

## Evaluation of antifungal activity of lactic acid bacteria from locally sourced yoghurts on isolates of *Candida Albicans* from cases of vaginitis

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### ABSTRACT

This study assessed antifungal activity of lactic acid bacteria from locally made yoghurts on isolates of *Candida albicans* from cases of vaginitis. Locally sourced yoghurt samples were cultured on MRS agar and incubated anaerobically at optimum temperature for the isolation of Lactic Acid Bacteria. The pH and total viable counts parameters of the isolates from the samples were determined. Isolates of *Candida albicans* from cases of vaginitis collected were biochemically confirmed after SDA plated. The isolates were exposed to antifungal agents with varied concentrations and thereafter, challenged with Lactic Acid Bacteria laden yoghurt samples prepared in varied concentration on MRS agar overlaid with SDA. Plasmid DNA of the LAB isolates were determined.

The isolates of *Candida albicans* exhibited 100% resistance to conventional antifungal agents exposed with the exception of *Candida albicans* 1 that were susceptible to all the antifungal agents while *Candida albicans* 4 was susceptible to amphotericin B, fluconazole, and nystatin. Three(3) of the four (4) isolates of *Candida albicans* challenged with Habib (LABHAY) and Tunik(LAB TY) yoghurt were 100% susceptible the LAB laden yoghurt samples while *Candida albicans* isolates 3 (*Ca3*) were (100%) resistant to LABHAY and LAB TY. Of the the four(4) isolates of *Candida albicans* were(100%) susceptible to Cedar (LABCY) and Fan milk(LABFM) yoghurt and while *Candida albicans* isolates number 3 (*Ca3*) exhibited resistance to LABCY and LABFM. Lactic Acid Bacteria in this study, exhibited remarkable antifungal activity on *Candida albicans* isolates from cases of vaginitis. This property could be exploited as a bio-protective and therapeutic option, for cases of *Candida albicans* and fungi with related features.

**Keywords:** Lactic Acid Bacteria, locally sourced yoghurt samples, *Candida albicans*, Vaginitis

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## Résumé

Cette étude a évalué l'activité antifongique des bactéries lactiques issues de yaourts fabriqués localement sur des isolats de *Candida albicans* provenant de cas de vaginite. Des échantillons de yaourt d'origine locale ont été cultivés sur gélose MRS et incubés en anaérobiose à une température optimale pour l'isolement des bactéries lactiques. Les paramètres de pH et de nombre total de bactéries viables des isolats des échantillons ont été déterminés. Les isolats de *Candida albicans* provenant de cas de vaginite collectés ont été confirmés biochimiquement après étalement sur plaque de SDA. Les isolats ont été exposés à des agents antifongiques à des concentrations variées, puis soumis à des échantillons de yaourt chargés de bactéries lactiques préparés à des concentrations variées sur gélose MRS recouverte de SDA. L'ADN plasmidique des isolats LAB a été déterminé.

Les isolats de *Candida albicans* ont montré une résistance à 100 % aux agents antifongiques conventionnels exposés à l'exception de *Candida albicans* 1 qui était sensible à tous les agents antifongiques tandis que *Candida albicans* 4 était sensible à l'amphotéricine B, au fluconazole et à la nystatine. Trois (3) des quatre (4) isolats de *Candida albicans* testés avec le yaourt Habib (LABHAY) et Tunik (LAB TY) étaient sensibles à 100 % aux échantillons de yaourt chargés de LAB tandis que les isolats de *Candida albicans* 3 (Ca3) étaient (100 %) résistants à LABHAY et LAB TY. Des quatre (4) isolats de *Candida albicans* étaient (100 %) sensibles au yaourt Cedaar (LABCY) et au lait Fan (LABFM) et tandis que les isolats de *Candida albicans* numéro 3 (Ca3) présentaient une résistance à LABCY et LABFM. Les bactéries lactiques de cette étude ont montré une activité antifongique remarquable sur des isolats de *Candida albicans* provenant de cas de vaginite. Cette propriété pourrait être exploitée comme une option bioprotectrice et thérapeutique, pour les cas de *Candida albicans* et de champignons présentant des caractéristiques apparentées.

**Mots clés :** bactéries lactiques, localement (NB: The abstract was translated from English to French by Google Translate and slightly edited by the Editor)

### Introduction

Lactic acid bacteria (LAB) constitute a broad spectrum of heterogeneous group of anaerobic bacteria that produce a large amount of lactic acid during carbohydrate metabolism and releases organic acid. It has been reported that metabolites releases possess anti-mycotoxigenic properties, that can be exploited for bio-protective and therapeutic purposes. Novel methodologies together with the development of molecular techniques have allowed the identification and discovery of new genera and species. Application of LAB has changed from traditionally fermented foods and beverages to rationally controlled food fermentation, in situ production of industrially relevant metabolites, and use as probiotics or as efficient microbial cell factories and antifungal agents (Gupta and Jeeveratnam, 2018).

Lactic acid bacteria (LAB) are capable of converting carbohydrate substrates into organic acids (mainly lactic acid) and producing a wide range of metabolites. Due to their interesting beneficial properties, LAB are widely used as starter cultures, probiotics, and microbial cell factories. Exploring LAB present in unknown niches may lead to the isolation of unique species or strains with relevant technological properties (Kobayashi *et.al.*, 2011).

Lactic acid bacteria (LAB) are naturally occurring in many food systems. They sometimes exhibit antifungal activity via inherent antagonistic metabolites. Several low molecular weight compounds have been isolated from LAB with the capacity to eliminate fungal growth either on their own or synergistically, including organic

acids, reuterin, fatty acids, proteinaceous compounds, and cyclic dipeptides( Bintsis,2018).

*Candida* species are commensal yeasts that are part of the normal human skin and gut microbiota and are detectable in up to 60% of healthy individuals. Colonization of *Candida* specie is regarded as a prerequisite for subsequent infections. Invasive candidiasis is not a single clinical entity but rather a disorder with myriad of clinical manifestations that can potentially affect any organ, as each *Candida* sp. possesses its own unique characteristics relative to its invasive potential, virulence and antifungal antagonism. *Candida albicans* can produce over 400 mycotoxins which can cause systemic illness, suppresses the immune system by decreasing the production of white blood cell and interfering with normal glutathione metabolism in the cell(Chmiewska, 2010).

Vaginitis is a medical term that describes various disorders that cause vagina to become infected or inflamed, while vulvovaginitis refers to inflammation of both the vagina and vulva. *Candida albicans* is the most common pathogen of vaginitis (in about 90% of cases), with most of the remaining cases caused by *Candida glabrata*. About 8% of women suffer recurrent candidal vulvovaginitis and 70% of women report having had candidal vulvovaginitis at some point in their lifetimes(Nomoto,2005). Women who are more likely to get vaginal candidiasis include those who are pregnant, use hormonal contraceptives (for example, birth control pills), have diabetes, are immunocompromized (for example, due to HIV infection or medicines such as steroids and chemotherapy) and are taking or have recently taken antibiotics (Dalie and Deschamps, 2010). Epidemiologic reports based on culture alone overestimate the disease, as 10% of women are asymptomatic with positive candidal cultures. Vaginitis is more common in women who are

sexually active, there is no evidence that candidal infection is sexually transmitted. Patients with recurrent candidal vaginitis have predisposing genetic factors that cause them to be susceptible to recurrent fungal infections( Francesca,1992).

Therefore, this study aimed at assessing the antifungal activity of lactic acid bacteria from locally sourced yogurts on isolates of *Candida albicans* obtained from cases of vaginitis as possible affordable and effective treatments for drug resistant candida albicans.

## Methods

### Collection of samples

Locally prepared and refined yogurt samples (in refrigerated form) identified by their labeling as Habib, Cedaa, Tunik and Fan milk yoghurt were purchased from the retail shops in Abadina community within the University of Ibadan, this community predominantly occupied by the non-teaching workforce of the university and their relatives. The samples purchased were immediately transferred to the laboratory for bacteriological analysis.

### Determination of pH of the yogurt samples.

The pH of yogurt was determined using pH meter (Hanna pH HI99164). The four brands of yogurt samples collected from different sources were poured into a beaker. The pH meter was calibrated using standard buffer solution and readings were taken at intervals of 0hr, 24hrs and 48 hrs.

### Preparation of the yoghurt for the isolation of Lactic acid bacteria (Serial Dilution)

Fourfold serial dilutions were performed by dispensing 1mL of the samples of the yoghurt samples in 9 mL of sterile distilled water and the procedure was repeated for four times. From each prepared dilutions, 0.2ml of the diluted sample was introduced to a sterile Petri-dishes and already prepared MRS was aseptically poured and

incubated at optimum temperature anaerobically for 48 hours. The colonies observed were sub-cultured to obtain pure isolates.

### **Viable count of LAB**

Ten-fold serial dilution was done for each sample from which  $10^{-2}$  and  $10^{-6}$  dilution factors were plated on MRS agar.

The viable count per ml were determined by dividing the actively growing colonies in each dilution factors with volume plated.

$$\text{cfu/ml} = \frac{\text{cfu} \times \text{dilution factor}}{\text{Volume plated (0.2ml)}}$$

### **Collection of clinical isolates.**

A total of ten (10) isolates of *Candida albicans* from patients diagnosed with vaginitis were collected from gynecology clinic in Ibadan. The organisms were immediately cultured on Sabouraud Dextrose Agar and biochemical tests was carried out on the isolates for confirmation. Of the ten isolates, 4 were found to be pure after cultural, morphological, and biochemical confirmation.

### **Antifungal activity of *Candida albicans* to conventional antifungal agents (Disc diffusion Assay)**

Sabouraud Dextrose Agar were prepared according to manufacturer specification and were allowed to set. Pure culture of *Candida albicans* isolates implicated from the cases of vaginitis were aseptically streaked on the prepared medium and thereafter impregnated with the following antifungal agents; Voriconazole(1 $\mu$ g) Itraconazole(10 $\mu$ g), Amphotericin B(10 $\mu$ g), Caspofungin(5 $\mu$ g), Fluconazole(25 $\mu$ g), Terbinafine(1 $\mu$ g), Griseofulvin (10 $\mu$ g), Nystatin (100units), Clotrimazole(10 $\mu$ g) and Ketoconazole(25 $\mu$ g) and then incubated at room temperature for 48 hours. The plates were observed for zones of growth inhibitions.

### **Antifungal Challenge test on Lactic Acid Bacteria (LAB) using the Agar Well Diffusion Method.**

The Antifungal activity of LAB isolate was determined using Agar well diffusion method. Plates containing Mueller Hinton Agar were prepared and overlaid with 5mls of SDA and the preparation was allowed to set. Each isolate of *Candida albicans* were streaked on the combined agar(MRS over layered with SDA) and 0.1ml broth of LAB was pipetted into the wells bored using a 6mm cork borer. The preparations were left on the table for pre-diffusion and then incubated aerobically for 48hrs at room temperature. Susceptibility of *Candida albicans* to viable LAB was observed.

### **Plasmid DNA extraction.**

The pure isolates of Lactic Acid Bacteria obtained from the stock were sub-cultured into nutrient broth and incubated for 24 hours, the culture of the bacteria cells was thereafter transferred into Eppendorf tubes centrifuged at 13,000rpm for 2 minutes after which supernatants were discarded. The pellet was suspended in the remaining broth by vortexing at high speed. The suspended pellet was treated as follows; 300 $\mu$ L of TENS (Tris25mM, EDTA 10mM, NaOH 0.1N and SDS 0.5 %) solution was added and mixed gently by inverting tubes until the solution becomes slimy. A volume of 150  $\mu$ L of 3.0 M sodium acetate (pH 5.2) was vortexed for about 10 seconds and the mixture was centrifuged at 13,000 rpm for 5 minutes. The supernatant was transferred into another 1.5 mL Eppendorf tube and 900  $\mu$ L of ice-cold absolute ethanol was added. This was vortexed and centrifuged at 13,000 rpm for 10 minutes after vortexing and centrifugation, the supernatant was discarded, and a white pellet was observed. 1000  $\mu$ L (1 mL) of ice-cold 70% ethanol was added to the observed white pellet and centrifuge at 13,000

rpm for 5 minutes without vortexing. The supernatant was discarded, and the pellets were totally air dried. The dried pellet was suspended in 40 µL of TE buffer (Tris 10 mM, 1 mM Na<sub>2</sub>EDTA).

**DNA agarose Gel electrophoresis.**

The agarose powder (0.8%) in x0.5 TBE buffer (Tris-borate, Na<sub>2</sub>EDTA) was dissolved by boiling. This was allowed to cool to about 60oC before adding 10 µL of ethidium bromide (1 mg/mL). After gentle swirling, it was poured into electrophoresis tank and comb inserted. After solidification (gelling) the comb was removed, and the gel totally submerged in x 0.5 TBE buffer. The sample (suspended pellet, 15 µl) was mixed with 2 µL of loading dye and carefully loaded into the wells created by the combs alongside 100 bp DNA ladder. This set up was connected to power pack and run at 100 V for 45 minutes. The gel was thereafter observed in gel photo documentation systems to read the plasmid bands waves.

**Table 2:Colony Count of Lactic Acid Bacteria**

Yoghurt Brand	CFU Dilution factor 10 <sup>-2</sup>	CFU/ml	CFU Dilution Factor 10 <sup>-6</sup>	CFU/ml
Habib	280	1.4 x10 <sup>1</sup>	200	1 x 10 <sup>3</sup>
Cedaa	52	2.6 x 10 <sup>1</sup>	40	2 x 10 <sup>-2</sup>
Tunik	288	1.44 x 10 <sup>2</sup>	160	8 x 10 <sup>2</sup>
Fan milk	50	2.5 x 10 <sup>2</sup>	30	1.5 x 10 <sup>3</sup>

Key: cfu/ml =  $\frac{\text{cfu} \times \text{dilution factor}}{\text{Volume plated (0.2ml)}}$

**Results**

The pH of the samples of yoghurts studied varied in relative with sampling time as shown in Table 1.

**Table 1.0: pH changes with Time.**

Sample	Time(hours)	pH
Hay <sub>1</sub>	0	4.0
Hay <sub>2</sub>	24	3.2
Hay <sub>3</sub>	48	2.0
Cy <sub>1</sub>	0	4.2
Cy <sub>2</sub>	24	3.5
Cy <sub>3</sub>	48	2.7
Ty <sub>1</sub>	0	4.1
Ty <sub>2</sub>	24	3.5
Ty <sub>3</sub>	48	3.0
Fm <sup>1</sup>	0	4.3
Fm <sup>2</sup>	24	3.0
Fm <sup>3</sup>	48	2.0

Key: Ha-Habib yoghurt, C-Cedaa yoghurt, T-Tunik yoghurt, Fm-Fan milk yoghurt.

Biochemical characterization of Lactic Acid Bacteria study elicited conventional reaction to the biochemical reagents used as shown in Table 3.

**Table 3: Biochemical characterization of Lactic Acid Bacteria**

Isolates	Gram staining	Catalase test	Citrate test	Methylred test	Oxidase	Glucose	Lactose	Fructose
Ha1	+	-	-	-	-	+	+	+
Ha2	+	-	-	-	-	+	+	+
Ha3	+	-	-	-	-	+	+	+
C1	+	-	-	-	-	+	+	+
C2	+	-	-	-	-	+	+	+
C3	+	-	-	-	-	+	+	+
T1	+	-	-	-	-	+	+	+
T2	+	-	-	-	-	+	+	+
T3	+	-	-	-	-	+	+	+
Fm1	+	-	-	-	-	+	+	+
Fm2	+	-	-	-	-	+	+	+
Fm3	+	-	-	-	-	+	+	+

Key: Hay: Habib yoghurt, Cy:Cedaa yoghurt, Ty:Tunik yoghurt, Fan milk Yoghurt.

The resistant patterns exhibited by the isolates of *Candida albicans* study to the conventional antifungal agents are shown in Table 4.

Isolates	VOR	ICZ	AMB	CAS	FCZ	TBF	GSF	NST	CLZ	KTZ
Ca 1	S	S	S	S	S	S	S	S	S	S
Ca 2	R	R	R	R	R	R	R	R	R	R
Ca3	R	R	R	R	R	R	R	R	R	R
Ca4	R	R	S	R	S	R	R	S	R	R

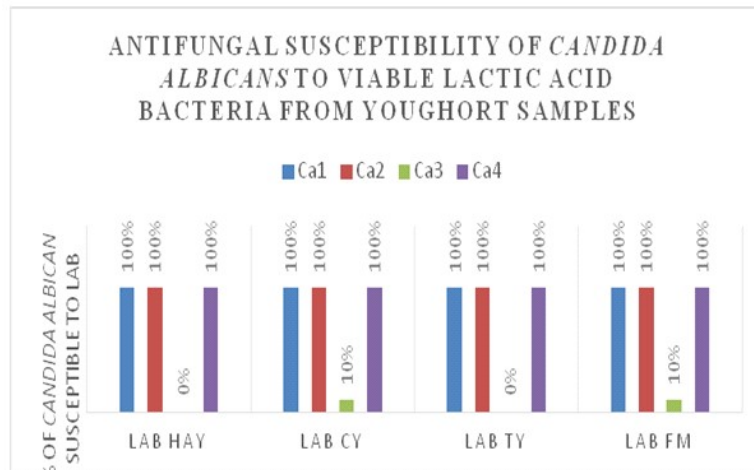
Key:VOR;Voriconazole(1µg) ICZ; Itraconazole(10µg) AMB; Amphotericin B(10µg) CAS; Caspofungin(5µg) FCZ; Fluconazole(25µg) TBF; Terbinafine(1µg) GSF; Griseofulvin(10µg) NST; Nystatin(100units) CLZ; Clotrimazole(10µg) KTZ; Ketoconazole(25ug)

Ca1-*Candida albican* isolate 1,Ca2-*Candida albican* isolate 2,Ca3-*Candida albican* isolate 3 ,Ca4-*Candida albican* isolate 4 . R- resistant, S- susceptible



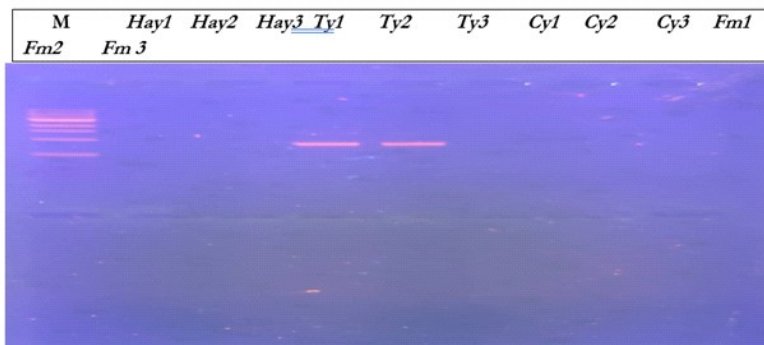
**Figure 1:** Antifungal activity of LAB on *Candida albicans* isolates (zones of growth inhibition from selected plates)

Antifungal activity of LAB on *Candida albicans* isolates elicited varied profiles of susceptibility as shown in Figure 2.



**Figure 2: Susceptibility of *Candida albicans* to viable Lactic Acid Bacteria from the yoghurt Samples**

Key: LAB HAY 1 :Lactic Acid Bacteria from Habib yoghurt, LAB CY:Lactic Acid Bacteria from Cedar yoghurt, LAB 3: Lactic Acid Bacteria from Tunik yoghurt, LAB FM Lactic Acid Bacteria from Fan milk yoghurt., Ca: *Candida albicans*.



**Figure 3: Plasmid DNA profiles of the isolates of LAB studied elicited the observable patterns**

Key: LAB (Hay):Lactic Acid Bacteria from Habib yoghurt. LAB (Ty):Lactic Acid Bacteria from Tunik yoghurt. LAB( Cy ): Lactic Acid Bacteria from Cedaa yoghurt

**Discussion**

In this study, Yoghurt samples from four selected local sources (Habib yoghurt, Cedar-yoghurts Tunik- yoghurt and Fan milk yoghurt) elicited growth on MRS agar. The pH parameters of each yoghurt samples elicited relatively close acidity range, between the minimum and maximum range of between 2.0 (as recorded in Hay 3 and Fm 3) and 4.2 as (elicited in Cy1) with time in Table 1, this could be attributed to

the kinetic of metabolic activity of the isolates inherent within the sample that dictate progressive acidity of fermentation as time advances, which corroborates the study of Solimama *et.al.*,(2018) on antibacterial activity of some Lactic Acid Bacteria isolated from Egyptian traditional fermented dairy products( Frank and Christien, 1992).

The total viable counts of the LAB isolates from the samples at two varied dilution factors selected as showed in (Table 2) varied considerably, which could be due to refrigeration and post acidification potential of the isolates as

time advances, this corroborates the findings of Laniewska-Trokenheim *et.al.*,2010 on parameters that could affect the viability of Lactic Acid Bacteria(Isolauri and Macpherson, 2010).

The isolates of LAB obtained were Gram positive, catalase negative, citrate negative, methyl red negative, oxidase negative, lactose positive and glucose positive. The cultural morphology and biochemical properties of the isolates studied corroborated the study of Purshotam(2008) on cultural and biochemical properties of microorganisms. The antifungal susceptibility of the *Candida albicans* explored exhibited alarming resistance to conventional antifungal agent exposed. Alarming resistance of 100% to voriconazole, itraconazole, caspofungin, terbinafine, grieseofulvin, clotrimoxazole and ketoconazole with the exception of *Candida albicans 1* that were susceptible to all the antifungal agents and *Candida albicans 4* that are also susceptible to amphotericin B, fluconazole and nystatin respectively as shown in Table 4. The phenomenon of degree of occurrence of susceptibility of these isolates of *Candida albicans*, could be attributed to inefficacy of the antifungals used in modulating virulence, development of resistance and poor penetration into the biofilm matrix, mishandling, poor storage, overuse of this conventional antifungal agents. This agreed with the study of Helman David *et al.*, (2023) focusing on a solution; molecular association of *Candida albicans* and vulvovagina candidiasis(McCarthy and Pappas,2016).

The same isolates of *Candida albicans* from patients with cases of vaginitis exposed to conventional antifungal agents were challenged with the four brand of yoghurt dilution samples by agar well diffusion techniques. Three(3) of the four (4) isolates of *Candida albicans* challenged with Habib(LABHAY) and Tunik(LAB TY ) yoghurt were 100% susceptible the yoghurt

samples while *Candida albicans* isolates number 3 (*Ca3*) were (100%) resistant to LABHAY and LAB TY. Three of the four(4) the same isolates of *Candida albicans* were(100%) susceptible to Cedaar (LABCY) and Fan milk(LABFM) yoghurt and while *Candida albicans* isolates number 3 (*Ca3*) exhibited resistance to LABCY and LABFM respectively as shown in Figure 2.0. In this very study, the phenomenon of occurrence, indicated the antifungal potential of the LAB laden yoghurt samples studied, this pattern of occurrence observed, could be due to strains variation or genetic variation of the *Candida albicans* isolates tested, because three(3) of the four(4) isolates were susceptible to the LAB laden yoghurt samples, with the exception *Candida albicans* isolates number 3 (*Ca3*) that exhibited resistance against the LAB laden sample studied. The preponderance of occurrence in this present study, corroborates the study of Aiping Liu *et al.*, (2022) antifungal mechanisms and application of lactic acid bacteria as antifungal tools in bakery products(Mokoena,2017).

Of the total number of the twelve isolates of LAB electrophoresed for plasmid DNA, two (2) isolates Ty2 and Ty3 were found to possess plasmid while the remaining twelve(12) elicited no plasmid DNA. The plasmid DNA could serve as a resistance armament for the 2 of the 12 isolates that possess it while resistance or susceptibility potential of the remaining 10 isolates that exhibited no plasmid could be chromosomal or any other genetic factors. The significance of plasmid DNA obtained is the ability to transmit pathogenically and environmentally relevant traits to the host bacteria, spread antibiotic resistance genes, promoting rapid evolution and adaptation to various environment. This agreed with the study of Werner Arber (1999 on genetic variation , molecular mechanisms and impact on microbial evolution( Nomoto,2005).



## Conclusion

Although antifungal activity of Lactic Acid Bacteria(LAB) on *Candida albicans* from the cases of vaginitis in this study unfolded, this could be attributed to metabolic secretion of organic acids with antagonistic potential from each of the isolates of LAB challenge( Salminen,2005).

Lactic Acid Bacteria inhibition of fungal growth in this study could offers significant health benefit and serves a feasible prophylactic management option for cases of *Candida albicans* mediated vaginitis when further purified. Further elucidation on antifungal potential of Lactic Acid Bacteria is therefore recommended.

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346