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EFFECT OF SUGARS AND AMINO ACIDS ON FROWTH AND SURVIVAL OF *ARCOBACTER BUTZLERI* IN HIGH SALINE MEDIUM

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The study of effect of sugars and amino acids on the growth and survival of four-strains mixture of Arcoabcter butzleri in tryptic soy broth (TSB) with different salt concentrations was carried out at 30 °C. A. butzleri was able to grow in TSB with 3.5 % NaCl whereas no viable counts were detected at 4.0 and 5.0 % after 0.5-day incubation. Sucrose, trehalose, alanine, glutamine or proline at 0.25 mmol levels were separately added to TSB with 3.5 % NaCl. The growth and survival parameters were estimated according to Baranyi and Weibull model, respectively. The growth of A. butzleri was affected by all the amino acids resulting in higher growth rate, lag phase and maximum population density in medium with 3.5 % NaCl. Slight increase in growth rate in the presence of sucrose and trehalose was only observed. On the other hand, bacteria better survived when sugars were used, increasing the first reduction time (δ). Comparing the results with TSB with natural content of NaCl (0.5 %), only sucrose enhanced the growth of A. butzleri, whereas both sugars and amino acids improved their survival resulting in higher δ.

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Introduction

Arcobacter butzleri, the potential water-borne and food-borne pathogen, has been attracting attention of many scientists since 1977, when it was re-arranged from aerotolerant campylobacters to the new genus *Arcobacter* [1]. Then various physical treatments have been evaluated to prevent growth or survival of *Arcobacter* species including temperature [2-4], pH [5], irradiation treatment [6], the effect of chlorination [7], organic acids and their salts [8,9] or various plant and herbal extracts [10-12]. It is evident from early published papers that some strains of *Arcobacter* are able to grow in the medium containing 2.0, 3.5 and 4.0 % of NaCl [13] or survive in the presence of 5% NaCl at suboptimal temperature [2]. Our recent study revealed that *A. butzleri* cells survived on various kitchen surfaces for a considerable period of time and even after the droplet of suspending medium has been visibly dried [14].

The bacteria undergoing such osmotic stress evoked by drying or presence of osmo-active molecules in environment often respond using various strategies to counteract the higher osmotic pressure that may otherwise lead to loss of cellular turgor pressure, dehydration and death [15-17]. The accumulation of low-molecular-weight organic compatible solutes presents one of the strategies. The compatible solutes are derived from relatively small groups of organic compounds including mainly amino acids, sugars and their derivates. The synthesis of compatible solutes is a gene-coding process, and not all the bacteria evolved genetic alterations for adaptation to the high saline environments. Therefore, the uptake and transportation of compatible molecule from surrounding environment to cytoplasm may be crucial for certain microorganisms [16]. To the best of our knowledge, there is no data about adaptation of arcobacters to osmotic stress.

The aim of this initial study was to determine the effect of sugars (sucrose and trehalose) and amino acids (glutamine, proline and alanine) on the growth and survival of *Arcobacter butzleri* in high saline laboratory medium at optimal temperature.

Materials and Methods

Chemicals

Sugars (sucrose and trehalose both analytical grade) and amino acids 99 % (namely glutamine, DL-proline, L-α-alanine) were obtained from Sigma-Aldrich Chemie, GmbH (Steinheim, Germany).

Bacterial Strains

A cocktail of four strains of *A. butzleri* (CCUG 30484 and three wild-type isolates) were used throughout the study. The strain CCUG 30484 was purchased from the Culture Collection at the University of Göteborg (Göteborg, Sweden). Three wild-type *A. butzleri* strains isolated from chicken and beef meat slaughterhouse environments were obtained from the culture collection of the Department of Biological and Biochemical Sciences at the University of Pardubice (Pardubice, the Czech Republic) [18]. The microorganisms were stored frozen at –80 °C in Luria-Bertani medium containing 30 % (vol/vol) glycerol as a cryoprotectant. For experimental purposes, the bacteria were separately activated by transfer into tryptic soy broth (TSB) purchased from Merck (Darmstadt, Germany) for 48 hours at 30 °C followed by an appropriate aerobic incubation in tryptic soy agar (TSA).

Preparation of Inoculum

The suspension of each strain was prepared in TSB from the bacteria freshly grown on TSA after aerobic incubation at 30 °C for 48 hours. The bacterial concentration was adjusted to approximately 1.0×10^8 CFU ml^{-1}, and then a mixture of four strains (1:1) was prepared by addition of an aliquot of each test suspension to the test tube. The strains mixture was used in order to obtain a general response of *A. butzleri*.

Growth of *A. Butzleri* in High Saline Medium

The high saline medium (HSM) was prepared from TSB with addition of NaCl (Merck) to give final concentration 2.0, 2.5, 3.5, 4.0 and 5 % (w/vol). TSB without added NaCl served as a control (containing naturally 0.5 % NaCl). This control will further be referred as normal saline medium (NSM). The arcobacter mixture was put into the TSB (250 ml) with different salt levels and incubated under aerobic conditions at 30 °C. The initial concentration of bacterial cells was approximately 4.0×10^4 CFU ml^{-1}. At appropriate time intervals, an aliquot of ten-fold serial dilutions was spread on the Petri dishes with TSA and the grown colonies were enumerated after 48-72 hours incubation at 30 °C.

Effect of Sugars and Amino Acids on Growth and Survival of *A. Butzleri* in High Saline Medium

TSB with different salt levels were used as described above. Sugars (sucrose and trehalose) and amino acids (alanine, proline, glutamine) solutions were prepared by dilution of appropriate amount of substances in distilled water. The solutions were filter-sterilized using 0.45 μm-pore-size cellulose membrane filters (Albet-Hahnemuehle, Barcelona, Spain). Appropriate volume of sugar or amino acid solution was aseptically added to sterile TSB (250 ml) with different sodium chloride concentrations to give various concentrations of each sugar or amino acid in TSB, i.e., 0.25 nmol, 0.25 μmol and 0.25 mmol ml^{-1}. Thereafter, arcobacters mixture was added to TSB-HSM batch followed by incubation at 30 °C under aerobic conditions. The initial concentration of bacterial cells was approximately 4.0×10^4 CFU ml^{-1}. An aliquot of ten-fold dilution was spread onto the surface of TSA at appropriate time intervals, and the concentration of culturables cells was determined after incubation at 30 °C. Normal saline medium inoculated with *A. butzleri* mixture was used as a control.

Statistical Analysis and Data Modelling.

The experiments were performed in two separate trials. Each trial consisted of three replicates (n = 6). Statistical differences were determined using student *t-test* at probability level $\alpha = 0.05$. The growth of *A. butzleri* was modelled using Baranyi primary model [19]

$$y(t) = y_0 \mu_{max} A(t) - \log_e \tag{1}$$

where $y_0 = \ln(x_0)$ $y_{max} = \ln(x_{max})$ and

$$A(t) = t + \frac{1}{v}\log_e\left(e^{-vt} + e^{-h_0} - e^{(-vt-h_0)}\right) \tag{2}$$

Under the assumption that the critical substances grow at the same specific rate as the cells in the exponential phase, $v = \mu_{max}$, resulting in four parameters of Baranyi model: y_0 and y_{max} (initial and maximum population, respectively), μ_{max} is the maximum specific growth rate (h^{-1}) and $h_0 = \lambda\,\mu_{max}$, where λ is lag phase duration (h) [19]. The model of Weibull, as modified by Peleg and Cole [20] was used for determination of survival rate

$$\log N = \log N_0 - \left(\frac{t}{\delta}\right)^p$$

where log$N$ and logN_0 are the the concentrations of viable cells (log(CFU ml^{-1})) at the time t and $t = 0$, respectively, δ is the first reduction time (days), i.e., the time to attain 1 – log reduction in the population number and p is the not-dimensional number which gives information about the shape of the curve. Baranyi model was fitted to the experimental data from initial time ($t = 0$) to time when maximum population density was obtained. The parameters for survival rate were determined using experimental data from the time when the maximum population density was determined to the end of the experiment, i.e., descending part of the curve. The fitted parameters were evaluated from each trial separately, followed by computation of growth (μ_{max} and λ) and survival characteristics (δ and p).

The growth curves and the estimation of parameters from Baranyi model were done using software DMFit 2.1 [19]. The parameters for survival experiment were evaluated using Gauss–Newton non-linear regression technique (QC.Expert 3.0, Trilobyte® Ltd., Pardubice, the Czech Republic). The goodness of fit was evaluated by the coefficient of determination (R^2) and mean relative percentage deviation (%P).

Results and Discussion

The mixture of four strains of *A. butzleri* grew in TSB with 3.5 % of NaCl but no viable bacterial cells were detected in TSB with 4.0 and 5 % of NaCl after 12 hours of incubation at 30 °C. Therefore, TSB with 3.5 % NaCl was used in further study as high saline medium (HSM). It is known that certain bacteria may adapt mechanisms which trigger morphological changes of the cells as a respond to environmental stresses. These changes lead to the formation of viable but non-culturable state (VBNC) [21]. *A. butzleri* may also formed cells which are viable but non-culturable in sea-water microcosmos [22]; however, it was the aim of this study to determine whether *A. butzleri* developed VBNC in 4 and 5 % NaCl media. Additionally, the organic substances tested in this study have no effect on the growth or survival of *A. butzleri* in medium containing 4.0 or 5.0 % NaCl, i.e., no viable cells were detected after 12 hours of incubation at 30 °C.

Both Baranyi and Weibull mathematical expressions of the growth and survival curves, respectively, fitted well to all the experimental data. The goodness of fit was characterized by $R^2 > 0.900$. The %P values ranged from 0.49 to 2.43 and from 0.54 to 6.83 for growth and survival experiments, respectively. The model can be accepted when %$P < 10.00$.

Three levels (0.25 nmol, 0.25 μmol and 0.25 mmol per ml) of sugars or amino acids were used in this study. Our results showed that the bacterial concentrations did not differ from those of control during the growth and survival experiments where nmol and μmol levels of sugars or amino acids were used. Therefore, the highest molar concentration of sugars and amino acids (i.e., 0.25

mmol ml^{-1}) were further discussed.

As shown in Fig 1, the effect of sucrose and threhalose on the growth of *A. butzleri* in TSB-NSM was similar to that of control (i.e., without addition of sugars), whereas significantly lower bacterial concentration was detected after 1-2 days of incubation at 30 °C in TSB-NSM with addition of amino acids.

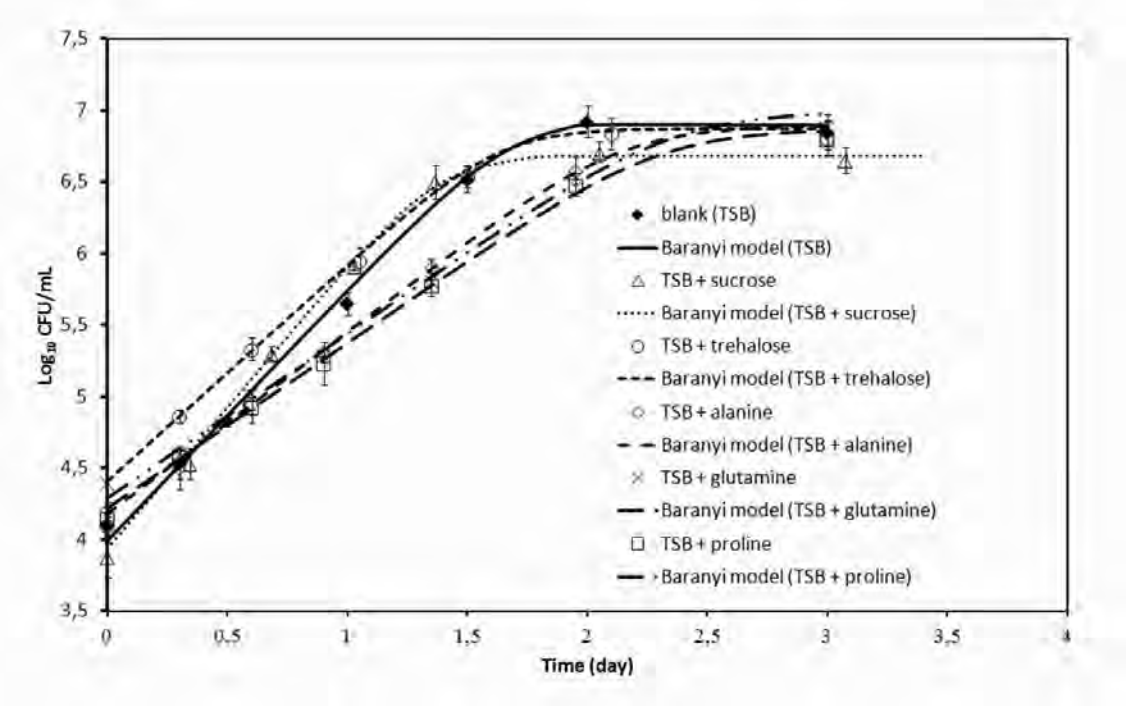

Fig. 1 Growth of *Arcobacter butzleri* four strains mixture in tryptic soy broth containing 0.5 % NaCl (control) and 0.25 mmol l^{-1} of different sugars and amino acids at 30 °C under aerobic conditions. The experimental values are expressed as means ± standard deviation (n = 6); the growth curves were predicted using Baranyi model

The growth curves of *A. butzleri* in TSB-NSM and the corresponding parameters of Baranyi model showed that the presence of amino acids in TSB-NSM decreased the growth rate compared with the control ($p < 0.01$) (Table I).

The similar maximum population density was detected after 3 days of incubation in all the batches. The addition of sucrose to TSB-NSM increased the growth rate ($p < 0.05$) of *A. butzleri* at 30 °C, whereas the effect of trehalose was similar to that of the control.

In high saline medium (3.5 % NaCl), the log counts of *A. butzleri* were apparently higher in the presence of trehalose after 0.3-1.8 days of incubation ($p < 0.05$), whereas sucrose did not seem to affect the growth of *A. butzleri* in TSB-HSM (Fig 2).

However, the estimated parameters of Baranyi model showed that both trehalose and sucrose increased the growth rate ($p < 0.05$) in comparison with the control. The lag phase (λ) was not significantly different. It was previously published that sucrose had protective effect on proteins and lipids *via* the changes

Table I The growth parameters of *Arcobacter butzleri* four-strains mixture in TSB with different NaCl concentration in the presence of 0.25 mM of low-molecular-weight organic compounds at 30 °C estimated by Baranyi model ($n = 6$)

NaCl % [w/vol]	TSB (control)	Sugars		Amino acids		
		Sucrose	Trehalose	Alanine	Glutamine	Proline
0.5						
μ_{max}	^{a}1.70±0.04	^{b}2.05±0.17	^{a}1.68±0.04	^{c}1.29±0.11	^{c}1.13±0.13	^{c}1.21±0.11
λ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MPD	6.90	6.68	6.82	6.85	6.90	6.81
R^2	0.999	0.932	0.996	0.984	0.979	0.982
%*P*	0.92	0.79	1.48	0.49	1.04	0.69
3.5						
μ_{max}	^{a}0.73±0.17	^{b}1.68±0.25	^{b}1.44±0.07	^{c}5.56±0.18	^{c}5.81±0.09	^{c}5.63±0.05
λ	^{a}0.94±0.08	^{b}1.23±0.12	^{a}0.89±0.09	^{c}1.69±0.09	^{c}1.75±0.11	^{c}1.70±0.08
MPD	7.85	4.78	4.96	6.06	6.13	6.12
R^2	0.947	0.997	0.979	0.965	0.989	0.997
%*P*	1.41	0.76	1.00	2.43	1.34	1.05

TSB, tryptic soy broth; μ_{max}, growth rate ($\log_{10}$ CFU ml^{-1} per day); λ, lag phase durativ (day); MPD, maximum population density; R^2, coefficient of determination; %*P*, mean relative percentage deviation; results expressed as means ± standard deviation; different small letters in superscript represent statistically significant differences in row ($p < 0.05$).

of membrane phase transition temperature of phospholipids [23] and many microorganisms possessed the uptake and regulatory mechanisms for distribution of sucrose [15]. Trehalose was identified as a compatible solute in many bacteria including *Escherichia coli* [24] or *Enterobacter sakazakii* [25] in response to osmotic or thermal stresses. *Campylobacter jejuni*, a closely related species to arcobacter, possessed pathway to formed excessive amount of trehalose in order to protect its cell structure against freeze temperatures [26]. All the amino acids had significant effect on the growth of *A. butzleri* in TSB-HSM at 30 °C. It is evident from the growth curves (Fig 2) that the presence of alanine, proline or glutamine in high saline environment had protective effect on the growth of *A. butzleri*. There is an enhanced lag phase, higher logarithmic phase of the growth and the higher maximum population density as compared with control (Table I). While the lag phase increased just about 0.7 day, the growth rate increased five times and the maximum population density increased about 1 log cycle in comparison with those in control. Our results showed that amino acids have a po-

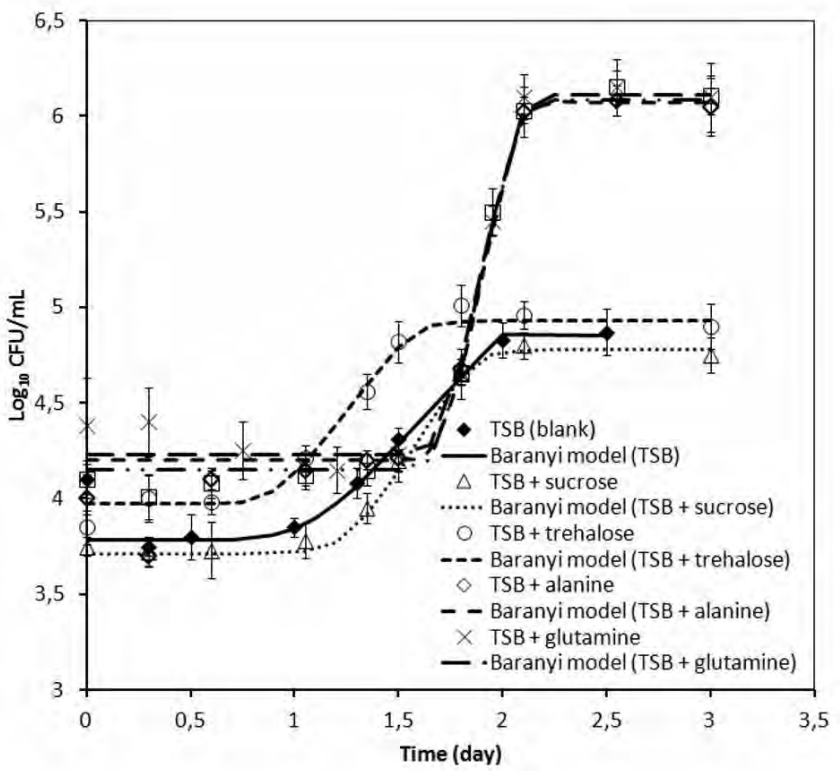

Fig. 2 Growth of *Arcobacter butzleri* four strains mixture in tryptic soy broth containing 3.5 % NaCl and 0.25 mmol l^{-1} of different sugars and amino acids at 30 °C under aerobic conditions. The experimental values are expressed as means ± standard deviation (n = 6); the growth curves was predicted using Baranyi model

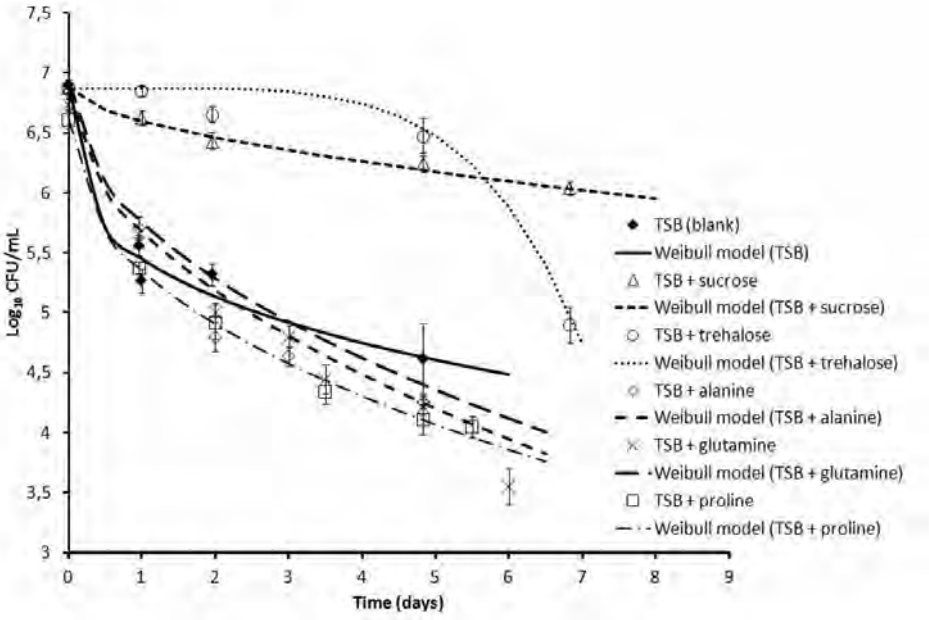

Fig. 3 Survival of *Arcobacter butzleri* four strains mixture in tryptic soy broth containing 0.5 % NaCl (control) and 0.25 mmol l^{-1} of different sugars and amino acids at 30 °C under aerobic conditions. The experimental values are expressed as means ± standard deviation (n = 6); the growth curves were predicted using Weibull model

tential to enhance the growth of *A. butzleri* in the presence of 3.5 % NaCl. It was previously described that *Campylobacter jejuni* exhibits unique nutritional requirements and utilize various amino acids as a carbon source [27]. In addition, the ability to metabolize glutamine, glutathione and asparagine may affect its ability to colonize specific host tissues [28].

The effect of sucrose and trehalose on the survival of *A. butzleri* in TSB-NSM at 30 °C is depicted in Fig 3.

It is apparent that the addition of sugars to the environment may protect the bacterial cells against osmotic pressure during survival. The concentration of *A. butzleri* cells during survival experiment in TSB-NSM with addition of sucrose and trehalose was higher by about 1.3 log cycle in the range of 0.96-4.83 days of incubation in comparison with the control. The effect of sucrose and trehalose on the survival of *A. butzleri* in TSB-NSM was similar up to 5 days of incubation. After the 7th day of incubation, the log count of *A. butzleri* decreased by about 1.5 log in the presence of trehalose, whereas only 0.21 log reduction was observed in

Table II The survival parameters of four-strains mixture of *Arcobacter butzleri* in TSB medium with different NaCl concentrations in the presence of 0.25 mM of low-molecular-weight organic compounds at 30 °C estimated by Weibull model (n = 6)

NaCl % [w/vol]	TSB[a] (control)	Sugars		Amino acids		
		Sucrose	Trehalose	Alanine	Glutamine	Proline
0.5						
δ	[a]0.28±0.14	[d]9.19±1.01	[c]6.02±0.11	[b]0.74±0.09	[b]0.72±0.07	[b]0.59±0.10
p	0.29	0.57	5.04	0.51	0.49	0.44
R^2	0.975	0.988	0.989	0.979	0.988	0.997
%P	1.77	1.85	1.14	2.73	6.83	0.79
3.5						
δ	[b]0.92±0.50	[c]4.62±0.15	[d]5.87±0.13	[a]0.26±0.13	[ab]0.48±0.64	[ab]0.43±0.19
p	0.38	1.70	5.68	0.16	0.26	0.24
R^2	0.955	0.994	0.938	0.996	0.959	0.991
%P	3.54	0.73	1.31	0.54	2.46	0.99

TSB, tryptic soy broth; δ, first reduction time (day); p, non-dimensional parameter of Weibull model; R^2, coefficient of determination; %P, mean relative percentage deviation; results expressed as means ± standard deviation; different small letters in superscript represent statistically significant differences in row ($p < 0.05$)

TSB-NSM with sucrose (Fig. 3). The first reduction times (δ) increased from 0.28 (in control) to 9.19 and 6.02 days for trehalose and sucrose, respectively. Amino acids used in this study also enhanced the survival in TSB-NSM with the parameter δ being significantly higher than in the control (Table II).

Similar response of *A. butzleri* cells to sucrose or trehalose was observed in TSB-HSM. The higher log counts of *A. butzleri* cells were detected in the presence of both sugars throughout the whole experiments in comparison with the control. A rapid decrease in the cells concentration after the first day of incubation followed by gradual reduction of bacterial counts in the control (TSB-HSM without addition of sugars or amino acids) and gradual decrease in the *A. butzleri* cells concentration during the beginning of the survival stage followed by rapid declining of bacterial counts in the presence of sugars resulted in a different shape of survival curves obtained from non-linear regression of Weibull model (Fig. 4).

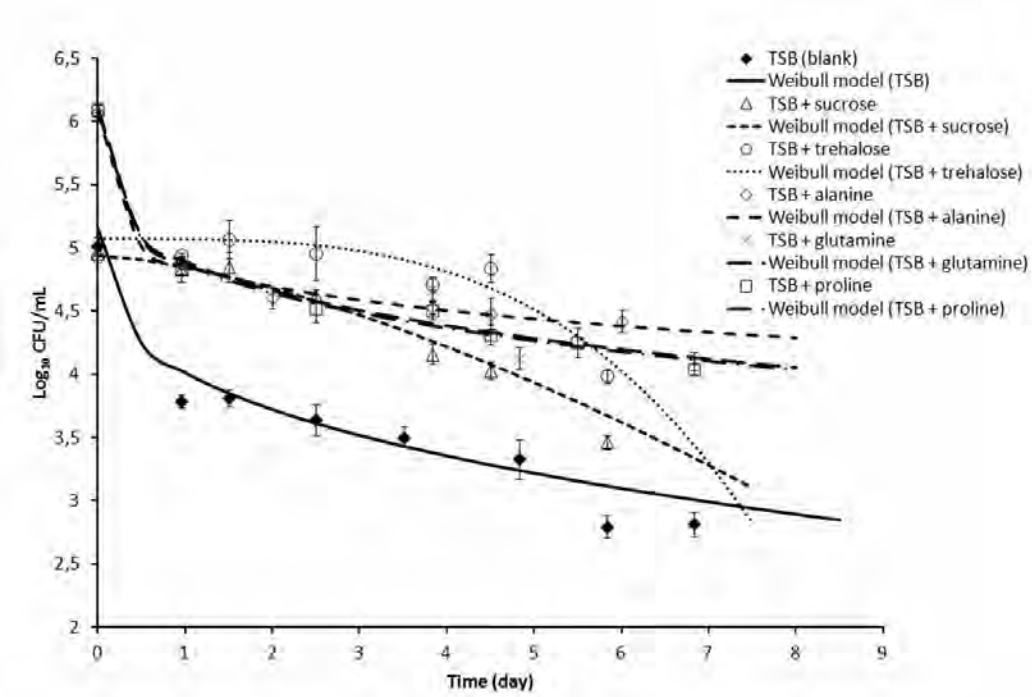

Fig. 4 Survival of *Arcobacter butzleri* four strains mixture in tryptic soy broth containing 3.5 % NaCl and 0.25 mmol l^{-1} of different sugars and amino acids at 30 °C under aerobic conditions. The experimental values are expressed as means ± standard deviation (n = 6); the growth curves were predicted using Weibull model

The first reduction time significantly increased from 0.92 (the control) to 4.62 and 5.87 days for sucrose and trehalose, respectively (Table II). The effect of amino acids on the survival of *A. butzleri* in TSB-HSM is also presented in Fig. 4. Since the initial bacterial count was significantly higher, we may also conclude that the shape of the survival curves is similar to that of control. It was found that sugars affected the resistance of *Lactococcus lactis* to conditions similar to those in gastrointestinal tract by different ways [29]. While sucrose significantly enhanced the growth of this probiotic bacterium, lactose promotes its survival.

We demonstrated that sucrose had improving effect on the growth of *A. butzleri* in tryptic soy broth. On the contrary, alanine, glutamine or proline declined the growth rate of *A. butzleri* cells in TSB with natural content of NaCl (0.5 %). We may also conclude that both the sugars and the amino acids used in this study significantly enhanced survival of *A. butzleri* in TSB-NSM at 30 °C but the presence of sugars in TSB medium resulted in a higher parameter δ. Higher concentration of sodium chloride (3.5 %) in TSB affected the growth of *A. butzleri* by decreasing the growth rate and increasing of lag phase. The addition of amino acids enhanced the growth of *A. butzleri* in TSB-HSM but did not affect the survival at the same temperature. On the other hand, sugars possessed protective effect on *A. butzleri* cells in high saline environment during survival.

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