at all concentrations (Fig. 7). When the effect of succinic acid on biofilm was investigated, it was discovered that the amount of succinic acid present in the medium had no effect on the speed criterion. The biofilm amount reached its peak in all concentrations at the 36th hour (Fig. 5). The biofilms were found to be insensitive to increases in rhamnose concentration, just like succinic acid; the rate of reaching the maximum concentration was slow, but the rate increased at the maximum concentration (Fig. 3). In the presence of citric acid in the medium, an inverse relationship

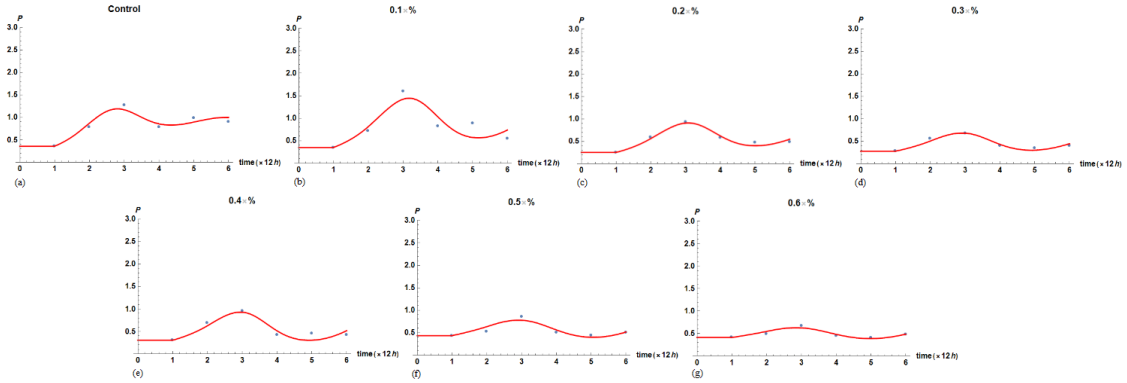


FIGURE 12. (a): Trajectory of *Salmonella* biofilm without any carbon source (b): Trajectory of *Salmonella* biofilm under 0.1% succinic acid (c): Trajectory of *Salmonella* biofilm under 0.2% succinic acid (d): Trajectory of *Salmonella* biofilm under 0.3% succinic acid (e): Trajectory of *Salmonella* biofilm under 0.4% succinic acid (f): Trajectory of *Salmonella* biofilm under 0.5% succinic acid (g): Trajectory of *Salmonella* biofilm under 0.6% succinic acid

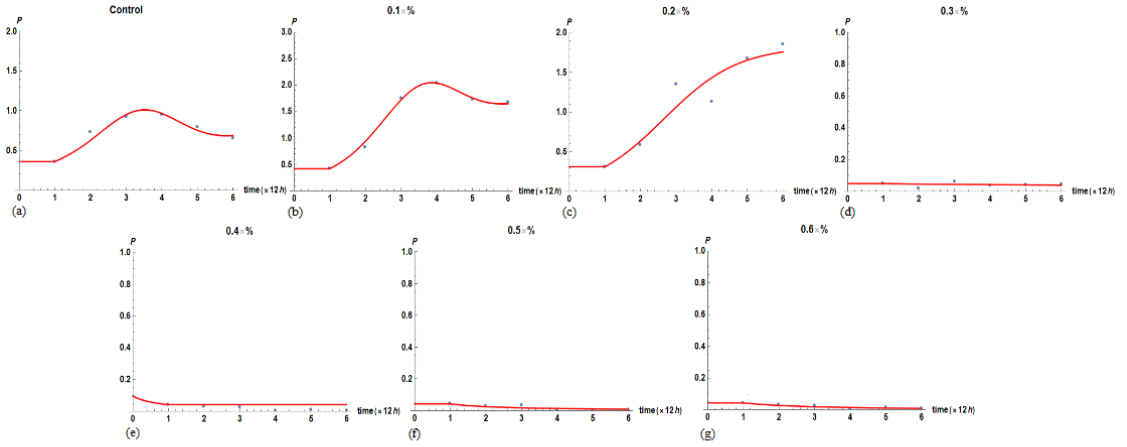


FIGURE 13. (a): Trajectory of *Salmonella* biofilm without any carbon source (b): Trajectory of *Salmonella* biofilm under 0.1% acetic acid (c): Trajectory of *Salmonella* biofilm under 0.2% acetic acid (d): Trajectory of *Salmonella* biofilm under 0.3% acetic acid (e): Trajectory of *Salmonella* biofilm under 0.4% acetic acid (f): Trajectory of *Salmonella* biofilm under 0.5% acetic acid (g): Trajectory of *Salmonella* biofilm under 0.6% acetic acid

was discovered between the rate of increasing concentration and the rate of reaching the maximum point of biofilm amount (Fig. 4). When the rate of glucose reaching the maximum point of biofilm amount was investigated, it was discovered that this rate was inversely proportional to concentration increase (Fig. 1). Glucose, in high concentrations, may cause drastic biofilm regulation alterations, making it unpredictable. This may be the cause of the difference between trajectories of the first experiment (Fig.S8) observed for higher concentrations of glucose. The model proposed here manages to adequately

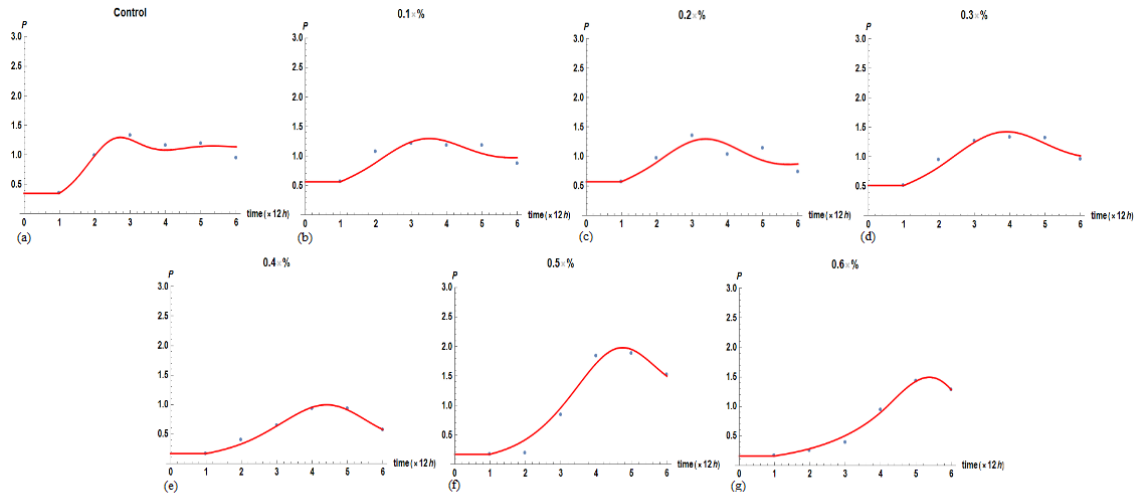


FIGURE 14. (a): Trajectory of *Salmonella* biofilm without any carbon source (b): Trajectory of *Salmonella* biofilm under 0.1% malic acid (c): Trajectory of *Salmonella* biofilm under 0.2% malic acid (d): Trajectory of *Salmonella* biofilm under 0.3% malic acid (e): Trajectory of *Salmonella* biofilm under 0.4% malic acid (f): Trajectory of *Salmonella* biofilm under 0.5% malic acid (g): Trajectory of *Salmonella* biofilm under 0.6% malic acid

represent the underlying process, but prediction errors (the deviation between the model line and the observations themselves) are observed to increase in the repeated experiment (Fig.S8). This means that as the amount of glucose in the surroundings of the biofilm increases, its regulation becomes more chaotic or unpredictable. The previous studies show that bacteria can regulate the biofilm formation by activating certain carbon metabolism mechanisms in high glucose environment and use this source to produce EPS or enhance the production of quorum sensing molecules which are critical for biofilm formation. However, in some cases, high glucose levels may lead to the accumulation of toxic byproducts and inhibit certain regulatory pathways that are essential in biofilm formation [20]. Thus, it can be said that in higher concentrations of glucose, the modeling of the biofilm regulation is more difficult considering only the past population as the predictor. This problem can be overcome by taking into consideration other variables (e.g. temperature or pH) when modeling the population. Furthermore, the error itself can be modeled using these variables. This route is suggested for further research. The presence of acetic acid in the medium was found to be exceptional when compared to other carbon sources, and the amount of biofilm was found to decrease sharply when a threshold concentration value (0.2%) was exceeded in the use of acetic acid (Fig. 6). This implies that acetic acid can be utilized as a biofilm reduction agent. For other types of carbon sources, the stage of the biofilm in a food product at any time can be predicted using these models if the time of initial contamination is known. Furthermore, as the same functional structure explains biofilm trajectory for a variety of carbon sources, these models can be applied across a broad range of food product. In addition to these, since majority of packaged food contains glucose or other carbon sources, the amount of these sources can be adjusted so as not to allow biofilm to develop.

The mathematical model presented in this paper ultimately shows that the process that generates the data, i.e. the biofilm formation and degradation processes, is significantly autoregressive and periodic. That is, the trajectory of the biofilm over time evolves depending primarily on the past amount of biofilm rather than the agents being applied. This conclusion is supported by the fact that all of the models created for varying carbon sources have the same temporal trajectory topology. Changes in carbon sources and the amount of these carbon sources affect the parameter values calculated for the model, but do not cause a change in the topology of the temporal trajectory, as mentioned before. Biofilm degradation (decrease in the amount of biofilm) has a slowing effect on the measurement time after a certain time,

and this delay is determined by the carbon sources and carbon source densities. Therefore, modeling biofilm formation and degradation processes only as a function of the time variable did not cause any loss of information. As noted by Verotta et al. [17], in future studies, model parameters can also be modelled as a function of environmental factors (as represented by carbon sources and carbon source intensities in this study) by constructing various models and examining the distribution of parameters fitted to the models, as was done in this study. Although the model is successful in describing and predicting the process, it is preferable that the context in which the model is used is close to the methodology used in this study. Firstly, the biofilm measurements in this study were obtained by spectrophotometry with linear regression in the background, so the stochastic nature of the measurements should not be ignored. In addition, the measurement frequency is 12-hour intervals, and although the model can be used to calculate intermediate values because it is a continuous function, it should be noted that the time input to the model should be scaled to suit the application area. This study abandons the statistical methodology that is widely preferred over mathematical methodology throughout the field, in order to focus more on explanatory modeling over predictive modeling. Furthermore, statistical methods that are generally used to explain this kind of data, such as Box-Jenkins methodology or machine learning methods which have grown in popularity in the last decade, require far larger volumes of data to be useful. Our model is preferable because it can be useful with a much smaller amount of data. Our study also reveals that biofilm trajectories possess an inherently oscillatory structure, regardless of exterior stimuli.

Supplementary Information To test whether the constructed models agree with empirical evidence, biofilm assays were repeated. The data obtained from these experiments are given as supplementary materials (Fig. S1-S7). As can be seen from these graphs, similar graph topologies were observed in the confirmation experiments, suggesting that the model applicability is replicable (Fig. S8-S14).

Declaration of Competing Interests The authors declare that they have no competing interests.

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