

Full Length Research Paper

Growth inhibitions of three fungal isolates from Zobo drink using sorbic and benzoic acids

Obioma E. Nwafor

Department of Microbiology, Delta State University, Abraka, Nigeria.
Email: obiomanwafor@yahoo.com; Tel: 08035878792.

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The growth inhibitions of *Penicillium citrinum*, *Rhizopus stolonifer* and *Aspergillus niger* isolated from locally prepared zobo drinks using sorbic and benzoic acids at room temperature were investigated for a period of eight weeks (56 days). A concentration of 0.05% (w/v) sorbic acid showed complete growth inhibition of *P. citrinum* and *R. stolonifer* on the 35th day and *A. niger* on the 49th day. The same concentration of benzoic acid inhibited the growth of *P. citrinum* and *R. stolonifer* on the 42nd day and *A. niger* on the 56th day. All combinations of the two preservatives demonstrated enhanced growth inhibitions to the three tested fungi. A combination of 0.05% (w/v) of sorbic and benzoic acids completely inhibited the growth of *P. citrinum* and *R. stolonifer* on the 21st day of storage. Similar inhibitory effect was observed for *A. niger* on the 25th day. The finding is an indication that sorbic acid and benzoic acid can be used to control the growth of these zobo drink spoilage microorganisms during storage.

Keywords: Growth inhibition, Sorbic acid, Benzoic acid, Storage.

INTRODUCTION

Zobo drink is a refreshing beverage obtained from dried reddish-purple petals of *Hibiscus sabdariffa*, a member of the family Malvaceae (Fasoyiro *et al*;2005). The drink is becoming very popular with acceptability cutting across many ethnic groups and socio-economic classes in Nigeria and other African countries. It is now served at various functions as non alcoholic drink. Zobo drink production is not currently industrialized due to its short shelf-life of about 1-4 days (11) The short shelf-life is associated with biodegradation activities of moulds and yeasts as with other beverages(Nwafor and Ikenebomeh ,2009).Moulds such as *Rhizopus stolonifer*, *Penicillium citrinum* and *Aspergillus niger* and their harmful roles in zobo drink stability during storage have been documented (Nwafor and Ikenebomeh,2009).

The use of antimicrobial agents such as sorbic and benzoic acids for the control of moulds associated with zobo drink in storage has not been given the deserved attention. Their use at low allowable concentrations either individually or in combinations will remove the risk associated with them (Leistnr, 1994). Furthermore, the role of *A. niger* in deleterious changes in phyto-chemical quality of zobo drink in storage has been reported (Nwafor and Ikenebomeh 2009).

Considering the significant position being assumed by zobo drink among Nigerians of all ages and other African countries, the need to control spoilage due to the spoilage organisms becomes very imperative.

This study is therefore designed to investigate the growth responses of *P.citrinum*, *R.stolonifer*, and *A. niger* to different concentrations of sorbic and benzoic acids in zobo drink during storage at room temperature with an aim of achieving a longer shelf life for the product.

MATERIALS AND METHODS

Preparation of zobo drink

Zobo drink was prepared following the recipe in magi family menu cook book. The drink was then divided into three batches with each batch having three subgroups and treated with different concentrations, 0.025, 0.05, and 0.075% (w/v) of sorbic acid and benzoic acid (BDH, England) per batch. Different concentrations each of the preservatives were also applied in combinations at 0.025 and 0.05% (w/v) concentration levels. They were then packaged in sterile glass bottles for further use.

Table 1. Effect of combinations of benzoic acid (BA) and sorbic acid (SA) on the growth of *Penicillium citrinum* (log₁₀cfu/ml) in zobo drink during storage at 29± 2° C.

Period of Storage (days)	Concentrations of BA and SA (% w/v)			
	Control	0.05+0.025	0.075+0.025	0.05+0.05
0	2.26±0.2	2.26±0.2	2.26±0.2	2.26±0.2
7	3.21±0.3	1.98±0.2	2.16±0.01	2.10±0.2
14	3.45±0.02	1.47±0.02	1.93±0.2	1.40±0.1
21	4.17±0.1	1.10±0.3	1.02±0.3	ND
28	5.49±0.3	ND	ND	ND
35	6.80±0.3	ND	ND	ND
42	7.36±0.2	ND	ND	ND
49	6.65±0.03	ND	ND	ND
56	5.33±0.3	ND	ND	ND

Note: Each value is the mean± standard deviation for triplicate determinations

BA = benzoic acid

SB = sodium benzoate

ND = not detected

Control = no preservative

Table 2. Effect of combinations of benzoic acid (BA) and sorbic acid (SA) on the growth of *Rhizopus stolonifer* (log₁₀cfu/ml) in zobo drink during storage at 29±2°C.

Period of storage (days)	Concentrations of BA and SA (% w/v)			
	Control	0.05+0.025	0.075+0.025	0.05+0.05
0	2.17±0.3	2.17±0.3	2.17±0.3	2.17±0.3
7	2.93±0.3	1.88±0.3	1.96±0.2	2.10±0.2
14	4.40±0.03	1.69±0.02	1.50±0.2	1.93±0.2
21	4.96±0.01	1.03±0.01	1.02±0.01	ND
28	5.54±0.3	ND	ND	ND
35	6.61±0.2	ND	ND	ND
42	7.70±0.2	ND	ND	ND
49	5.40±0.3	ND	ND	ND
56	4.95±0.03	ND	ND	ND

Note: Each value is the mean ± standard deviation for triplicate determinations

BA = benzoic acid, SA = sorbic acid,

ND = not detected

Control= no preservative

Inoculation of zobo samples with test organisms (challenge test)

Stock cultures of *Penicillium citrinum*, *Rhizopus stolonifer* and *Aspergillus niger* isolated from deteriorating zobo drink during storage and identified using morphological and cultural characteristics (Bounds *et al.*) were constituted according to the method of (Harrigan and McCance, 1976) The inoculum size was determined by pour plate method on yeast-malt agar (oxid) supplemented with chloranphenicol. The zobo drink samples (20mls in 100mls conical flasks.) treated as indicated above were inoculated with 1ml of 4hs old broth cultures of *P. citrinum*, *R. stolonifer* and *A. niger* for each batch, aseptically sealed with hand sealing machine (Super master, Japan) and kept at 29±2°C for a period of 8 weeks. Microbial growth was monitored at weekly (7 days) intervals.

Microbiological Analysis

Viable counts in various zobo samples were determined as described by Vanderzant and Splittsoesser, (1992). A 1ml portion of the sample was aseptically added to 9ml of 0.10%(w/v) sterile peptone water and allowed to stand for 3 minutes with occasional stirring using clean sterile glass rod. This was serially diluted (1:10) and viable counts enumerated and expressed as colony forming unit per millilitre (cfu/ml).

STATISTICAL ANALYSIS

The data obtained were subjected to statistical analysis of mean, standard deviation and analysis of variance (ANOVA). The significant value was determined by t-distribution test using appropriate computer software

Table 3. Effect of combinations of sorbic acid (SA) and benzoic acid (BA) on the growth of *Aspergillus niger* (\log_{10} cfu/ml) in zobo drink during storage at $29\pm 2^{\circ}$ C.

Period of Storage(days)	Concentrations of BA and SB (% w/v)			
	Control	BA+SA 0.05+0.025	BA+SA 0.075+0.025	BA+SA 0.05+0.05
0	2.11 \pm 0.2	2.11 \pm 0.2	2.11 \pm 0.2	2.11 \pm 0.2
7	3.40 \pm 0.3	2.10 \pm 0.03	2.02 \pm 0.2	1.63 \pm 0.3
14	3.49 \pm 0.3	1.98 \pm 0.01	1.99 \pm 0.3	1.02 \pm 0.02
21	4.17 \pm 0.2	1.04 \pm 0.1	1.02 \pm 0.2	ND
28	5.49 \pm 0.1	1.00 \pm 0.2	1.01 \pm 0.01	ND
35	6.80 \pm 0.3	ND	ND	ND
42	7.36 \pm 0.3	ND	ND	ND
49	6.65 \pm 0.1	ND	ND	ND
56	5.33 \pm 0.2	ND	ND	ND

Note: Each value is the mean \pm standard deviation for triplicate determinations

BA = benzoic acid

SA = sorbic acid

ND = no detected

Control = no preservative

(Ogbeibu,2005).

RESULTS

The growth inhibitions of *P. citrinum*, *R. stolonifer* and *A. niger* to using different concentrations of sorbic acid and benzoic acid in zobo drink during storage at $29\pm 2^{\circ}$ C are shown figures 1-6. Different concentrations of the preservatives inhibited the growth of the tested microorganisms to varying degrees. For instance 0.05% (w/v) showed complete growth inhibition of *P. citrinum*, *R. stolonifer* on the 35th day (5th week) of storage(fig 1 and 2) but showed the same effect on *A. niger* on the 49th day (7week, figure 3). On the other hand 0.05% (w/v) of benzoic acid showed complete inhibition of growth of *P. citrinum* and *R. stolonifer* on the 42 day (6th week) of storage (fig 4 and 5) while complete growth inhibition of *A. niger* was observed on the 56th day (8th week) of storage (figure 6).

The least concentration (0.025%) of the tested preservatives showed similar trend in growth inhibition of *P.citrinum* and *R. stolonifer* which was different for *A. niger* where complete inhibition of growth did not occur until the last day of storage. The highest concentration (0.075%) showed complete growth inhibition of the tested microorganisms on the 35th day (5th week).

The growth inhibitions of the tested microorganisms using combinations of the preservatives are shown on tables 1-3. All the combinations demonstrated enhanced growth inhibitions of the three tested fungi.Complete growth inhibition was observed on the 21th day (3rd week) of storage when a combination 0.05%(w/v) both of sorbic and benzoic was applied on zobo drink samples

inoculated with *P. citrinum* and *R. stolonifer* (tables 1 and 2). With *A. niger* the same combination showed complete inhibition of growth on the 28th day (4th week) of storage (table 3). On the other hand, there was steady increase in microbial count from $2.26\pm 0.2\log_{10}$ cfu/ml on the first day to $5.33\pm 0.3\log_{10}$ cfu/ml on the 56th day (8th week) of storage for *P. citrinum*. The trends were the same for the *R. stolonifer* and *A. niger*.

DISCUSSION

One of the intended goals in the use of preservatives in food is the inhibition of growth and elimination of the undesirable activities of microorganisms thereby extending the shelf life under defined conditions. This study has revealed the capacity of different concentrations of sorbic and benzoic acids to inhibit the growth of *P. citrinum*, *R. stolonifer* and *A. niger* which are three major spoilage microorganisms of zobo drink during storage at $29\pm 2^{\circ}$ C. The decrease in microbial counts and subsequent cessation of growth observed suggest that the preservatives have created unfavourable microenvironment for the test organisms. They may have created hurdles which the organisms were not able to overcome. Similar reports have been documented (Okoro, 2003). The presence of the preservatives may have caused extensive distortion of the homeostasis of the organisms leading to death as a result of their inability to overcome the resultant shock. This is in consonance with earlier reports by(Effiuwewewere and Efi 1999)).

The stepwise and gradual decrease in microbial counts observed in zobo samples treated with 0.025%(w/v) sorbic acid (fig 2 and 3) and benzoic acid (fig 5 and 6)

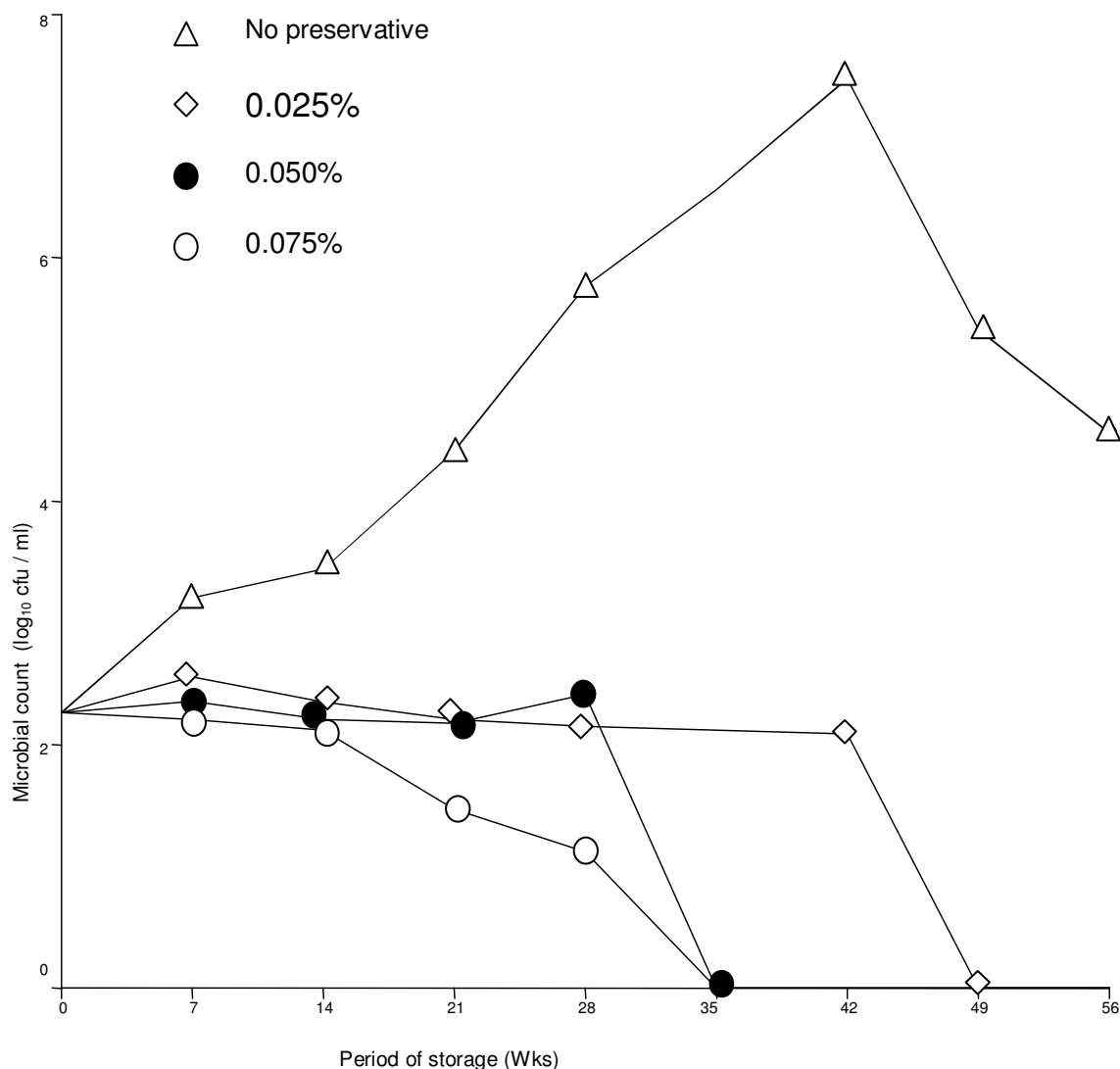


Figure 1. Effect of sorbic acid on the growth of *Penicillium citrinum* (log₁₀cfu/ml) in zobo drink during storage at 29±2°C

throughout the period of storage is an indication of the difficulty encountered by the vegetative cells of the surviving organisms. This may be due to an unfavourable microenvironment created by the preservatives at that concentration. It implies that the organisms may have survived to some extent, the effect of the preservatives but further attempt to overcome the hurdles may cause them to become metabolically exhausted, hence the continuous decrease in population observed. The corroborates some earlier reports by Leistner (1994), Nwafor and Ikenebomeh (2009). The differences in degree of inhibition at higher concentrations as compared with lower ones in all the cases suggests that the effect is concentration dependent. This is in line with the report of (Pokorn, 1994).

Enhanced antimicrobial effect observed when the preservatives were used in combinations (Table 1-3) may be due to increased effect of the preservative hurdles, which makes it difficult for the tested microorganisms to "jump over" easily. Synergistic effect of combinations of antimicrobial agents have been reported (Leistner, 2000). Combinations of preservative hurdles create additional homeostatic imbalance for microorganisms and repairs of distorted homeostasis demand more energy (Leistner, 1994). It is also possible that the hurdles are acting at different targets (multi-target effect) in the microbial cells. These targets may be cell membrane, enzyme system, protein synthesis or DNA, thus distorting homeostasis in different respects which can make repair process difficult resulting in the death of the

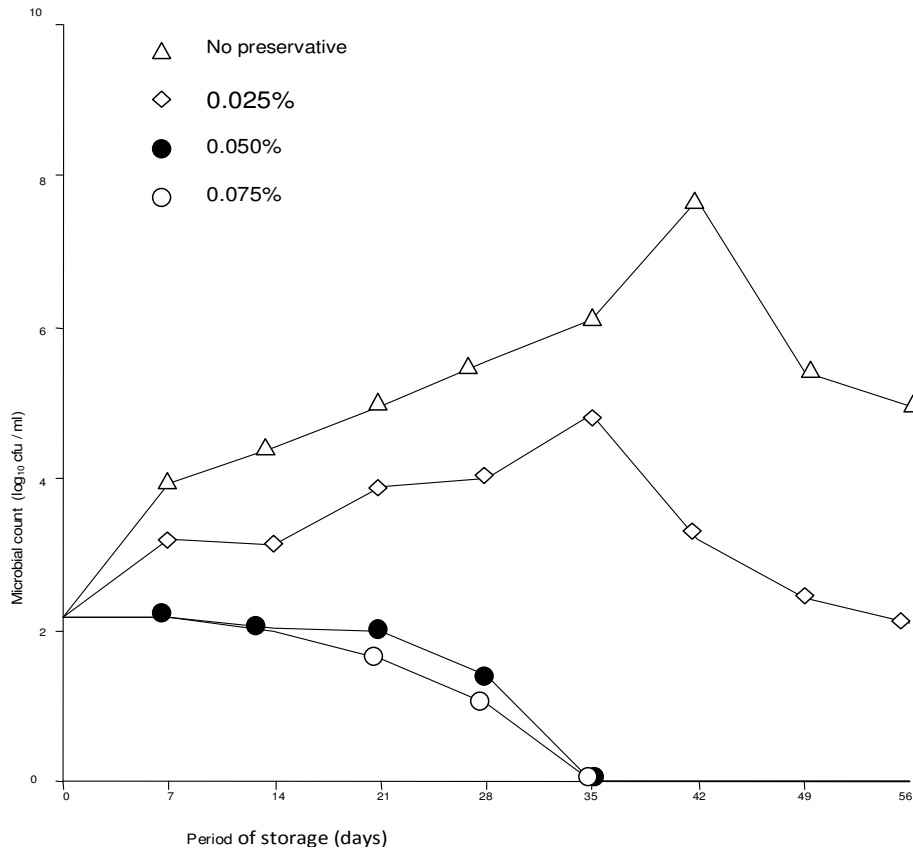


Fig 2: Effect of sorbic acid on the growth of *Rhizopus stolonifer* (log₁₀cfu/ml) in zobo drink during storage at 29 ± 2°C

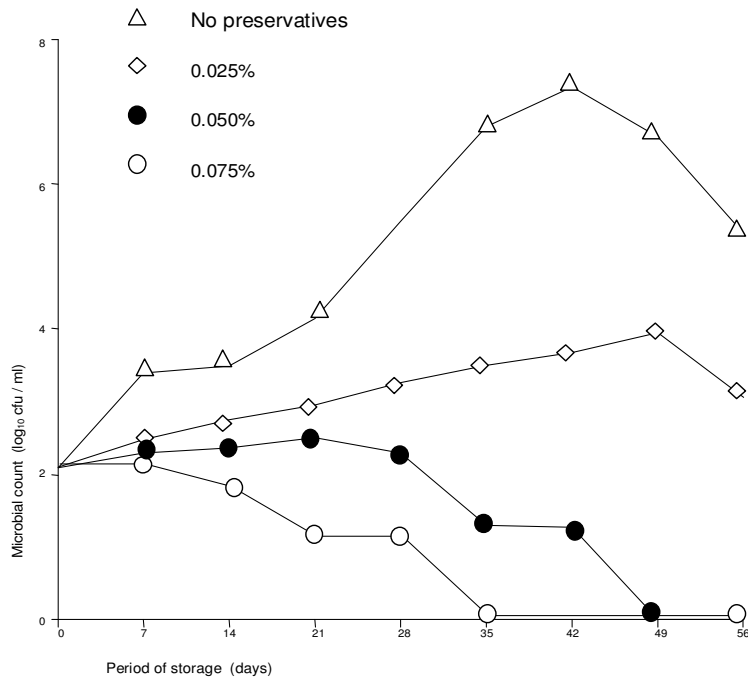


Figure 3. Effect of sorbic acid on the growth of *Aspergillus niger* (log₁₀cfu/ml) in zobo drink during storage at 29±2°C

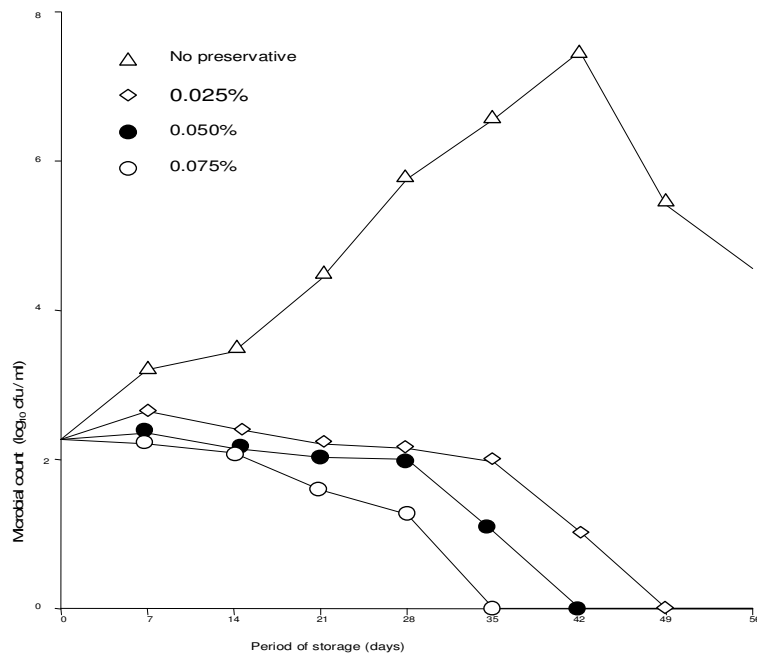


Figure 4. Effects of benzoic acid on the growth of *Penicillium citrinum* (log₁₀ cfu/ml) in zobo drink during storage at 29±2°C

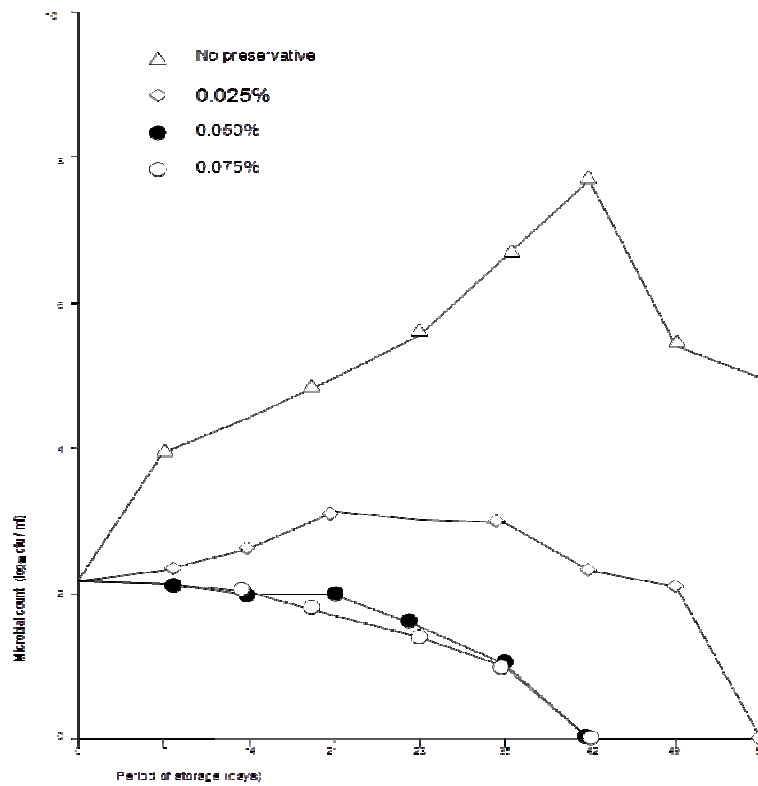


Figure 5. Effects of benzoic acid on the growth of *Rhizopus stolonifer* (log₁₀ cfu/ml) in zobo drink during storage at 29±2°C

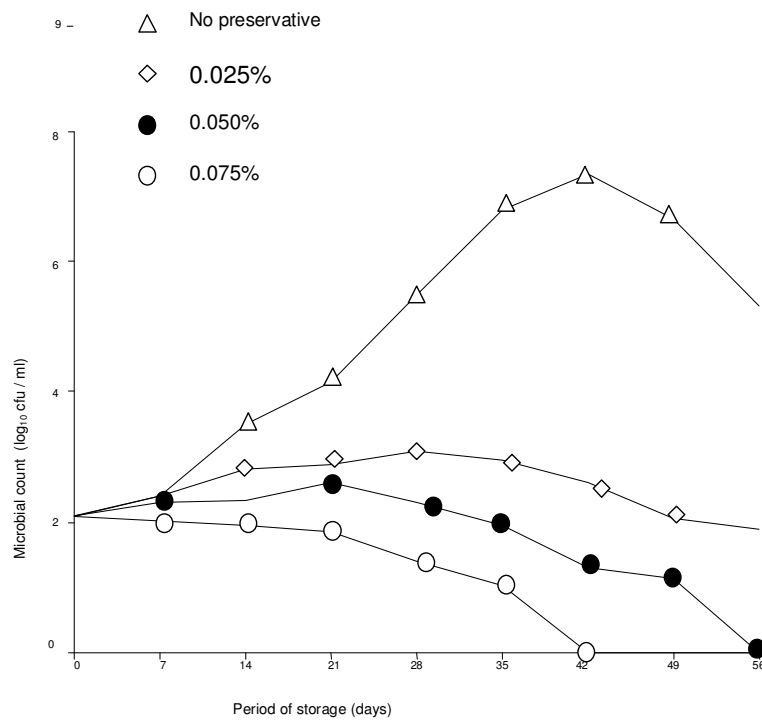


Figure 6. Effects of benzoic acid on the growth of *Aspergillus niger* (\log_{10} cfu/ml) in zobo drink during storage at $29\pm 2^\circ\text{C}$

microorganisms (Leistner, 1994)

The steady increase in population observed in the control experiments may be attributed to presence of the organisms in suitable microenvironment which makes it easy for the organisms to grow and proliferate. On the other hand the decrease in counts observed on the 49th day (7th week) of storage may be due to depletion in nutrient contents of the medium, competition for limited space or release of deleterious metabolites as have been previously reported (Ogiehor and Nwafor 2004; Ogiehor and Nwafor 2008).

In summary, this study has shown the ability of sorbic acid and benzoic acid to control the growth and proliferation of *P. citrinum*, *R. stolonifer* and *A. niger* which are common spoilage microorganism of zobo drink in storage. Findings may be useful in the establishment of indices for predicting zobo drink safety during processing, handling and storage and hence will enhance its local and international commercial potentials.

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