

Removal of aflatoxin B1 from experimentally contaminated whole milk using a pool of probiotic strains of lactic acid bacteria and baker's yeast *Saccharomyces cerevisiae*

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Abstract: The contamination of food and animal feed with AFB₁ is a worldwide problem. Aflatoxin B1 (AFB₁), the most toxic AF the aim of the present study was to evaluate the ability of both commercial product Ecolife aqua® (a pool of commercially available LAB strains, with actinomycete and some enzymes) and Baker' yeast *Saccharomyces cerevisiae* alone or in combination to remove AFB₁ from whole milk, as experimental media. several parameters were tested for removal of AFB₁ from whole milk, (time: 0, 12, 24, 36 and 72 hr – temperature: 5°C, 37°C, 50°C, 60°C and 100 °C. pH: 2.5, 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 - concentrations: Ecolife 0.5, 3.0, 5.0 ml/L and *S. cerevisiae* 1x10⁹ cells/ml, 5x 10⁹ cells/ ml and 7x 10⁹ cells/ ml⁻¹ and combination of *S. cerevisiae* 5x 10⁹ cells/ ml & Ecolife 3.0 ml/L and AFB₁. Quantification of AFB₁ in milk was performed using HPLC. Present results showed that both commercial products can remove AFB₁ from milk solution but combination between two products can remove efficiently AFB₁, followed by baker's yeast followed by Ecolife aqua® depending on time, temperature, pH and concentrations of used products.

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Key words: AFB₁; HPLC; Ecolife aqua®; Baker' yeast; *Saccharomyces cerevisiae*; whole milk.

1. Introduction:

Aflatoxins are a group of a growing list of fungal secondary metabolites which are recognized as being of economic and health importance. They are produced by two toxic strains of *Aspergillus*, namely *A. flavus* and *A. parasiticus*. Aflatoxin B1 is a well-known carcinogen and is classified by the International Agency for Research in Cancer as a class 1 human carcinogen. Therefore, reducing its bioavailability is of great interest for human health. They are potent hepatocarcinogens in several species of animals and human (Eaton and Callagher, 1994; El-Nezami *et al.*, 1998),

The contamination of food and animal feed with AFB₁ is a worldwide problem. Aflatoxin B1 (AFB₁), the most toxic AF, is of particular interest because it is a frequent contaminant of many food products and one of the most potent naturally occurring mutagens and carcinogens known to man (Teniola *et al.*, 2005). AFB₁ is currently of great interest because of their toxic, carcinogenic and mutagenic potential on human and animal health. For this reason, there is a great demand for novel strategies to reduce or inactivate (Serrano-Niño *et al.*, 2013)

It has been reported that many micro-organisms, including bacteria, yeasts, moulds, actinomycetes and algae are able to remove or degrade small amounts of aflatoxin in foods and feeds but biological detoxification of aflatoxin has not been established in practice. A number of studies have screened these microorganisms for the ability to bind to Aflatoxine and have reported a wide range of genus, species and strain specific binding capacities (El-Nezami *et al.*, 1998; Lee *et al.*, 2003;

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Hwang *et al.*, 2005; Zinedine *et al.*, 2005; Shahin, 2007).

Saccharomyces cerevisiae is effective for binding AFB₁ (Shetty & Jespersen, 2006; Corassin *et al.*, 2013). Several lactic acid bacteria (LAB) strains have shown different capabilities for binding AFM₁ in solutions and in milk (Bovo, *et al.*, 2012; El-Nezami, *et al.*, 1998; Haskard, *et al.*, 2001; Kabak & Var, 2008; Pierides, *et al.*, 2000). There is no previous report on the use of Baker's yeast *S. cerevisiae* strain for decontamination of milk containing AFB₁.

Therefore, the aim of the present study was to evaluate the ability of both commercial product Ecolife aqua® (a pool of commercially available LAB strains, with actinomycete and some enzymes) and Baker' yeast *Saccharomyces cerevisiae* alone or in combination to remove AFB₁ from whole milk, as experimental media for removal AFB₁

2. Material and methods:

2.1. Ecolife aqua (a pool of lactic acid bacteria & enzymes):

EcoLife® is commercial product used in aquaculture as probiotic, it is brown solution, FDA approved dietary product, the safest for human, animals, fish and plants, made from fermented green tea leaves by extracting 14 different kinds of beneficial vegetable lactic acid bacteria, actinomycete and some enzymes. EcoLife is much more effective complex lactic acid bacteria, in which includes *Lactobacillus* and other 13 different kinds of lactic acid bacteria plus actinomycete (beneficial white fungi) and some enzymes, unlike other

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probiotic products which are mostly composed of single Lactobacillus and enzymes. EcoLife® used in present study kindly supplied from KorSun International, Inc. Abdulrahman Alhakbani Trading.

2.2. Baker's yeast preparation for experiment:

Commercial Baker's yeast *S. cerevisiae* was obtained from Baker shop and preserved in refrigerator (4 °C) till used in the experiment. The lyophilized yeasts were reactivated in sterile water and activated at 23 °C. The number of yeast cells in the suspension was determined by light microscopy using Neubauer chamber. The suspension was diluted with sterile water until reaching a cell concentration of 1.0×10^9 cells/mL. (Corassin *et al.*, 2013).

2.3. Preparation of collected whole milk:

Fluid milk samples were centrifuged at 3500 g and 20 °C for 10 min. After discarding the upper cream layer, the lower phases were used for quantitative test of aflatoxine B1 samples were prepared according to El Khoury *et al.*, (2011).

2.4. Preparation of AFB1 standard solution:

Aflatoxin B1 Powder (Sigma Chemical Co., St Louis, MO, USA) was dissolved in a mixture of HPLC grade benzene/acetonitrile (97:3 v/v) to a concentration of 10 mg/mL. Subsequently, the standard solution was prepared by diluting the mixture in PBS (0.5 M, pH 7.2). The benzene/acetonitrile was evaporated by heating 80 °C, 10 min. in a water bath (Haskard, *et al.*, 2001). The final concentration of the standard solution (5 mg/mL) was calculated using the Lambert Beer equation ($A = \epsilon c l$) using the absorbance (A) at 354 nm, a molar absorptivity $\epsilon_{354} = 19.950 \text{ M}^{-1} \text{ cm}^{-1}$ and the path length traversed by light in the medium (l) (Zinedine, Faid, & Belemlih, 2005). An aliquot (1 mL) of this standard solution was diluted in PBS (pH 7.2)

to reach a concentration of 10 ng/mL and to perform the AFB1 removal assay.

2.5. Preparation of artificially AFB1 contaminated milk:

Methanol (50 ml) was added prior to making up to volume with aqueous solution. The ionic strength of AFB1 solutions (pH) was adjusted by adding NaCl. The pH of AFB1 solutions was adjusted using HCl or NaOH. All other AFB1 solutions were prepared in phosphate buffered saline (PBS) Haskard *et al.* (2000).

One mL of AFB1 standard (5 mg/mL) prepared as previously described, was re-suspended in 1 mL of methanol to a concentration of 2.5 mg of AFB1/mL, then 0.2 mL were taken from this solution and diluted in 50 mL of investigated experimental liquid milk to a concentration of 10 ng/mL of AFB1.

2.6. AFB1 removal assay

Cells of the prepared strains named with dose in Table 1 were centrifuged at 4000 rpm for 15 min and resuspended in 1.5 ml PBS containing AFB1. Bacterial and yeast suspensions were incubated at 37°C for 24h. The samples were centrifuged and the supernatant fluid were analysed by HPLC. Test strains were examined for their ability to remove AFB1 at different conditions: (time: 0, 12, 24, 36 and 72 hr – temperature: 5°C, 37°C, 50°C, 60°C and 100 °C. pH: 2.5, 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5, concentrations: Ecolife 0.5, 3.0, 5.0 ml/L and *S. cerevicea* 1×10^9 cells/ml, 5×10^9 cells/ml and 7×10^9 cells/ml and combination of *S. cerevicea* 5×10^9 cells/ml & Ecolife 3.0 ml/L and AFB1 concentration 5 mg/ml). In all treatments, positive and negative controls were included; for the positive control, PBS was substituted for the bacterial cells and for the negative control PBS was substituted for El-Nezami *et al.* (1998).

Table 1: showing used strains and dose used for treatment

Strain	Dose of treatment
Ecolife	0.5 ml/L
	3.0 ml/L
	5.0 ml/L
Baker's yeast	1×10^9 cells/ml
	5×10^9 cells/ml
	7×10^9 cells/ml
Combination of E and B	B 5×10^9 cells/ml & E 3.0 ml/L
+ ve control	PBS + AFB1
- ve control	Strain + PBS

2.7. Quantification of AFB1 by HPLC:

The HPLC procedure used for the analysis of AFB1 was performed according to El-Nezami *et al.* (1995) with slight modifications. No extraction of AFB1 from samples of supernatant fluid was required, 70 ml supernatant was injected directly into HPLC. The HPLC

system (Applied Biosystem, CA, USA) was fitted with a dual pump model 400 solvent delivery system, a model 980 programmable fluorescence detector and a220_4.6 mm, 5 mm ODS Spheri-5 Brownlee col- umn fitted with C18 guard column. Water-acetonitrile-methanol (60:30:10, by vol) was used as the mobile phase with a

flow rate of 1 ml/min. Detection was by excitation at 365 nm and emission at 418 nm. The retention time was 9.5 min. Chromatograms were recorded at chart speed of 0.3 cm/min and peak width of 0.4 min. The percentage of AFB1 bound by the investigated strains suspension was calculated using the following formula: $100 \times (\text{Peak area of AFB1 in the supernatant} / \text{peak area of AFB1 in the positive control})$.

2.8. Statistical:

To determine significant differences in the results, a one way ANOVA and Tukey's means comparison test ($p = 0.05$) were performed, using the statistical package Minitab v. 15. All experiments were performed in duplicate making the mean \pm SD.

3. Results:

3.1. Factors affect binding of AFB1 from milk solution:

3.1.1. Time factor:

The results revealed that Ecolife aqua® at the dose 0.5 ml/L at the time of 24 h was the highest removal while at 72 h was the lowest removal, also at the dose 3.0 ml/L the highest removal was at 24 h and the lowest removal was at 72h, while at the dose 5.0 ml/L the highest removal was at 12h. and the lowest removal was at 72h. *S. cerviacea* at the lower dose 1×10^9 cells/ml, the highest removal percentage was at 72h. and the lowest removal was at 0h. also the doses 5×10^9 cells/ml and 7×10^9 cells/ml the removal removal was at 72h. while the lowest removal was at 0h. while the combination between Ecolife and *S.cerviacea* (E. 3.0 ml/L & S. 5×10^9 cells/ ml) the highest removal was at 36 h. and the lowest removal was at 72h table 2.

Table 2: Showing strains concentration corresponding to % of removed AFB1 from milk solution. In different times after incubation at 37°C

Tested strain	Strain conc.	0h.	12h.	24h.	36h.	72h.
Ecolife	0.5 ml/L	53 \pm 2.65a	65 \pm 3.25b	81 \pm 4.05b	79 \pm 3.95b	48 \pm 2.40a
Ecolife	3.0 ml/L	61 \pm 3.05b	73 \pm 3.78b	96 \pm 4.8c	93 \pm 4.65c	56 \pm 2.80a
Ecolife	5.0 ml/L	83 \pm 4.15c	94 \pm 4.70c	91 \pm 4.55b	89 \pm 4.45c	78 \pm 3.65b
<i>S.cerviacea</i>	1×10^9 cells/ml	39 \pm 1.94a	54 \pm 2.70a	57 \pm 2.85a	67 \pm 3.35a	82 \pm 4.12b
<i>S.cerviacea</i>	5×10^9 cells/ml	71 \pm 3.55c	86 \pm 4.30b	84 \pm 4.20b	89 \pm 4.12c	99 \pm 4.93c
<i>S.cerviacea</i>	7×10^9 cells/ml	64 \pm 3.20b	75 \pm 3.78b	79 \pm 3.95b	87 \pm 4.23c	98 \pm 3.73c
Combination (E+S)	E. 3.0 ml/L & S. 5×10^9 cells /mL	92.75 \pm 4.63c	95.33 \pm 4.46c	98.67 \pm 4.94c	100.00 \pm 5.00b	76.14 \pm 3.80b
control	PBS + AFB1	00.00 \pm 00.0d	00.00 \pm 00.0d	00.00 \pm 00.0	00.50 \pm 0.025d	3.00 \pm 0.15d

Treatments with different letters in each column are statistically different for each strain ($p < 0.05$). (each value is a mean \pm SD for 2 samples)

3.1.2. Temperature factor:

Removal percentage of AFB1 from milk solution as experimental media, present study displayed that Ecolife at the dose 0.5 ml/L at the incubation temperature of 37°C was the highest removal while at temperature of 5°C was the lowest removal. also at the dose 3.0ml/L and 5.0 ml/L the highest removal was at temperature 37°C and the lowest removal was at

temperature 5°C. *S. cerviacea* at the dose 1×10^9 cells/ml and 5×10^9 cells /ml the highest removal was at 100°C and the lowest removal was at 5°C, while the dose 7×10^9 cells/ml, the highest removal was at 50 °C, and the lowest removal was also at 5°C. while the combination between Ecolife and *S.cerviacea* (E. 3.0 ml/L & S. 5×10^9 cells/ ml) the highest removal was at temperature 100 °C and the lowest removal was at 5°C table 3.

Table 3: showing strain concentration corresponding to % of removed AFB1 from milk solution After incubation at Different temperatures for 24 h.

Tested strain	Strain conc.	5°C	37°C	50°C	100°C
Ecolife	0.5 ml/L	22 \pm 1.10a	92 \pm 4.60a	87 \pm 4.43a	43 \pm 2.16a
Ecolife	3.0 ml/L	31 \pm 1.55b	96 \pm 4.82a	89 \pm 4.12a	65 \pm 3.25a
Ecolife	5.0 ml/L	29 \pm 1.47b	98 \pm 3.61a	96 \pm 4.20a	76 \pm 3.87b
<i>S.cerviacea</i>	1×10^9 cells/ml	19 \pm 0.95a	63 \pm 3.15b	74 \pm 3.73b	85 \pm 4.25b
<i>S.cerviacea</i>	5×10^9 cells/ml	24 \pm 1.23a	77 \pm 3.85b	82 \pm 4.13b	91 \pm 4.33c
<i>S.cerviacea</i>	7×10^9 cells/ml	23 \pm 1.15a	84 \pm 4.24b	96 \pm 4.89a	95 \pm 4.23c
Combination (E+S)	E. 3.0 ml/L & S. 5×10^9 cells/ml	27 \pm 1.35b	89 \pm 4.36a	84 \pm 4.23b	91 \pm 3.42c

control	PBS + AFB1	00.00±00c	00.00±00c	10.40±0.52c	17.67±0.88d
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Treatments with different letters in each column are statistically different for each strain ($p < 0.05$). (each value is a mean±SD for 2 samples)

3.1.3. pH factor:

Removal percentage of AFB1 from milk solution as experimental media, present study displayed that Ecolife at the dose 3.0 ml/L at the incubation temperature of 37°C for 24h the highest removal at pH 8.5 while the lowest removal was at pH 4.5. *S. cereviceae*

at the dose 5×10^9 cfu/ml at the incubation temperature of 37°C for 24h, the highest removal was at pH 6.5 and the lowest removal was at pH 3.5 while the combination between Ecolife and *S.cerviacea* (E. 3.0 ml/L & S. 5×10^9 cells ml) the highest removal was at pH 4.5 and the lowest removal was at pH 8.5 fig 1.

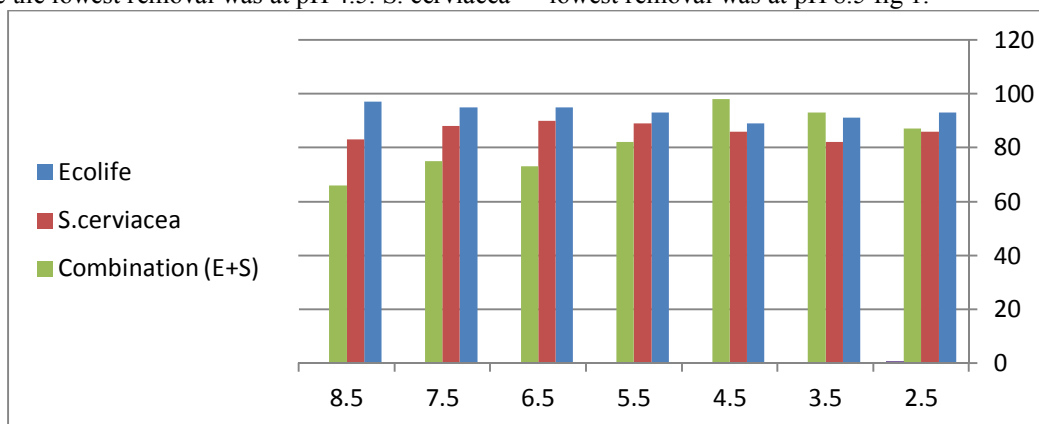


Fig 1; showing percentage of removed AFB1 from liquid milk after incubation at different pH at 37°C for 24 h.

4. Discussion:

The present study aimed to evaluate the ability of both commercial product Ecolife aqua® (a pool of commercially available LAB strains, with actinomycete and some enzymes) and Baker' yeast *Saccharomyces cerevisiae* alone or in combination to remove AFB1 from whole milk, as experimental media.

Our investigation has been fedocused on the binding ability of commercial products; Ecolife aqua® to bind food carcinogen AFB1. It is used in aquaculture as probiotic enhancing non-specific immune system and weight gain for marine and freshwater fishes also used for improving water parameters. A potential function of these specific strains may be the capacity to reduce the carcinogenic or toxic effect of food carcinogens by binding to them or metabolically transforming them into less toxic and carcinogenic degradation products.

EcoLife aqua® used in the present study is commercial product used in aquaculture as probiotic, it is FDA approved as dietary product, the safest for human, animals, fish and plants, made from fermented green tea leaves by extracting 14 different kinds of beneficial vegetable lactic acid bacteria. The genus *Lactobacillus* was LAB, frequently involved in the antifungal activity of LAB. The antifungal strains have been isolated from different environments such as sourdough (Corsetti *et al.*, 2008), grass silage (Magnusson & Schnürer, 2001; Magnusson *et al.*, 2003) and vegetable products (Sathe *et al.*, 2007)

The pool of LAB (Ecolife aqua®) used in present study was shown to be efficient to remove AFB1 from whole liquid milk with different degrees depending on time of incubation temperature of incubation and pH the results nearly agree with the results of (Kankaanpää, *et al.*, 2000; Gratz *et al.*, 2004) who reported that several LAB have been found to be able to bind aflatoxin B1 in vitro and in vivo with an efficiency depending on the bacterial strain (Shah & Wu, 1999). El-Nezami, *et al.* (1998) have evaluated the ability of five *Lactobacillus* to bind aflatoxins in vitro and have shown that probiotic strains such as *Lb. rhamnosus* GG and *Lb. rhamnosus* LC-705 were very effective for removing aflatoxin B1, with more than 80% of the toxin trapped in a 20 lg/ml solution (Haskard, *et al.*, 1998). According to Magnusson *et al.* (2003), three mechanisms may explain the antimicrobial efficiency of LAB: the yield of organic acid, competition for nutrients and production of antagonistic compounds (Dalié *et al.*, 2010).

Present study also revealed that *S.cerevisiae* can removed AFB1 from whole milk, the results agree with that obtained by some literature who indicate that other organisms than LAB, such as *Saccharomyces cerevisiae* have the potential to bind aflatoxin B1 (Haskard *et al.*, 2000; Santin *et al.*, 2003; Baptista *et al.*, 2004). In order to clarify the in vitro aflatoxin B1 removal by LAB and *S. cerevisiae*, The yeast *S. cerevisiae* was reported to be the most efficient microorganism for aflatoxin B1 removal (Bueno *et al.*, 2006) that is agree

with the present study. *S. cerviacea* at the dose 1×10^9 cells/ml and 5×10^9 cells/ml d highest removal was at 100°C and the the lowest removal was at 5°C , while the dose 7×10^9 cells/ml, displayed that highest removal was at 50°C , and the lowest removal was also at 5°C . while the combination between Ecolife and *S.cerviacea* (E. 3.0 ml/L & S. 5×10^9 cells/ml) the highest removal was at temperature 100°C and the lowest removal was at 5°C . present study nearly agree with that of **Corassin et al. (2013)** who reported that Comparing to the LAB pool, *S. cerevisiae* cells had higher ($P < 0.05$) capability to bind AFM1 in milk for 30 min and 60 min, respectively), they also added that When using *S. cerevisiae* in combination of LAB pool, a significant increase ($P < 0.05$) was observed in the percentage of AFM1 bound in the contact times, which values were 91.7 0.5% (30 min) and 100.0% (60 min).

Concerning the factors affect removal of AFB1, present study revealed that both products; Ecolife aqua and Baker's yeast have the ability to remove AFB1 from whole milk also combination of two products posses potential ability to remove AFB1 whole liquid milk but these ability greatly affected with several factors, time of incubation, temperature, and pH.

Present study revealed that the time is very important factor for removal percentage of AFB1 from milk solution as experimental media, the results revealed that Ecolife aqua® at the dose 0.5 ml/L at the time of 24 h was the highest removal while at 72 h was the lowest removal, also at the dose 3.0 ml/L the highest removal was at 24 h and the lowest removal was at 72h, while at the dose 5.0 ml/L the highest removal was at 12h. and the lowest removal was at 72h. *S. cerviacea* at the lower dose 1×10^9 cells/ml, the highest removal percentage was at 72h. and the lowest removal was at 0h. also the doses 5×10^9 cells/ml and 7×10^9 cells/ml the removal was at 72h. while the lowest removal was at 0 h. while the combination between Ecolife and *S.cerviacea* (E. 3.0 ml/L & S. 5×10^9 cells/ml) the highest removal was at 36h. and the lowest removal was at 72h. It is clear that time is important factor for removal AFB1 from milk solution, previous studies also indicted that, time is essential factor in these process, this may be due to that incubation time for removing organisms. It is also important that take in mind that the process of binding is reversible (**Bueno, et al., 2006**) who reported that, in vitro binding of aflatoxin B1 by LAB was described as a fast (no more than 1 min) and reversible process and it is strain- and dose-dependent (**Kankaanpää et al., 2000**).

Present study displayed that Ecolife at the dose 0.5 ml/L at the incubation temperature of 37°C was the highest removal while at temperature of 5°C was the lowest removal. Also at the dose 3.0 ml/L and 5.0 ml/L the highest removal was at temperature 37°C and the lowest removal was at temperature 5°C . The current

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study confirmed that there are several factors help to determine the amount of removal of AFB1 from liquid media (whole milk), temperature and incubation period (time) are essential factors that modulate LAB growth and significantly affect the amounts of antifungal metabolites produced which it is one of methods that by which AFB1 removed from any media. Another mechanism of removal is binding to the wall of organism lactic acid bacteria LAB or *S. cervisea* (**Dalié et al., 2010**). Mainly temperature of incubation play great role, make LAB affect by 2 ways. The results nearly agree with that obtained by **EI-Nezami et al. (1998)** study the 24-hr-old cultures of both LBGG and LC705 removed about 80% AFB1 within 24 hr. they also added that optimal removal was achieved at 37°C . This finding is similar to that of **Lillehoj et al. (1967)** where the maximal removal of AFB1 by *Flavobacteria aurantiacum* occurs at 35°C . this may be due to that temperature directly activate the production of antifungal metabolites produced by LAB. As illustrated by **Sathe et al. (2007)** who demonstrated that antifungal activity of *Lb. plantarum* CUK501 was maximal (1280 AU/ml) at 30°C , when the culture was at the end of its logarithmic phase. Over 48 h of incubation, a decrease in antifungal activity was observed when the culture entered the stationary phase. Also **Batish et al., (1990)**, who observed that the antifungal activity of a *Lb. acidophilus* strain was maximal at 30°C after 48 h incubation.

Regarding the effect of pH on removal of AFB1 from whole milk, Ecolife at the dose 3.0 ml/L at the incubation temperature of 37°C for 24h the highest removal at pH 8.5 while the lowest removal was at pH 4.5. *S. cerviacea* at the dose 5×10^9 cells/ml at the incubation temperature of 37°C for 24h., the highest removal was at pH 6.5 and the lowest removal was at pH 3.5 while the combination between Ecolife and *S.cereviace* (E. 3.0 ml/L & S. 5×10^9 cells/ml) the highest removal was at pH 4.5 and the lowest removal was at pH 8.5. Present investigation confirmed that removal percentage of AFB1 from milk solution greatly affect with pH, the results agree with the results of **Batish et al., (1997)** and **Reddy & Ranganathan, (1985)** who reported that *Lc. lactis* subsp. diacetylactis was able to produce substantial amounts of antifungal substance in a narrow range of pH (5.5–7), although maximal production occurred at pH 6.8 also **Corsetti et al. (1998)** reported an optimal production of inhibitory substances by *Lactobacillus sanfrancisco* CB1 at pH 6. The pH effect was shown to be linked to many factors such as substrate, incubation period, temperature, mould strains and the occurrence of competing microflora (**Gourama & Bullerman, 1995**).

From the present study we can concluded that it is first time to use ready coomercial products to remove the potential carcinogen AFB1 milk solution. Both

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Ecolife aqua and Baker's yeast *S.cerevisia*. able to bind efficiently AFB1 from whole milk but combination between the two products was more efficient depending on time temperature, pH and concentration of strain used. It is very important to perform other or further studies on another natural commercial products for removal of aflatoxins from solution of food products to be more safe.

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