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Toxicity Enhancement of Clostridium Argentinense by non O1, non O139 Vibrio Cholerae in Co-culture

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Abstract: The growth and botulinic toxin production of *Clostridium argentinense* G 89 HT in co-culture with non O1, non O139 *Vibrio cholerae* were studied using three culture systems, operated under different aeration conditions. Growth in homogeneous co-culture with aerobic and anaerobic stage presented a dual-phase progression and 2000 LD_{50} of botulinum toxin was obtained. This value represented an increase of 20 times compared with the other culture systems studied and high levels of protease activity were achieved. The remarkable increase on the toxicity of *C. argentinense* G 89 HT was attributed to activation of the toxin by non O1, non O139 *Vibrio cholerae* proteases. The heterogeneous co-culture obtained with a dialysis membrane physically separating both bacteria, thereby eliminating the protease activity, produced low levels of activity toxigenic.

Keywords: *Clostridium argentinense;* Non O1, non O139 *Vibrio cholerae*; Co-culture; Protease activity; Toxin activation

1. Introduction

Clostridium botulinum is an anaerobic, gram-positive, spore-forming rod-shaped microorganism that produces a potent neurotoxin causing botulism in man and animals. There are seven serotypes of botulinum neurotoxins (BoNTs), designated BoNT/A-G. Several related clostridial species (e.g., *C. baratii* and *C. butyricum*) can produce some BoNT as well [1-2].

The geographical distribution of *C. botulinum* has been studied extensively in Europe, Asia, North America and Argentina. They have isolated from diverse soils, freshwater sediments, and marine environments where they share their ecological niche with other anaerobic and aerobic bacteria [3-4]. *C. botulinum* type G (strain 89) was first isolated in 1969 by Giménez and Ciccarelli, from a cornfield in the Mendoza Province of Argentina [5]. Later, Sonnabend et al. [6-7], isolated type G microorganisms from necropsy specimens in adults and an 18-week-old infant in Switzerland. Type G botulinal toxin was demonstrated in the serum of these individuals. *C. argentinense* comprises all strains of *C. botulinum* type G and some non-toxigenic strains previously identified as *C. subterminale* and *C. hastiforme* [8-9]. *C. argentinense* strain 89 produced very little toxin, even in special media, is weakly proteolytic, asaccharolytic, not produce lipase and lecithinase, poor sporulator in the common media and its toxin is activated by trypsin [10]. Centorbi OP et al. [11], isolated a highly toxigenic and sporulating strain obtained by selection of the prototype strain G89, called *C. argentinense* G89 HT. Two plasmids, of 83 and 170 MDa were isolated, whereas the prototype strain G89 has only an 81 MDa plasmid [12].

C. argentinense G89 growth and toxin production is dependent on environmental factors, presence of nutrients and biological interactions with other microorganisms, which produce profound changes in behaviour [13-14].

The Gram-negative bacterium non O1, non O139 *Vibrio cholerae* is morphologically and biochemically identical those belonging to serogroups *V. cholerae* O1 and *V. cholerae* O139, the causative agents of cholera, but differs in antigenic, epidemiological and clinical terms. Nor has the factors of pathogenicity of *V. cholerae* O1 as cholera toxin and colonization factor, but synthesizes a heat-stable enterotoxin, which is the main virulence factor. Therefore it is able to produce human disease, such as sporadic outbreaks of watery diarrhea, inflammatory enterocolitis and fatal sepsis. This microorganism has a worldwide distribution and is commonly found in aquatic environmental sources [15-16-17].

The aim of the present study was to investigate the effect of non O1, non O139 *V. cholerae* on the growth and toxicity of *C. argentinense* G89 HT. A comparative study of different parameters of growth, toxin production and protease activity in single

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cultures and co-cultures between both microorganisms was performed.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

C. argentinense strain G89 HT, obtained by the methods described previously and preserved by lyophilization in skim milk [11]. In order to produce spores of *C. argentinense* strain G89 HT, the lyophilisate was resuspended in phosphate buffered saline (PBS), 500 µL was inoculated in chopped meat medium (CMM) and incubated overnight at 35 °C into GasPak jar system (Oxoid). Afterwards, 2 mL of CMM culture was transferred to 100 mL of the sporulation medium containing: 5% tryptone (Difco), 1% $(NH_4)_2SO_4$, pH 7.0. After 7 days of anaerobic incubation at 30 °C, spore production was checked microscopically by Gram coloration and then harvested by centrifugation at 10000 x g for 20 min and washed three times with PBS, pH 7.2. The pellet was resuspended in 40 mL of sterile physiological salt solution and stored at 4 ºC. This suspension was diluted 1:200 in distilled water and subsequently used as inoculum.

Non O1, non O139 *V. cholerae* strain 289, was isolated from environmental water source in the Córdoba Province of Argentina during the 1991-1996 period under a cholerae surveillance program carried out at the "Centro de Enterobacterias-División Laboratorio Central de Salud Pública de la Provincia de Córdoba" [15]. The strain was grown in Luria-Bertani (LB) medium containing 1% tryptone (Difco), 0.5% yeast extract (Difco), and 1 % NaCl and was stored at -70 °C in 20 % (vol/vol) glycerol. Strain from glycerol stock cultures was streaked on LB agar plates and incubated overnight at 37 °C. A loopful of cells from a plate was inoculated into 5 mL of LB medium in 18-mm-diameter 15-cm-long culture tubes and was grown at 37°C with shaking. This culture was diluted 1:1000 in fresh LB medium and subsequently used as inoculum.

The evaluation of the toxin production by *C. argentinense* G89 HT was performed using an optimized medium (OM) of the following composition (g/L): proteose peptone (Difco), 30; yeast extract (Merck), 5; trypticase (Difco), 5; glucose, 11. The pH of the media was adjusted to 7.6 and sterilized at 121 ºC for 15 min.

2.2. Culture Systems

2.2.1 Homogeneous culture system anaerobic (HoCSA)

C. argentinense G 89HT, non O1 non O139 *V. cholerae* single cultures and co-culture between both microorganisms, were developed in 800 mL capacity fermenters containing 700 mL of OM without aeration or agitation. Inoculum size for single cultures consisted of 3.5 mL of *C. argentinense* G89 HT spore suspension and 3.5 mL of non O1, non O139 *V. cholerae*, and a mixture of the same volume of each microorganism to co-culture. The fermenters were inoculated immediately after autoclaving to prevent reoxygenation. After inoculation single cultures and co-culture were incubated at 37 ºC for 100 h in static conditions.

2.2.2 Homogeneous culture system in two stages (HoCSTS)

Single cultures and co-culture were developed in the same fermenters previously described, but containing 350 mL of OM. After inoculation, the cultures were incubated at 37 °C for 20 h under aerobic conditions using a sterile air flow of 15 L/h with constant shaking at 80 rpm (Aerobic stage). At this time were added 350 ml fresh OM and the incubation was continued under static conditions to complete the 100 h of culture.

2.2.3 Heterogeneous culture system in two stages (HeCSTS)

Another experiment was carried out by incorporating a tubular dialysis membrane (Spectra/Por, MWCO 12–14 kDa) into the fermenter to perform a co-culture with physical separation of both microorganism but sharing the same nutritional environment. Inoculum of non O1, non O139 *V. cholerae* was seeded inside the dialysis membrane, while *C. argentinense* G 89 HT was seeded outside. The fermenter was operated in aerobic and anaerobic conditions as described previously.

2.3. Quantification of biomass and residual glucose

Duplicate samples were taken at various times throughout the culture period to monitor the optical density (OD), levels of glucose and protease activity. Before sampling in static conditions, the cultures were homogenized by magnetic stirring at 50 rpm for 5 min flushing N2 in the headspace to prevent reoxygenation. OD measurements were made in a Spectronic 20 Genesis (Spectronic Instruments) spectrophotometer at 600 nm (OD_{600}) and glucose was quantified using the glucose-oxidase peroxidase method in culture supernatants. Biomass was estimated at the start and end of the exponential growth phase by dry weight measurement of samples centrifuged at 10000 x g for 20 min, washed twice with distilled water and dried at 100 °C for 16 h. The specific growth rate (µ) was calculated using the expression: $\ln x2/x1 \cdot t^{-1}$, where x1 and x2 were two determinations of biomass in the exponential growth

phase at time t.

2.4. Determination of protease activity

Protease activity was assayed in cell culture supernatants using azocasein (Sigma Chemical Co. St. Louis, U.S.A.) as substrate at a concentration of 0.5% dissolved in 100 mM Tris/HCl buffer (pH 8.0). After centrifugation at 10000 x g for 20 min, 100 µL of supernatant was added to 100 µL of substrate solution and the mixture was incubated at 37 ºC for 1 h. The reaction was stopped by the addition of 400 µL of 10 % (w/v) trichloroacetic acid. The mixture was centrifuged at 7000 x g for 10 min and trichloroacetic acid supernatant was mixed with 700 µL 525 mM NaOH, followed by absorbance estimation at 442 nm (OD_{442}) calibrating with a control tube. The control was prepared in a similar manner to the test tube except that the sample was first inactivated with trichloroacetic acid before the addition of the substrate. One unit azocasein (UA) was defined as the amount of enzyme producing an increase of 0.01 OD_{442} unit per h at 37 ºC. The protease activity was expressed as UA/mL.

2.5. Botulinum toxin assay

The mouse bioassay was conducted in single and co-culture supernatants obtained by centrifuging at 10000 x g for 20 min to estimate the toxin production. Rockland strain mice weighing between 18 and 21 g were injected intraperitoneally with 0.5 mL dilutions of culture supernatants. For those subsequently developing typical symptoms of botulism, the LD_{50}/mL was calculated by the Up-and-Down-Procedure [18].

2.6. Statistical analyses

All assays were performed in duplicates in at least three different experiments. The data are presented as mean ± standard deviation.

3. Results

3.1. Single culture of *C. argentinense* G89 HT, non O1 non O139 *V. cholerae* and co-culture between both microorganisms in HoCSA

The results obtained in growth experiments using the same culture medium are shown in Fig. lA. *C. argentinense* G89 HT single culture presented a lag period of about 10 h corresponding to the time of germination of spores, followed by a rapid growth phase and a subsequent period of decline and cell lysis, which lasted until the end of the culture. Similar growth kinetics was observed with non O1, non O139 *V. cholerae*, although the maximum biomass and specific growth rate were lower that obtained by *C. argentinense* G89 HT (Table 1). In this case, the lag period was due to the adaptation of this microorganism to be transferred from LB medium to OM. Approximately, 40 % of the glucose available was consumed by *C. argentinense* G89 HT, but in non O1, non O 139 *V. cholerae* culture the glucose consumption was 30% (Fig. 1B). Co-culture growth kinetics showed a similar behaviour to that described for both microorganisms in individual cultures, but with a higher specific growth rate and the maximum biomass obtained was slightly higher than the sum of the individual cultures biomass (Fig.1A and Table 1). The increased use of glucose occurred between 10 and 20 h corresponding to the exponential growth phase, with a total consumption of carbon source about 60% (Fig. 1B). Similar values of protease activity were detected both in single culture of *C. argentinense* G 89 HT as in co-culture, however in individual culture of non O1, non O 139 *V. cholera*e protease activity was not observed (Table 1). Most toxigenic activity was obtained at 100 h, and toxin levels both for single culture of *C. argentinense* G 89 HT as co-culture did not differ. (Table 1).

3.2. Single culture of *C. argentinense* G89 HT, non O1 non O139 *V. cholerae* and co-culture between both microorganisms in HoCSTS

The HoCSTS was not suitable for the individual growth of *C. argentinense* G89 HT, however this microorganism was able to grow in co-culture with non O1, non O139 *V. cholerae* in this culture system. The single culture growth of non O1, non O139 *V. cholerae* and co-culture with *C. argentinense* G 89 HT in HoCSTS are shown in Fig. 2A. Both growth kinetics showed similar behaviour in the first 20 h (aerobic phase), manifesting a lag period of 10 hours followed by a phase of rapid growth reaching the maximum biomass at 20 h of culture with a similar specific growth rate (Table 2). In both cases glucose available was completely consumed (Fig. 2B) and 690 UA/mL of protease activity was detected in this phase of the cultures. At this time fresh medium was added, glucose levels increased to 5.5 g/L, biomass was halved, aeration was stopped and started growing anaerobic stage. The co-culture presented a second lag period of 10 h followed by an exponential growth phase between 30 and 40 h of culture corresponding to growth of *C. argentinense* G89 HT (Fig. 2A). This correlated with the glucose consumption (Fig. 2B). Toxin level obtained at the end of co-culture was 2000 LD_{50}/mL which represented a 20 times increase respect to co- culture in the HoCSA. Similarly, protease activity detected in HoCSTS was about 6 times higher to that observed in the HoCSA co-culture (Table 1 and Table 2), due to the contribution of non O1, non O139 *V. cholerae* proteases.

3.3. Co-culture between *C. argentinense* G89 HT and

non O1, non O139 *V. cholerae* in HeCSTS

Estimated parameters in HeCSTS for the co-culture between non O1, non O139 *V. cholerae* and *C. argentinense* G 89 HT were measured outside the dialysis membrane. In this system, the growth exhibited a prolonged lag period of approximately 30 h followed a similar pattern to that observed for single culture of *C. argentinense* G 89 HT in HoCSA (Fig. 3A). Toxicity and protease activity obtained in HeCSTS were approximately 100 LD_{50}/mL and 76 UA/mL, respectively (Table 2). Glucose was completely consumed at 20 h of culture due to the growth of non O1, non O139 *V. cholerae* within the dialysis membrane. After the addition of fresh medium, a second period of glucose consumption was observed between 30 and 40 h of co-culture (Fig. 3B). Protease activity estimated within dialysis membrane at the end of the culture was 3650 UA/mL. This high value was due to the inability of the protease to diffuse out of the dialysis membrane.

4. Discussion

The results obtained manifested a strong influence of non O1, non O139 *V. cholerae* on the growth and toxicity of *C. argentinense* G 89 HT in co-culture, although this effect was dependent on the culture system used. Experience in HoCSA showed that non O1, non O139 *V. cholerae* was able to grow in reduced environments, adapting their metabolism to this condition with low biomass production compared to HoCSTS. The slightly higher level of co-culture biomass and specific growth rate, respect to single cultures in HoCSA, suggests the existence of some synergistic effect between both microorganisms. The ability to grow of *C. argentinense* G 89HT in HoCSTS and HeCMTS co-culture, demonstrated the important metabiotic effect exerted by non O1, non O139 *V. cholerae* on *C. argentinense* G 89HT. This effect is probably due to depletion of dissolved oxygen after aerobic culture stage generating anaerobic conditions, allowing the growth of *C. argentinense* G89 HT. The term "metabiotic effect" indicates the ability of one species to change otherwise unfavorable conditions and thereby allows a second species to grow. For example, *Bacillus licheniformis* exerted a metabiotic effect causing oxygen depletion and pH elevation, allowing *C. botulinum* can grow and produce toxin [19]. Some strains of the lactic acid bacteria and *Pediococcus pentosaceus* produce bacteriocins that are inhibitory to the growth of proteolytic and nonproteolytic *C. botulinum* types A and B [20-21-22]. *P. mendocina* is a microorganism that grows preferably under aerobic conditions but showing a remarkable metabolic versatility in reduced environments being able to redirect its anabolic flux towards the synthesis of uronic acids or alginate, which are released in the extracellular medium. When *P. mendocina* is anaerobically co-cultivated with *C. argentinense* G 89 HT, these compounds can be used by this microorganism as a non-readily available carbon source when glucose is exhausted, improving its growth at very low specific growth rates. The toxin production by *C. argentinense* G 89 HT is clearly enhanced, which indicate the existence of an important interaction between both microorganisms [23].

Glucose consumed in HoCSTS and HeCSTS co-cultures was mainly used by non O1, non O139 *V. cholerae* to produce biomass. High protease activity observed under these conditions suggests that glucose plays an important role in the production of proteases by non O1, non O139 *V. cholerae*. Previous studies indicated that *V. cholerae* O1 produces a hemagglutinin protease (Hap), which is synthesized in response to nutrient, and specifically glucose limitation. Hap production is subject to a dual regulation which involves the quórum-sensing regulator HapR and the cAMP receptor protein [24-25]. Hap produced by *V. cholerae* O1 can proteolytically activate cholera toxin (CT) A subunit, El Tor cytolysin, hemolysin and hydrolyze several physiologically important proteins, such as mucin, fibronectin, lactoferrin [26]. Proteolytic activity of non O1, non O139 *V. cholerae* is also due to Hap which has been purified, cloned, and sequenced. The *hap* gene consisted of 1827 nucleotides with a predicted molecular mass of 69.3 kD [27]. A comparative study of non O1 *Vibrio cholerae* Hap with those of *Vibrio cholerae* O1 showed that soluble protease activities were identical in heat stability, immunodiffusion, inhibition by antiserum, and electrophoretic análisis [28]. Similarly, *V. cholerae* O139 produces a protease which is indistinguishable from that Hap of *V. cholerae* O1 and non O1 *V. cholerae* [29].

In this work, glucose repression was confirmed in HoCSA experiments where residual glucose caused inhibition of protease synthesis by non O1, non O139 *V. cholerae*, while HoCSTS and HeCSTS the carbon substrate was completely consumed in the first 20 h of both single culture or co-culture with *C. argentinense* G89 HT, obtaining higher levels of protease activity. However, this effect was not observed with *C. argentinense* G89 HT where protease activity was independent of glucose levels, indicating that regulatory mechanisms of production would be different from non O1, non O139 *V. cholerae*. The study of the growth curves both single culture *C. argentinense* G89 HT or co-cultures with non O1, non O139 *V. cholerae*, indicated that BoNT /G was actively excreted into the external environment after the exponential growth phase associated with cell lysis. This toxin is initially synthesized as large molecules with comparatively low biological activity owing to masking of active

chemical groupings and must be partially degraded by the proteolytic enzymes before manifesting their full toxic potentialities. Is produced in culture as a relatively large protein complex (L complex ~500 kDa) consisting of a neurotoxin and three neurotoxin-associated proteins (NAPs): two hemagglutinins (HA17 and HA70) and a nontoxic-nonhemagglutinin (NTNH) component. [30]. Therefore, BoNT/G produced by *C. argentinense* G89 HT in HoCSA and HeCSTS was activated by proteases specific *C. argentienense* G89 HT. The significant increase in toxicity observed in HoCSTS co-culture strongly suggests that the activation of the toxin is due to the sum of specific proteases and those produced by non O1, non O139 *V. cholerae*. This conclusion is supported by the results obtained in HoCSA and HeCSTS co-cultures, where in the absence, or unavailability of non O1, non O139 *V. cholerae* proteases retained by the dialysis membrane, did not increase toxigenic activity of *C. argentinense* G 89 HT.

Whereas both microorganisms are widespread in the environment with similar natural habitats, different ecological relationships can be established between them. Our results may provide an explanation for the increased cases of avian botulism produced by strains of *C. botulinum* type C observed in certain regions of the world as the Neusiedler See lake in Austria where, due to global warming, there is a high population of non O1, non O139 *V. cholera*e [31].

Conclusions

The optimal conditions for growth and toxin production by *C. botulinum* strains have been well described in the literature. However, there are few reports describing the effect of other bacteria on toxin activation when they share the same habitat with *C. botulinum*. The interaction between *C. argentinense* G 89 HT and non O1, non O 139 *V. cholerae* in co-culture was properly characterized in terms of growth and toxin parameters using different culture systems. The studies using homogeneous and heterogeneous co-cultures indicated the important role that non O1, non O 139 *V. cholerae* proteases exerted on the activation of the toxin produced by *C. argentinense* G 89 HT.

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Conflict of interest
All authors disclose any conflict of interest.

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Table 1. Growth parameters, toxicity levels and protease activity of single cultures of *C. argentinense* G89 HT, non O1 non O139 *V. cholerae* and co-culture between both microorganisms in the HoCSA.

Parameters	*C. argentinense* G89 HT	Non O1, non O139 *V. cholerae*	Co-culture
Higher Biomass (g/L)	0.71 ± 0.07	0.38 ± 0.03	1.30 ± 0.10
Specific growth rate (h^{-1})	0.21	0.18	0.22
Toxicity (LD_{50}/mL)[a]	100 ± 10	ND	110 ± 10
Protease Activity (UA/mL)[a]	74 ± 7	ND	78 ± 7

[a] Parameters calculated at 100 h of culture.
ND: non detected
All assays were performed in duplicates in at least three different experiments. The data are presented as mean ± standard deviation.

Table 2. Growth parameters and toxicity levels of single cultures of non O1, non O139 *V. cholerae* in HoCSTS and co-culture with *C. argentinense* G89 HT in HoCSTS and HeCSTS.

Parameters	Non O1, non O139 *V. cholerae*		Co-culture	
	HoCSTS[a]	HeCSTS[b]	HoCSTS[a]	
Higher Biomass (g/L)	4.51 ± 0.22		4.74 ± 0.23	0.69 ± 0.07
Specific growth rate (h^{-1})	0.35		0.35	0.20
Toxicity (LD_{50}/mL)[c]	ND		2000 ± 200	100 ± 10
Protease Activity (UA/mL)[c]	340 ± 34		455 ± 45	76 ± 7

[a] Homogeneous culture system in two stages
[b] Heterogeneous culture system in two stages
[c] Parameters calculated at 100 h of culture
ND: non detected
Note: HeCSTS co-culture parameters were estimated outside the dialysis membrane
All assays were performed in duplicates in at least three different experiments. The data are presented as mean ± standard deviation

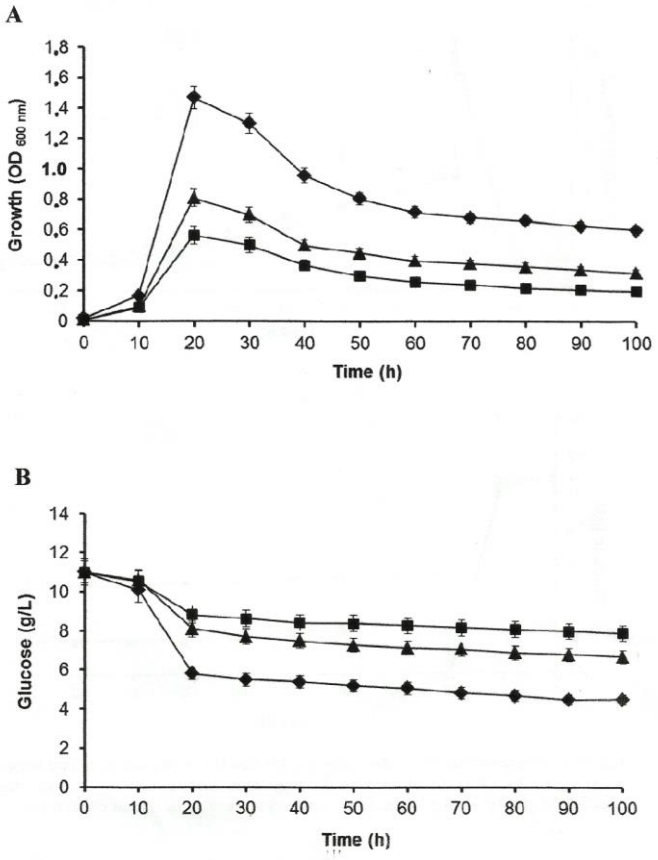

Figure 1. Single cultures of *C. argentinense* G89 HT (▲), non O1 non O139 *V. cholerae* (■) and co-culture between both microorganisms (◆). (A) Growth in HoCSA. (B) Residual glucose. Error bars reflect the standard deviation of the means.

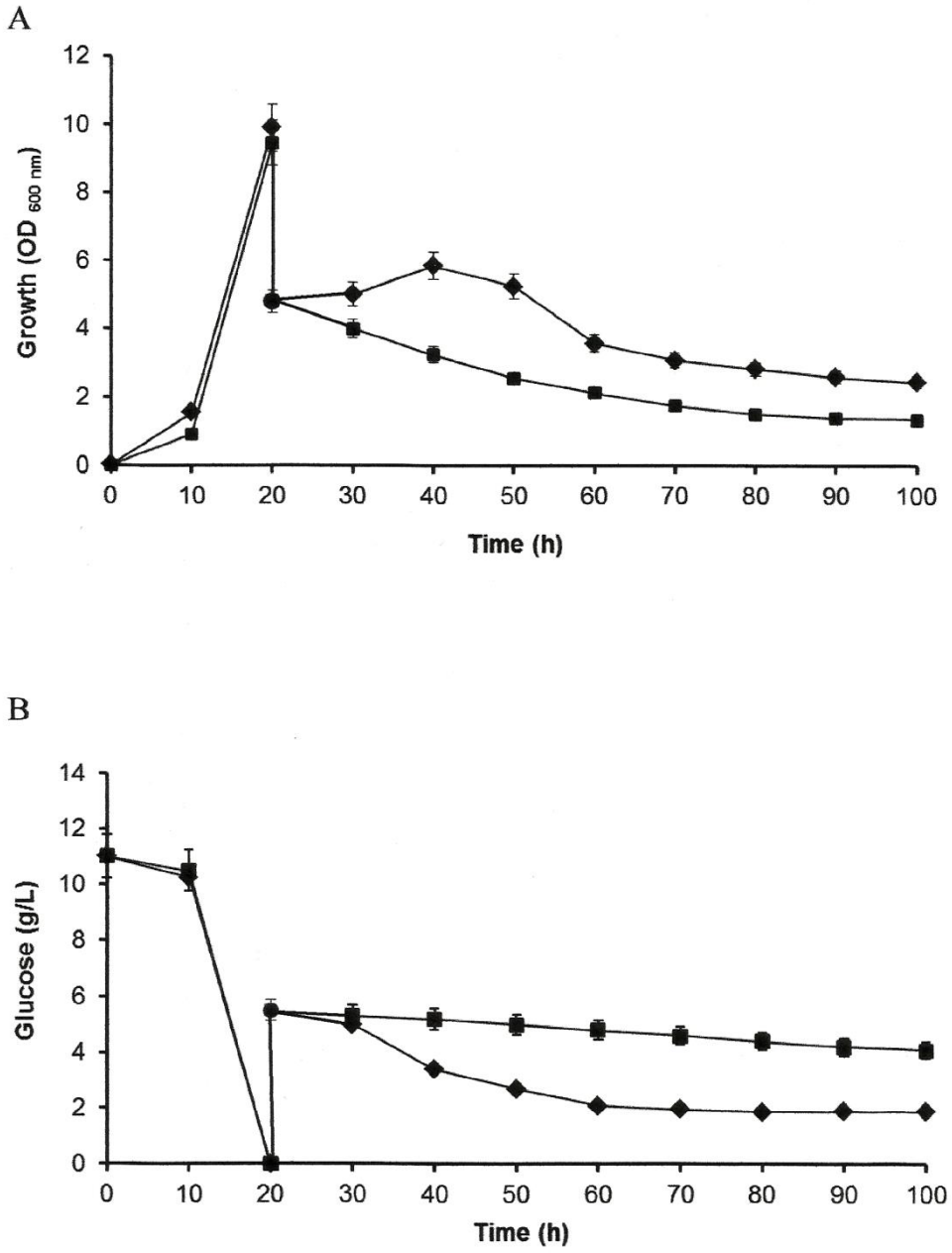

Figure 2. Single culture of non O1, non O139 *V. cholerae* (■) and co-culture with *C. argentinense* G89 HT (◆). (A) Growth in HoCSTS. (B) Residual glucose. Error bars reflect the standard deviation of the means.

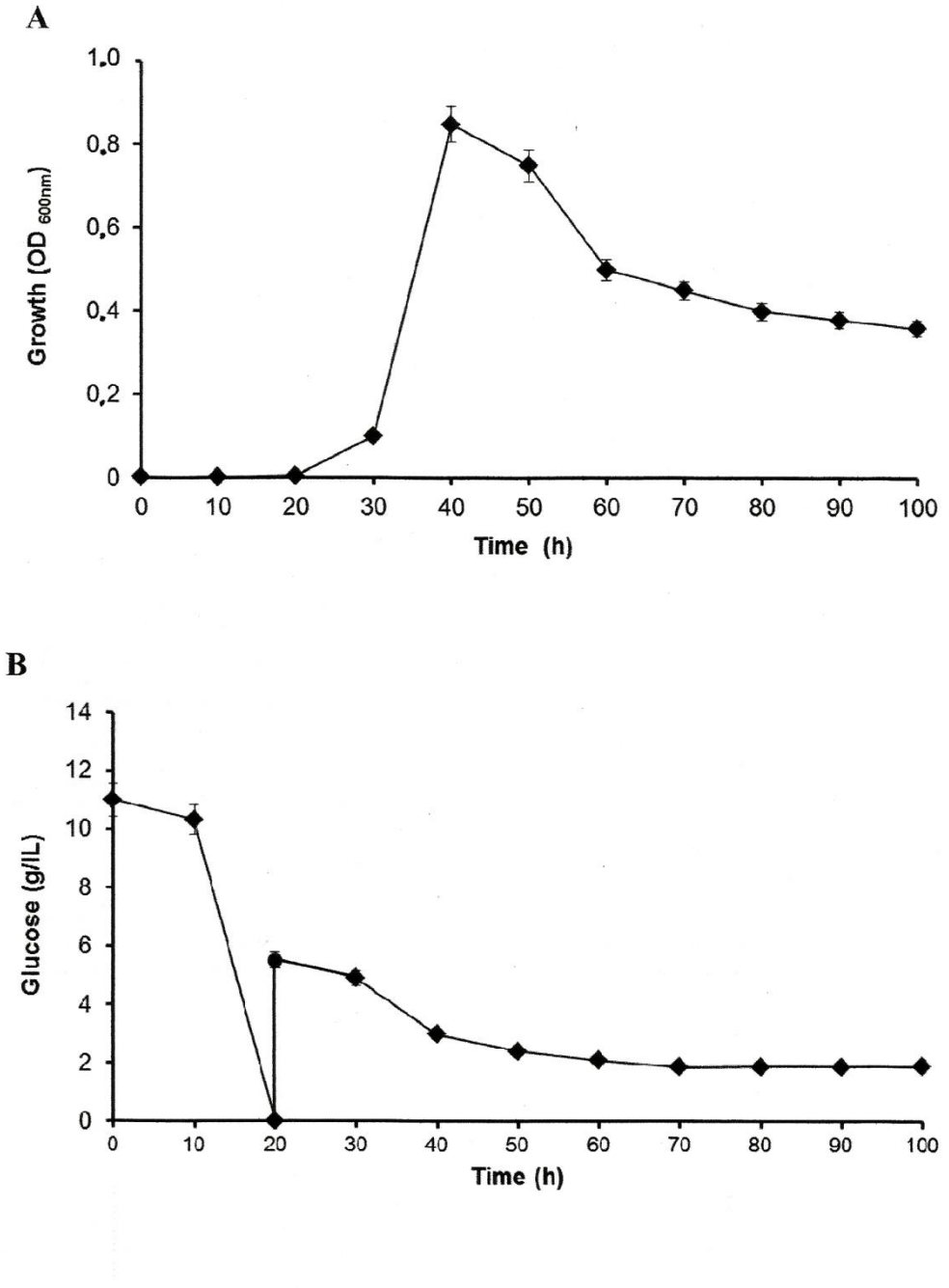

Figure 3. Co-culture between *C. argentinense* G89 HT and non O1, non O139 *V. cholerae* (◆). (A) Growth in HeCSTS. (b) Residual glucose. Error bars reflect the standard deviation of the means.