Shelf life improvement of the biocontrol agent *Candida sake* CPA-1 by suitable package and storage conditions

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Abstract

Agrochemical companies are increasing their interest in the production of biopesticides based on biocontrol agents (BCAs), mainly due to the need to reduce the synthetic fungicide application and the consequent benefits for the environment and for human health. The efficacy of the BCA *Candida sake* CPA-1 has been demonstrated against the main postharvest diseases on pome fruit, and *B. cinerea* and sour rot in grapes control on field. Recently, two effective fluidised-bed spray-dried formulations based on *C. sake* CPA-1 and biodegradable compounds were described as an effective biocontrol product. However, the maintenance of the effectiveness and survival of these formulations during a long shelf life is a crucial aspect to the success of its marketing. For this reason, the aim of this study was to select the best packaging and storage conditions to maintain a long shelf life of two fluidised-bed spray-dried *C. sake* CPA-1 formulations. Viability and a_w (water activity) of CPA-1 formulations stored in different packages (bottles and bags), temperatures (− 20 °C and 4 °C) and atmospheric conditions (vacuum and air) were tested for 22 months. Both formulations maintained their initial viability (around 4×10^9 CFU ml^{-1}) when they were stored at − 20 °C regardless of the packages or atmospheric conditions. In contrast, the viability of CPA-1 formulations stored at 4 °C in bags than in bottles. Both formulations could be stored for at least 21 months at − 20 °C, and only one formulation could achieve a 1-year shelf life stored in bottles at 4 °C. *C. sake* CPA-1 formulations stored for 12 months at the most suitable conditions significantly reduced *B. cinerea* on grapes by 25 to 56%.

Keywords

Fluidized-bed spray-dried
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Formulation
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Electronic supplementary material

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Introduction

Biocontrol agents have achieved a relevant role within the strategies to control fungal diseases, in both pre- and postharvest as a suitable alternative for the development of low cost, sustainable and eco-friendly management approaches (Syed Ab Rahman et al. 2018). Currently, the big companies are developing production and formulation programs based on BCAs as an alternative to fungicides. However, a fundamental issue for the commercialization of BCAs is the maintenance of cell viability (Droby et al. 2016), which is recommended from 6 months (Spadaro et al. 2014) to 18 months (Keswani et al. 2016). The efficacy of the commercialised BCAs also tends to be a limitation, especially under field conditions (Ribes et al. 2018).

The antagonist efficacy of the yeast *Candida sake* CPA-1 has been demonstrated against the major postharvest diseases on pome fruit (Viñas et al. 1998). *C. sake* efficacy was also demonstrated in preharvest against *Botrytis cinerea* (Calvo-Garrido et al. 2017) and sour rot (Calvo-Garrido et al. 2013) in grapes.

Several formulations of *C. sake* CPA-1 have been developed since the yeast was isolated from apples, however some of them were not suitable due to their low efficacy compared with fresh cells (Abadias et al. 2001); short self-life (Torres et al. 2003); or low survival of the yeast after the drying process (Abadias et al. 2005). Notwithstanding, a liquid formulation in an isotonic solution maintained the efficacy of fresh cells after 7 months (Abadias et al. 2003b) and three solid formulations stored for 1 year (Carbó et al. 2017a) or 6 months at 4 °C (Carbó et al. 2017b) maintained the efficacy similar to that of fresh cells. Additionally, the previous cited studies demonstrated that *C. sake* CPA-1 lost its viability when it was stored at room temperature, either for liquid or solid formulations, so cold storage temperatures are required to maintain the viability of CPA-1. Despite it usually being accepted that dried microorganisms could be stored at ambient temperatures (Droby et al. 2016), also other dried yeasts such as *Metschnikowia pulcherrima* and *Pichia guilliermondii* (Kinay and Yildiz 2008) or two microencapsulated bifidobacteria (Hsiao et al. 2004) required cold temperatures to maintain their viability.

Previously, two fluidised-bed spray-dried formulations of *C. sake* CPA-1 optimised by Carbó et al. (2017a, b) were demonstrated to be interesting products for commercial use due to their efficacy against *B. cinerea* on grapes under field conditions (Carbó et al. 2019), and also due to the coating additives included in the formulated products to improve the yeast survival. Also, these formulations were tested under the effect of climate change (Carbó et al. 2018b) and their ecological niches and environmental resilience were predicted using the Bioscreen C (Carbó et al. 2018a). Therefore, several typical problems of BCAs, such as formulation, performance in practical conditions or the lack of curative effect (Usall et al. 2016) were overcome.

Nevertheless, commercialization and marketing purposes will require further steps to consider. In this way, the optimisation of the storage conditions and the selection of the best packaging to prolong the viability of the BCAs that play an important role to accelerate the commercialization process, prevent the variation in moisture content and the contamination of the product, and maintain the formulation as effective as fresh cells (Powell 1992). Previous studies pointed out that *C. sake* CPA-1 formulations should be stored inside high water vapour barrier material because the water activity (a_w) of the powder is critically conditioned with the cell viability (Marín et al. 2017).

The main objective of the present study was to optimise the packaging and the storage conditions for two fluidised-bed spray-dried formulations of *C. sake* CPA-1 to achieve a long shelf life. Specifically, different packages, atmospheric conditions and storage temperatures were studied. Furthermore, the efficacy of both CPA-1 formulations against *B. cinerea* on grapes was checked

after 12 months of storage in those packaging and storage conditions that CPA-1 maintained its viability.

Materials and methods

Biocontrol agent

Candida sake strain CPA-1 used in this study belongs to the Collection of Postharvest Pathology Group of IRTA (Lleida, Catalonia) and it was deposited in the Colección Española de de Cultivos Tipo (CECT-10817) at the University of Valencia, Burjassot, Spain. CPA-1 stock cultures were stored in Criobilles tubes (Criobilles AEB 400100, AES Laboratory, Comburg, France) at − 80 °C for long term storage. Then, yeast cells were sub-cultured on nutrient yeast dextrose agar plates (NYDA: nutrient broth, 8 g l^{-1}; yeast extract, 5 g l^{-1}; dextrose, 10 g l^{-1}; and agar, 15 g l^{-1}) and stored at 4 °C. When required, yeast cells were sub-cultured on NYDA plates at 25 °C for 48 h.

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Mass production and formulation

Candida sake was produced in two liquid fermentation systems with 5-l working volume each one (BIOSTAT-A modular fermenter, Braun Biotech International, Germany) with an initial concentration of 10^6 CFU ml^{-1} for 40 h as described by Abadias et al. (2003a, b). For formulation, CPA-1 cells from the 5-l bioreactors were centrifuged (Avanti™, Beckman, Fullerton, CA, USA) at 8631×*g* and 10 °C for 10 min and the resulting pellet was resuspended in potassium phosphate buffer (pH 6.5; KH_2PO_4 0.2 mol l^{-1}, 70 ml; K_2HPO_4 0.2 mol l^{-1}, 30 ml and deionized water, 300 ml) to achieve a concentration of 6 to 8 × 10^9 CFU ml^{-1}.

Two fluidised-bed spray-dried formulations of CPA-1 were used in the present study to test the effect of different packages and storage conditions on CPA-1 viability and efficacy: (i) potato starch formulation, and (ii) maltodextrin formulation. Both formulations were optimised previously by the addition of biodegradable coatings (Carbó et al. 2017b), and the dehydration process by fluidised-bed spray-drying system was done using the protocol described by Carbó et al. (2017b) with some modifications to obtain a higher amount of formulate. Briefly, for each formulated product, approximately 450 g of yeast suspension plus 8.2 g of binder and the corresponding protective compounds were homogenised and sprayed onto 700 g of suspended carrier.

Types of packaging

Four types of plastic packages were tested to achieve the longest shelf life for both solid formulations of *C. sake* CPA-1: (i) high density polyethylene bottles; (ii) multilayer bottles with ethylene–vinyl alcohol copolymer barrier; (iii) aluminium-based bags 12/12/100; and (iv) transparent plastic bags. For details about the composition and technical specifications of each package see Table 1.

Table 1

Properties of packaging materials used in the present study

Code	Packages	Properties	Provided by
HD	Monolayer high density polyethylene bottles	White plastic polyethylene; high density (> 0.940 g cm^{-3}) 250 ml	Alción Plásticos, SL Aldaia (Valencia, Spain)

Code	Packages	Properties	Provided by
EV	Multilayer bottles with ethylene–vinyl alcohol copolymer (EVOH)	White plastic; coextruded 250 ml	Alción Plásticos, SL Aldaia (Valencia, Spain)
AL	Aluminium based bags 12/12/100	12 µm aluminium polyester plus 12 µm polyethylene plus 100 µm amino/polyethylene co-extruded film Oxygen permeability: 0.76 cm^3 m^{-2} day^{-1}	Plastienvase, SA Córdoba (Spain)
TR	Transparent plastic bags	20 µm polyamide plus 50 µm polyethylene Oxygen permeability: 8 cm^3 m^{-2} day^{-1}	W.K. Thomas España, SL Rubí (Barcelona, Spain)

Storage conditions

Plastic bags were hermetically sealed (Engarvac Basis, Vacarises, Barcelona, Spain) at two different atmosphere conditions: air and 99.9% vacuum. Each tested bag contained 2 g of fluidised-bed spray-dried *C. sake* CPA-1, then bags were stored at 4 °C and − 20 °C. Two bags were stored for each tested condition and package. On the other hand, two bottles of each type were stored air packaged at 4 °C, and two at − 20 °C. Then, to simulate a commercial packaging, one bottle of each type were used to sample the first 8 months of storage and the others were kept closed and sampled from 8 months of storage to the end of the assay. Twenty grams of CPA-1 were contained in each bottle. This experiment was repeated twice.

Additionally, to confirm the possible effect of sampled bottle on the viability of *C. sake* CPA-1, another assay was carried out for 15 months with formulations stored at 4 °C in EV bottles. EV bottles were selected for this assay due to their excellent gas barrier properties, which probably influenced the water activity of the product. Duplicate samples were prepared and one bottle was sampled during the first 12 months and the other was opened only after 1 year to compare their shelf life.

Candida sake CPA-1 viability and water activity (a_w) during storage

Viability, moisture content and a_w of *C. sake* CPA-1 formulations in bags and bottles were tested after 1, 2, 4, 6, 8, 10, 12, 15, and 18 months. Additionally, bottles containing potato starch or maltodextrin formulation stored at − 20 °C were sampled after 21 and 22 months, respectively.

For each time all the content of the bags was used, whereas bottles were sampled and stored again. First, a_w was determined with an Aqualab (Decagon Devices Inc, Pullman, WA, USA) a_w-meter. Then, three replicate samples (0.5 g) were rehydrated with sterile water as described by Carbó et al. (2017a, b) and survival was checked by plating on NYDA and incubating at 25 °C for 48 h.

Efficacy of *Candida sake* CPA-1 formulations after 1 year of storage

The efficacy of stored formulations was evaluated against *B. cinerea* on grapes after 1 year for all those conditions which maintained a reasonable viability. *B. cinerea* strain BC03 from the IRTA Culture Collection was used in this study to test the efficacy of the BCA. It was originally isolated from infected grapes from a local vineyard that is located in Lleida (Catalonia, Spain) and it was deposited at the Colección Española de Cultivos Tipo (CECT-20973) at the University of Valencia,

Burjassot, Spain. The pathogen was grown on potato dextrose agar (PDA) for 15 days at 20 °C with a daily 14 h photoperiod of near-ultraviolet light and 10 h dark to induce sporulation. Efficacy of stored formulations was compared with: (i) a negative control (water); (ii) fresh cells of CPA-1; (iii) and both freshly made formulations without storage (potato starch and maltodextrin formulations). Fresh cells were obtained following growth in 100-ml conical flasks as described by Carbó et al. (2017a, b). On the other hand, another efficacy assay was carried out to compare the efficacy of maltodextrin formulation stored in closed and sampled EV bottles at 4 °C for 12 months.

Table grape berries were previously washed with tap water. Once grapes were dried, they were cut with the pedicel attached and injured with sandpaper. Then, berries were placed into a grid and treated with the *C. sake* CPA-1 at 5×10^7 CFU ml^{-1} using an air brush. After air drying, a suspension at 10^3 conidia ml^{-1} of *B. cinerea* was also sprayed for 3 s and left to dry again. Each treatment was replicated four times with five berries per replicate. Afterwards, berries were incubated under controlled conditions at 20 °C and 85% RH for 7 days or 9 days, depending on the assay. Incidence and severity were evaluated as the percentage of rotted berries and the percentage of berry surface covered with *B. cinerea* mycelium, respectively.

Statistical analysis

The efficacy of CPA-1 treatments was analysed using a generalised linear model (GLM). Incidence analysis was based on a binomial distribution and logit-link function, whereas severity analysis was based on a normal distribution and identity-link function. Means separation for GLM were performed by orthogonal contrasts and differences at $P < 0.05$ were considered as significant. Data were analysed using JMP8 software (SAS Institute INC., Cary, NC, USA).

Results

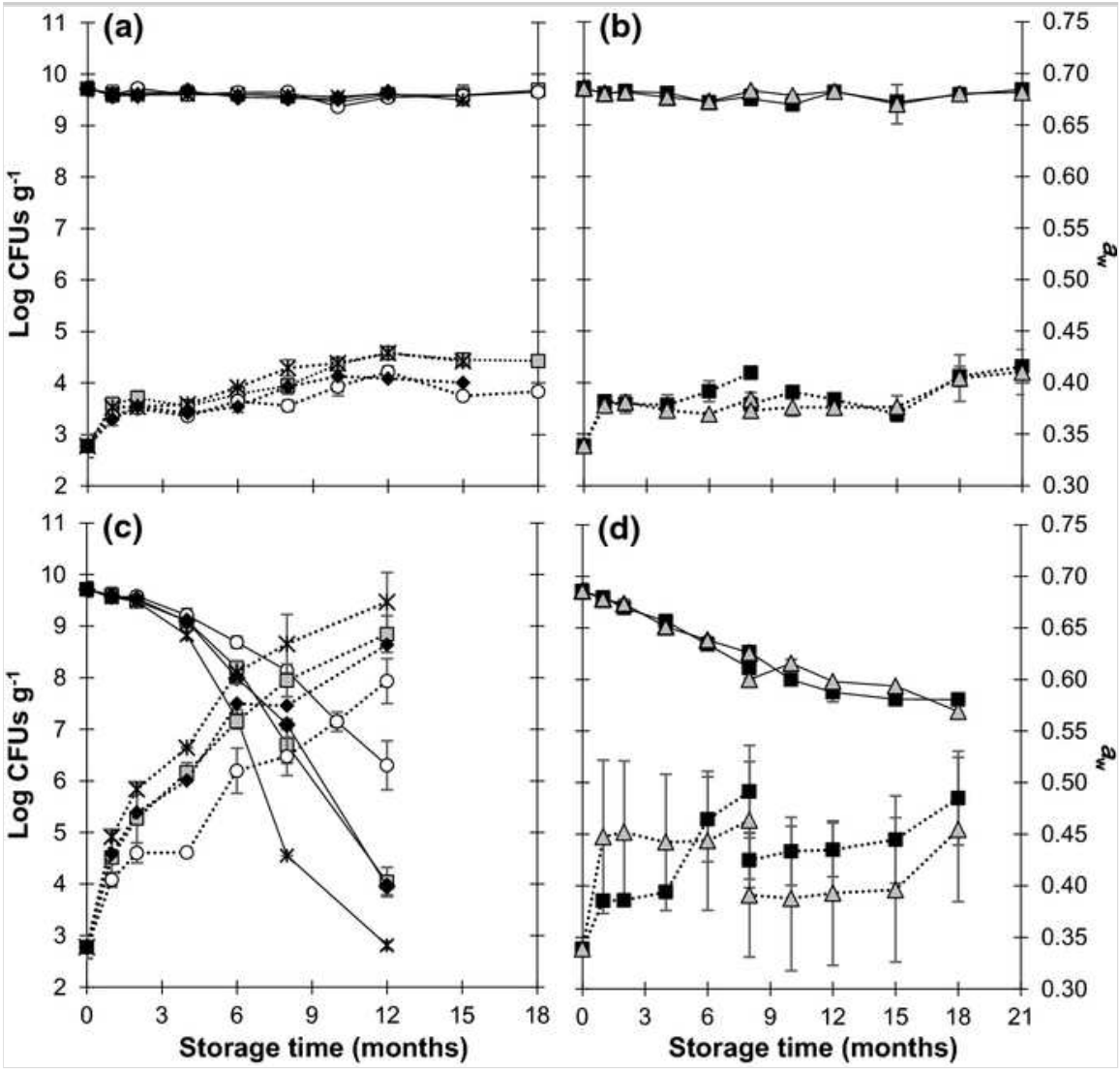
Viability and a_w of *Candida sake* CPA-1 formulations after storage

Potato starch formulation

Viability and a_w of CPA-1 formulated in potato starch under different packaging and storage conditions along the time are shown in Fig. 1. CPA-1 formulations stored at − 20 °C for 18 months in bags (Fig. 1a) or 21 months in bottles (Fig. 1b) maintained viability. However, at 4 °C, the viability of *C. sake* decreased progressively in bottles (Fig. 1d) or sharply in bags (Fig. 1c), showing reductions from 2.1 to 2.3 Log or from 3.4 to 6.9 Log respectively. Moreover, at 4 °C, the shelf life assay finished after 12 months of storage because several bags were rejected due to caking of the product or the loss of vacuum conditions.

Fig. 1

Viability (solid line) and water activity (dotted line) of fluidised-bed spray-dried *Candida sake* CPA-1 formulation in potato starch stored for 21 months in different packages and also at different temperatures: **a** bags at − 20 °C; **b** bottles at − 20 °C; **c** bags at 4 °C; and **d** bottles at 4 °C. Different composition of the packages and atmospheric conditions for bags are represented: AL bag air stored (grey square); AL bag vacuum stored (open circle); TR bag air stored (asterisk); TR bag vacuum stored (black diamond); HD bottles (black square); and EV bottles (grey triangle). Discontinuous lines were represented for the bottles a_w (**b**, **d**) to show the differences on the a_w caused due to the opening of the sample. Viability results are expressed as Log CFU g^{-1}. Error bars represent the SE. When bars are not visible, they are smaller than the symbol size



In bags stored at 4 °C (Fig. 1c), viability reduction of CPA-1 was affected by a_w increase. Packaging and storage conditions which maintained the viability of the yeast showed a minor variation on the a_w, being the most adequate value around 0.400 a_w. The a_w values of closed bottles for 8 months were lower than a_w values of the sampled bottles, mainly at 4 °C (Fig. 1d). Specifically, a_w values of closed bottles were around 0.410 after 8 months of storage, whereas sampled bottles reached a_w values of 0.480 in the same time.

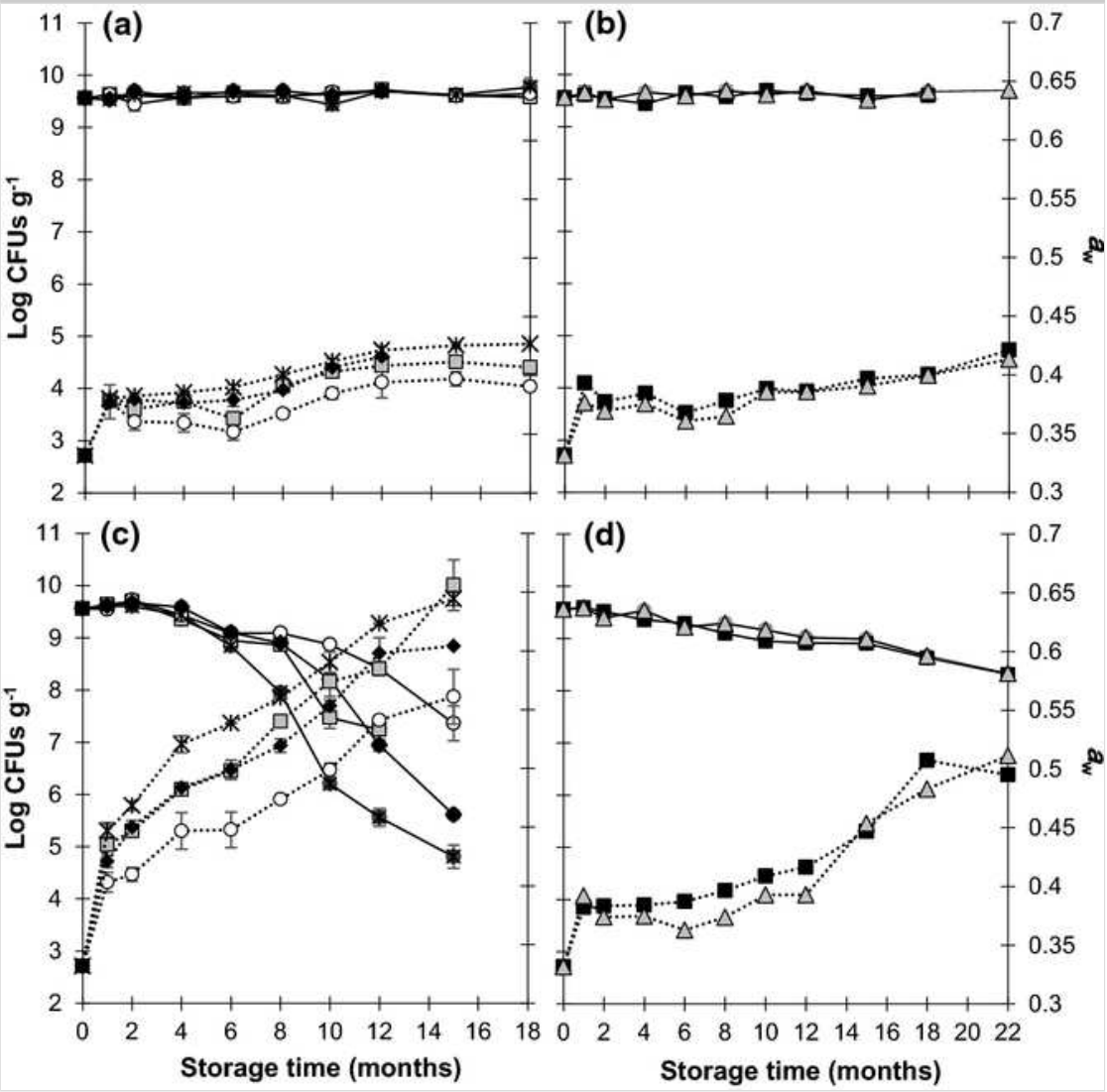
Regarding the package properties and atmosphere conditions, in general CPA-1 viability and a_w values followed the same trend. The highest differences were observed in the bags stored at 4 °C (Fig. 1c), where AL bags under vacuum conditions achieved the lowest viability reduction (3.4 Log) and the lowest a_w rise (0.258) after 12 months of storage.

Maltodextrin formulation

Viability and a_w of *C. sake* formulated in maltodextrin and stored under different packaging and storage conditions along the time are shown in Fig. 2. Similar to potato starch formulation, bags and bottles maintained CPA-1 viability at − 20 °C for 18 months (Fig. 2a) and 22 months (Fig. 2b), respectively. *C. sake* viability decreased by approximately 0.5 Log after 1 year of storage and by around 1.2 Log after 22 months. In contrast, viability was reduced from Log 2.2 to Log 4.8 when maltodextrin formulation was stored in bags at 4 °C (Fig. 2c). Moreover, the shelf life assay for bags at 4 °C finished after 15 months due to caking of the product or the loss of vacuum conditions.

Fig. 2

Viability (solid line) and water activity (dotted line) of fluidised-bed spray-dried *Candida sake* CPA-1 formulation in maltodextrin stored for 22 months in different packages and also at different temperatures: **a** bags at − 20 °C; **b** bottles at − 20 °C; **c** bags at 4 °C; and **d** bottles at 4 °C. Different composition of the packages and atmospheric conditions for bags are represented: AL bag air stored (grey square); AL bag vacuum stored (open circle); TR bag air stored (asterisk); TR bag vacuum stored (black diamond); HD bottles (black square); and EV bottles (grey triangle). Viability results are expressed as Log CFU g^{-1}. Error bars represent the SE. When bars are not visible, they are smaller than the symbol size



A negative relationship between CPA-1 viability and a_w values was also evident for maltodextrin formulation, especially with bags at 4 °C (Fig. 2c). However, no differences were observed between the closed bottles for 8 months and the sampled bottles. In general, maltodextrin formulation maintained its cell viability when a_w values were around 0.400.

CPA-1 viability and a_w followed the same tendency regardless of the package's properties or atmospheric conditions. Nevertheless, some variations could be appreciated after 15 months of storage in bags at 4 °C (Fig. 2c) despite of the loss of viability and the increase in the a_w values for all conditions. Specifically, CPA-1 viability at 4 °C after 15 months in AL bags under vacuum

conditions decreased 2.2 Log and their a_w was 0.561, whereas viability reduction in air packaged TR bags was 4.8 Log and its a_w raised to 0.644.

Efficacy of *Candida sake* CPA-1 formulations

Efficacy of *C. sake* CPA-1 formulations against *B. cinerea* on table grapes after 1 year of storage under different packaging and storage conditions are shown in Fig. 3. All the treatments reduced significantly the incidence and severity of *B. cinerea* compared with the control (CK) (Table 2).

Fig. 3

Effect of packaging and storage conditions on the efficacy of *Candida sake* CPA-1 formulations against *B. cinerea* on table grapes (cultivar Vitoria) after 12 months in EV or HD bottles, and in TR or AL bags under vacuum conditions (Vac.) or air packaged (Air). Efficacy of fresh cells of CPA-1 (CS), and freshly made formulations (NS) are also represented. A negative control was treated with water (CK). Incidence (grey square) and severity (black circle) were evaluated. Error bars represent the SE

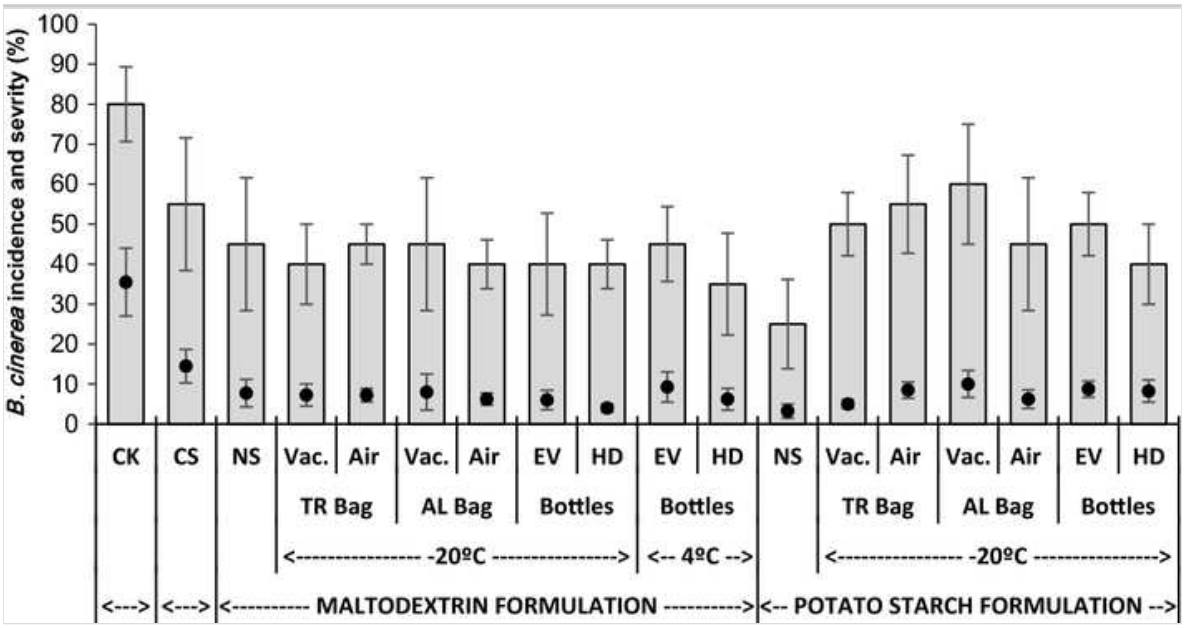


Table 2

Orthogonal contrasts testing the effects of packaging and storage conditions on the efficacy of *Candida sake* CPA-1 formulations reducing *Botrytis cinerea* incidence on grapes under controlled conditions

Effect test[a]	Efficacy statistics					
	Incidence			Severity		
	df	χ^2	P-value	df	χ^2	P-value
C. sake control	1	10.17	0.0014*	1	90.18	< 0.0001**
C. sake dehydration	1	0.99	0.3202	1	7.23	0.0072*

See coefficients of contrasted values in supplementary table S1

* Significant P < 0.05; ** significant P < 0.001

[a]*Candida sake* control: comparing with or without *C. sake* CPA-1; *C. sake* dehydration: comparing fresh cells with formulated cells; Formulation storage: comparing not stored with stored formulations; MAL: comparing not stored MAL with stored MAL; PS storage: comparing not stored PS with stored PS; Formulation: comparing MAL with PS; Storage temperature: comparing − 20 °C with 4 °C; Packaging: comparing bags with bottles; Air condition: comparing vacuum with air; Bottle properties: comparing EV with HD

Effect test[a]	Efficacy statistics					
	Incidence			Severity		
	df	χ^2	P-value	df	χ^2	P-value
MAL dehydration	1	1.30	0.2545	1	7.11	0.0077*
PS dehydration	1	0.55	0.4569	1	6.48	0.0109*
Formulation storage	1	1.57	0.2106	1	0.71	0.4001
MAL storage	1	0.10	0.7465	1	0.11	0.7347
PS storage	1	4.02	0.0451*	1	2.43	0.1187
Formulation	1	0.61	0.4328	1	0.03	0.8523
Storage temperature	1	0.47	0.4907	1	0.08	0.7726
Packaging	1	0.83	0.3616	1	0.02	0.8761
Atmosphere condition	1	0.10	0.7510	1	0.07	0.7927
Bag properties	1	< 0.0001	0.9974	1	0.11	0.7425
Bottle properties	1	0.55	0.4601	1	0.67	0.4138
See coefficients of contrasted values in supplementary table S1						
* Significant P < 0.05; ** significant P < 0.001						
[a]*Candida sake* control: comparing with or without *C. sake* CPA-1; *C. sake* dehydration: comparing fresh cells with formulated cells; Formulation storage: comparing not stored with stored formulations; MAL: comparing not stored MAL with stored MAL; PS storage: comparing not stored PS with stored PS; Formulation: comparing MAL with PS; Storage temperature: comparing − 20 °C with 4 °C; Packaging: comparing bags with bottles; Air condition: comparing vacuum with air; Bottle properties: comparing EV with HD						

Contrast analysis (Table 2) indicated that treatments with fluidised-bed spray-dried formulations had significantly less *B. cinerea* severity percentage than *C. sake* fresh cells. Moreover, although no differences were observed among formulations efficacy, maltodextrin formulation maintained its efficacy after 12 months of storage, whereas potato starch formulation reduced its efficacy controlling *B. cinerea* incidence after the same time of storage.

Although the highest reduction of *B. cinerea* (56%) was obtained with maltodextrin formulation stored in HD bottles at 4 °C, storage temperature (− 20 °C or 4 °C), packaging (bags or bottles), bottle properties (EV or HD), bag properties (AL or TR), or atmospheric conditions (vacuum or air) did not affect the efficacy of *C. sake* CPA-1 after 1 year of storage.

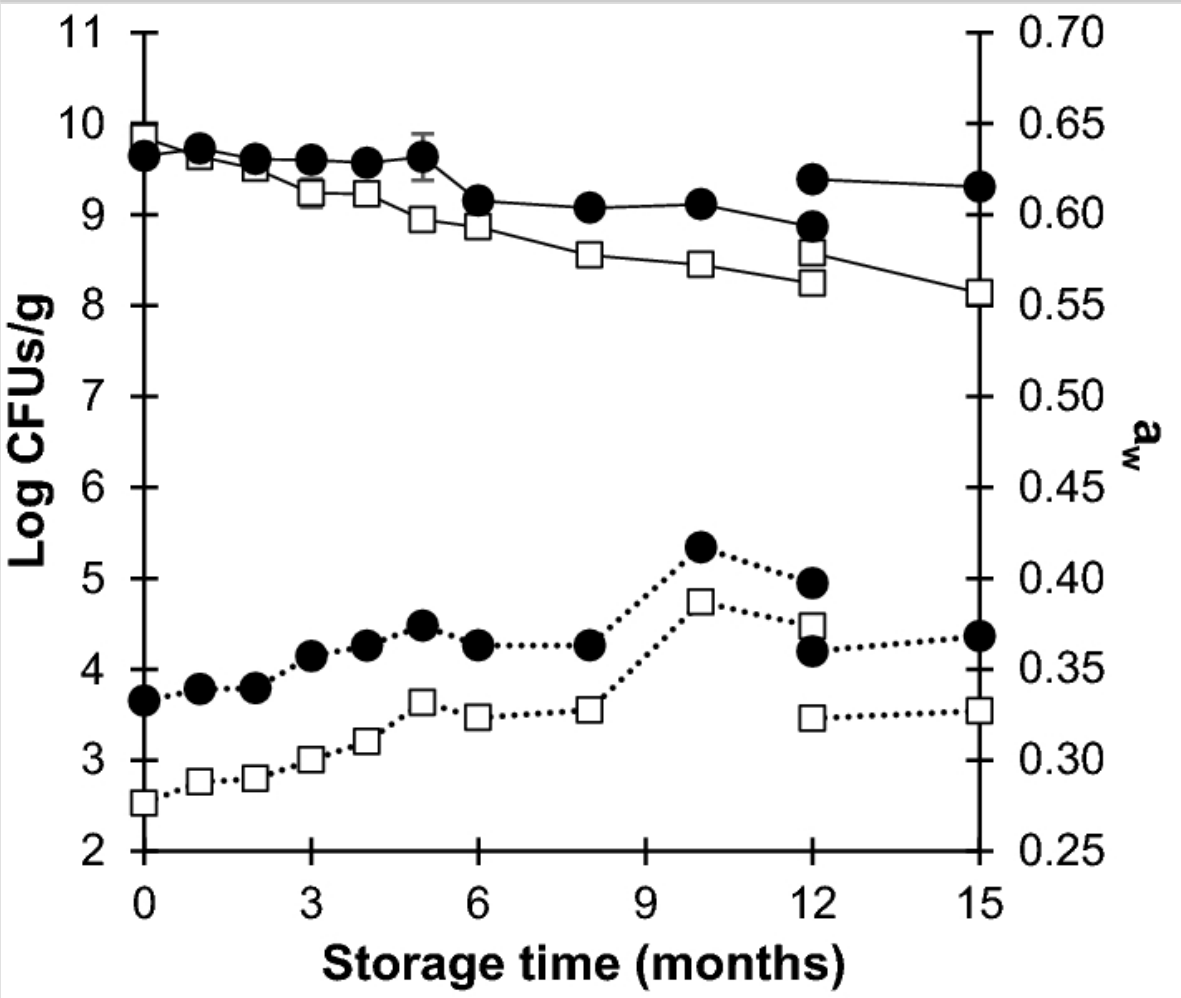
Preservation of *C. sake* CPA-1 formulations on closed bottles after 1 year

Effect of sampled bottle on the viability and a_w of *C. sake* CPA-1 formulations

The viability and a_w of both studied CPA-1 formulations stored in EV bottles at 4 °C are shown in Fig. 4. The viability of cells of both formulations decreased progressively during the first year in the sampled bottles at the same time that a_w was increasing. However, closed bottles for 1 year showed a better survival. In particular, viability reduction of CPA-1 on maltodextrin formulation after 1 year at 4 °C was 0.8 Log in the sampled bottle and only 0.3 Log in the closed bottle. The same occurred for potato starch formulation, whose viability decreased 1.6 Log in the sampled bottle and only 1.3 Log in the closed bottle.

Fig. 4

Viability (solid line) and water activity (dotted line) of fluidised-bed spray-dried *Candida sake* CPA-1 of Maltodextrin (black circle) and Potato starch (open square) stored in EV bottles at 4 °C for 15 months. One bottle was used for the first 12 months, and another one was opened after 12 months of storage to check any possible effect of sampled bottle. Viability results are expressed as Log CFU g^{-1} and represent the mean of two repetitions. Error bars represent the SE. When bars are not visible, they are smaller than the symbol size



After 1 year at 4 °C, a_w values of sampled bottles also differed from closed bottles. The initial a_w of maltodextrin formulation was 0.333, and this value increased to $a_w = 0.397$ in the sampled bottles, whereas it was $a_w = 0.360$ in the closed ones. For potato starch formulation, the initial a_w was 0.273 and after 1 year it augmented to $a_w = 0.374$ in the sampled bottles, but a_w was 0.323 in that bottle which was maintained closed.

The viability and a_w of closed bottles for 1 year were also evaluated after 15 months of storage. In comparison to 12 months, the viability of cells showed a decrease and a_w increased after 15 months for both formulations.

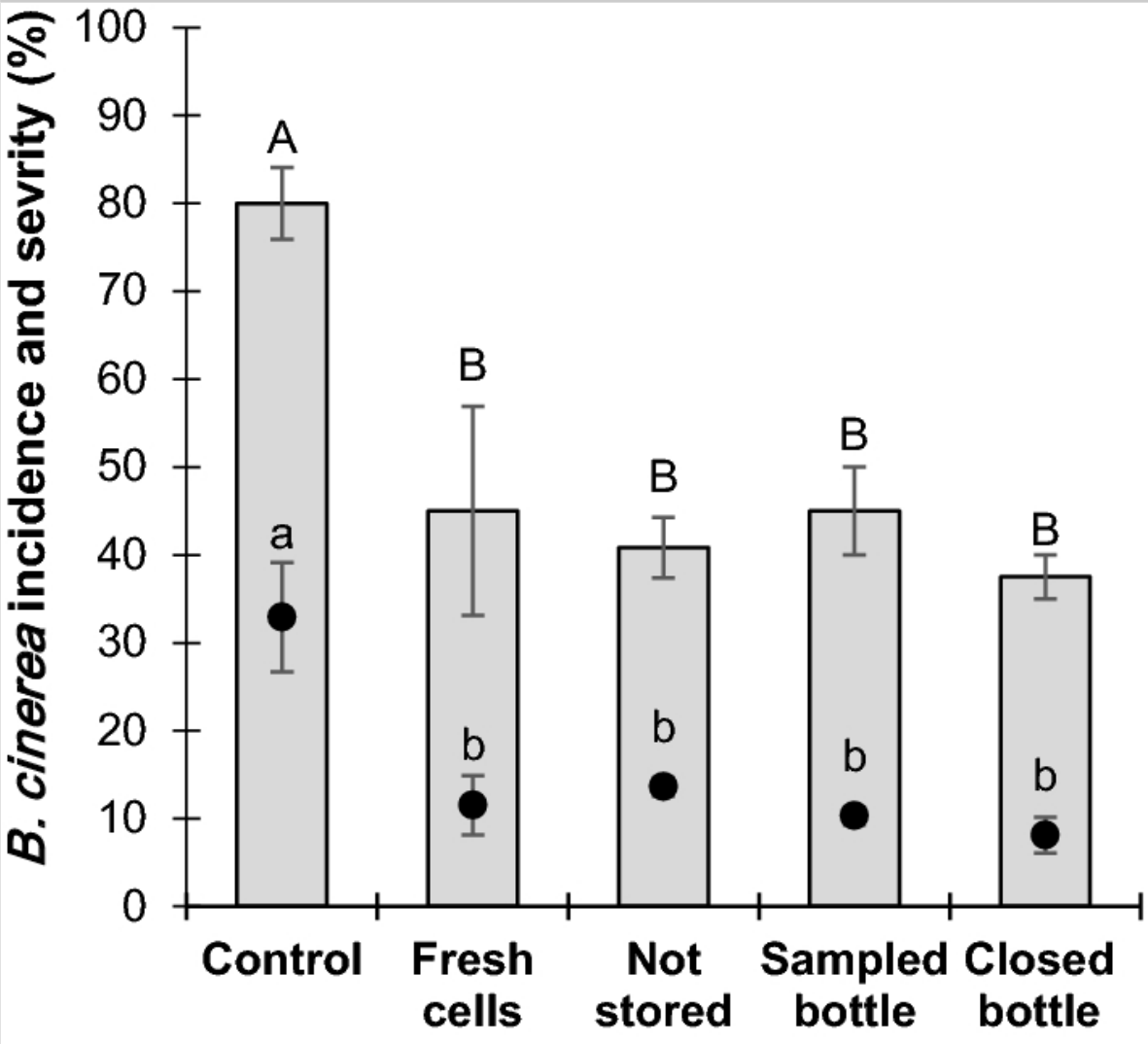
Effect of sampled bottle on the efficacy of *C. sake* CPA-1 formulations

Effect of sampled bottle on the efficacy of stored CPA-1 was only tested on maltodextrin formulation due to the loss of viable cells on potato starch formulation. All tested *C. sake* CPA-1 treatments reduced the incidence and severity percentages of *B. cinerea* on table grapes significantly (incidence: df = 1; $\chi^2 = 19.38$; $P < 0.0001$; severity: df = 1; $\chi^2 = 35.04$; $P < 0.0001$) (Fig. 5) and no significant differences were observed among treatments (incidence: df = 3; $\chi^2 =$

0.64; $P = 0.8863$; severity: df = 3; $\chi^2 = 2.16$; $P = 0.5399$). Neither the drying process (incidence: df = 1; $\chi^2 = 0.18$; $P = 0.6697$; severity: df = 1; $\chi^2 = 0.05$; $P = 0.8147$) nor the storage conditions (sampled or closed bottles) (incidence: df = 1; $\chi^2 = 0.46$; $P = 0.4954$; severity: df = 1; $\chi^2 = 0.24$; $P = 0.6227$) affected *C. sake* efficacy against *B. cinerea* on grapes.

Fig. 5

Effect of sampled bottle on the efficacy of *Candida sake* CPA-1 Maltodextrin formulation against *Botrytis cinerea* on table grapes (cultivar Sweet Globe). The formulation was stored in EV bottles at 4 °C for 12 months. Incidence (grey square) and severity (black circle) were evaluated. Different letters (uppercase letters for incidence and lowercase letters for severity) indicate significant differences ($P < 0.05$) according to orthogonal contrasts analysis and error bars represent the SE



Discussion

Overcoming the commercialization process could seem easy after several steps such as the isolation of the BCA, its mass production, or the optimization of handling and effective formulation. However, the commercialization is the most difficult stage in the development of a biocontrol product (Droby et al. 2016).

Two effective fluidised-bed spray-dried formulations of *C. sake* CPA-1 with biodegradable compounds on their composition were recently reported as a potential biocontrol product against *B. cinerea* on grapes (Carbó et al. 2017b). However, achieving success will depend on the formulations' shelf life, whose study will suppose the last step to prove their consistency and

performance. *C. sake* CPA-1 does not develop resilient spores and one of the most challenging barriers to overcome in these cases is the loss of cell viability during storage (Slininger et al. 2003). For this reason, the present study tested several packaging strategies for both fluidised-bed spray-dried CPA-1 formulations.

Our results have demonstrated that the shelf life of *C. sake* depends on the storage temperature. Both CPA-1 formulations achieved a long-term shelf life when they were stored at − 20 °C. At freezing conditions, potato starch and maltodextrin formulations could be stored in bottles for at least 21 and 22 months, respectively. Additionally, maltodextrin formulation reached a reasonable shelf life when it was stored in bottles at 4 °C, showing only a decrease of 0.5 Log after 1 year and 1.2 Log after 22 months. Other fluidised-bed spray-dried formulations of *Bacillus amyloliquefaciens* CPA-8 did not required freezing temperatures and maintained their viability after 15 months at 4 °C and also at 22 °C (Gotor-Vila et al. 2017) but other BCAs need cold temperatures to achieve the preservation of microorganisms (Kinay and Yildiz 2008; Sabuquillo et al. 2010; Torres et al. 2014). These low temperatures maintain the BCAs in a state of low metabolic activity and then the storage stability increases (Selmer-Olsen et al. 1999).

Compared with other shelf life of *C. sake* CPA-1 formulations, the package and storage conditions studied in the present study achieved the longest shelf life for a CPA-1 formulation so far. Specifically, a liquid formulation of CPA-1 achieved a shelf life of 12 months at 4 °C (Abadias, unpublished data) and fluidised-bed dried CPA-1 cells maintained their viability after 12 months (Carbó et al. 2017a). In contrast, the viability of freeze-dried CPA-1 cells decreased 1 Log (CFU ml^{-1}) after 2 months of storage (Abadias et al. 2001).

At 4 °C, the viability of *C. sake* CPA-1 was closely related to a_w of the formulation and the increase in a_w values came with a diminution in cell viability. The stability of CPA-1 formulations as the function of water activity was previously studied, and formulations lost cell viability when they were stored at a_w < > 0.43 (Marín et al. 2017). The viability of other dried BCAs such as *Cryptococcus flavescens* (Dunlap and Schisler 2010) or *Fusarium oxysporum* (Elzein et al. 2004) also decreased when the a_w of the formulations increased. In fact, an increase in the water availability for the yeast could revert the dormant state and then death occurs because the available water and nutrients are insufficient (Marín et al. 2017).

Moreover, in the present study, the increase in the a_w of the formulations involved some additional problems such as the caking of the product or the loss of vacuum conditions. These problems were only observed when the formulations were stored in bags at 4 °C, probably due to the permeability properties of the bags allowing the increase of the a_w. Comparing both formulations, the caking of the product and the loss of vacuum conditions were less remarkable for maltodextrin formulation, with initial and final a_w values slightly lower. In contrast, the caking of the product was observed in refrigerated bottles for the BCA *B. amyloliquefaciens* CPA-8 (Gotor-Vila et al. 2019).

Regarding package properties or atmospheric conditions, no differences were observed because both bottles, both bags and both atmospheric conditions followed the same tendency as the viability results. However, CPA-1 viability at 4 °C was always higher with AL bags than TR bags, apparently it was due to the bags permeability (0.76 $cm^3\ m^{-2}\ day^{-1}$ and 8 $cm^3\ m^{-2}\ day^{-1}$, respectively). Torres et al. (2014) highlighted the importance of using packages with low oxygen permeability and high moisture barrier for long-term storage of the BCAs and Marín et al. (2017) suggested high water vapour barrier packages for *C. sake* CPA-1 formulations storage because the a_w of the powder conditioned CPA-1 viability.

Efficacy of formulated *C. sake* CPA-1 after storage was not dependent on the packaging or storage conditions. Nevertheless, a higher amount of powder was required in those conditions that CPA-1 viability decreased, such as for maltodextrin formulation stored in bottles at 4 °C.

Interestingly, freshly made fluidised-bed spray-dried formulations and the most formulations stored under different package and storage conditions were more effective at reducing *B. cinerea* severity than CPA-1 fresh cells. Biocontrol improvement of the solid formulations was probably due to the addition of biodegradable compounds in their composition which developed a film on fruit surfaces, improved the survival of the BCA and, in the end, increased the biocontrol efficacy (Carbó et al. 2017b). The incorporation of coating compounds with the BCA treatments usually have a beneficial effect on BCA survival (Calvo-Garrido et al. 2014; Aloui et al. 2015) or efficacy (Parafati et al. 2016).

The effect on sampled bottles was relevant for potato starch formulation, mainly at 4 °C, whereas no effect was observed on maltodextrin formulation. Nevertheless, after 12 months of storage, sampling effect was perceptible in both formulations. In fact, sampled bottles came with a gradual increase in the a_w of the formulations due to the reasonable humidification of the bottle atmosphere. Fortunately, this effect could be disregarded on the commercialisation level because the package will only be opened to be used and did not influence the BCA efficacy.

The current study optimised the packaging and storage conditions to reach a shelf life of more than 20 months for two fluidised-bed spray-dried formulations of the biocontrol agent *C. sake* CPA-1, which should be stored in plastic bottles or bags at − 20 °C. Alternatively, to avoid freezing temperatures, maltodextrin formulation could be stored at 4 °C for 12 months. Therefore, maltodextrin formulation seems to be the most recommendable formulation to become an alternative to synthetic fungicides. Henceforth, this study points out different options of packaging and storage conditions to commercialise biocontrol-based products of *C. sake* CPA-1.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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