Verification of the presence of *Candida albicans* in the gastrointestinal tract in healthy subjects, after supplementation of the lactic yeast *Kluyveromyces marxianus fragilis B0399*, through the examination of the feces. (Trial No. 129)

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0 - Summary:

The object of this study was to verify and evaluate the presence of *candida albicans* in the feces of 23 patients, before and after treatment with *Kluyveromyces marxianus fragilis B0399.*
The statistical analysis of the results obtained shows that there is a significant decrease in the presence of *Candida albicans* ---from those found in the pre-treatment analyses to those found in the post-treatment analyses. This confirmed that *Kluyveromyces marxianus B0399* has the capacity to limit the development of *Candida albicans* at the intestinal level.
We know that the intestine constitutes the natural receptacle for this fungus and that the importance of this type of trial *in-vivo* is self-evident.
Since this probiotic may be taken simultaneously with antibiotics thanks to its particular resistance, its presence would inhibit the phenomenon of re-colonization of C*andida albicans* (spore-forming) after the treatment with fungicides and antibiotics have ended, thus avoiding the impoverishment of the intestinal flora.

1 - Structures employed in the experimentation

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2 - INTRODUCTION:

Candida albicans is a spore-forming fungus which is normally present in the intestine of humans in a non-pathogenic form. The intestine constitutes the principal receptacle for the *Candida*.
As far back as 1978 it was demonstrated and established that the transition from the spore-forming form to the pathogenic form of the *candida* was determined by the alteration or destruction of the normal intestinal native probiotic flora. In these conditions, the polymorphic fungus, which is highly invasive when it has no antagonists (probiotic yeasts and bacteria), colonizes the areas of the body in which the conditions of the environment are favorable. This happens when the beneficial intestinal flora is reduced or destroyed for various reasons causing its lactic acid production to diminish and the pH level to become less acidic and more basic and therefore suitable to the colonization of the fungus. When this happens, the *Candida* becomes pathogenic a migrates from the intestine to other parts of the body.
The treatments with fungicides and antibiotics in an acute phase eliminate the *Candida* and the serious symptomatology. However, these drugs significantly alter the intestinal flora, destroying its equilibrium. It's not unusual to have a situation in which, once the treatment is finished, the spores of the C*andida* (which are highly resistant) take advantage of the intestinal flora's imbalance and once again attack the more sensitive areas. The addition of treatment with probiotics in conjunction with the fungicides and antibiotics is vital in recuperating and maintaining an equilibrium.
Often, Candida is referred to as a "silent epidemic," given the long series of pathologies which have often been misdiagnosed or not recognized by the anamnesis and are related to infections caused by this fungus.
One of elected sites for migration of Candida in its pathogenic form , is the vaginal mucosa. The resulting pathology, Candida albicans Vaginitis, is a particularly disturbing infection, both for the difficulty in eradicating the pathogenic agent and for the relapses which may occur over time.

A treatment with selected probiotic yeasts and bacteria, capable of normalizing the beneficial bacterial flora, impeding the transformation of *Candida albicans* into the pathogenic form and directly blocking its development in the vagina, has gained the recognition today as being a fundamental and vital defense.
Probiotic organisms are defined by the guidelines of the FAW/WHO (Cordoba, Argentina 2001) as living organisms which, when taken in an adequate quantity, create beneficial effects in the subject.
Various studies underline the effectiveness of probiotics:

- in the modulation of the immune system (Matsuzaki T. [1]),
- in the prevention and treatment of imbalance of microbial intestinal flora which can provoke diarrhea or syndromes caused by the degeneration of inflammatory reactions (e.f. Crohn's Disease, Irritable Bowel Syndrome) (Castagliuolo M.S. et al.[2], Gorbach S.L.[3]),
- in the reduction of the development of allergic phenomena like asthma and eczema in children (Benn C. et al.[4]) if used by the mother when pregnant.

In the reduction of the risk of infections in the genital-urinary tract (Senok A.C. et al. [5], Reid G. et al. [6], Reid G. et al. [7], Reid G. [8]).

It has furthermore been demonstrated that many yeasts show a significant killer-type activity against pathogenic fungi of clinical importance (Sugisaki Y. et al. [9], Walzer G.M. et al. [10],Cerikcioglu N. [11]). This activity was attributed to the production of proteinic-type toxins.
In particular, a possible anti-fungus action of di *Kluyveromyces marxianus B0399* has been reported: it is a homo-fermenting yeast of human diet origin, used as a probiotic for persons affected by intestinal problems stemming from a imbalance of microbial intestinal flora (meteorism, constipation alternating with diarrhea, difficulty in assimilating, etc.) and/or lactose intolerance. The yeast, in fact, produces the enzyme β galactosidase.

Objective of the study

The objective of this study was to verify and evaluate the presence of *Candida albicans* in human feces before and after taking *Kluyveromyces marxianus fragilis B0399* at the dosages recommended by the distributing company.

A few notes regarding the active ingredient in the trial.
K*luyveromyces marxianus fragilis B0399* is a lactic yeast with characteristics which differ from those of bifidobacter and the yeast *Saccharomyces*. It has been used for some time now as a probiotic in the zootechnical field as well (Commission Regulation EC 773/06). It is a eukaryotic cell (Lachance M.A [12]) equipped with an elevataed lactasic enzymatic activity ((β-galactosidase). It therefore ferments lactose, producing lactic acid. The enzymatic activity, in anaerobic conditions typical of the intestine, is homo-fermenting, in that it transforms all the resulting glucose into lactic acid, without the production of gas (CO2) (ex.
Saccharomyces) (Vananuvat-Kinsella[13] ,Wasserman-Hopkins-Porges[14]). This contributes in modulating the intestinal environment, reducing the pH. It has also proven to be particularly resistant to antibiotic action (Voughan [15])

Kluyveromyces marxianus fragilis B0399 is able to resist gastric shock. This ability was first tested *in vitro* (Susmel and Stefanon[17]) through the measurement of the fermentative capacity before and after gastrointestinal digestion. The resistance to digestion proved to be elevated.

The capability of intestinal colonization after treatment with *Kluyveromyces B0399* was later demonstrated through the examination of human feces (trial 130 Mustacchi at all,[16]) .
The ability of bypassing the gastric barrier was also confirmed in *in vivo* trials on monogastric animals such as piglets (which have a digestive system very similar to that of humans) (Lovrovich P. [18]) and horses, by testing the presence of *Kluyveromyces B0399* in the feces and the modification of the pH in the colon (Lowell R. S. [19]. Susmel-Stefanon [20],Bosi P. [21].)

The benefits of *Kluyveromyces B0399* have also been demonstrated by the various studies

on cases of colon disorders. (Andreoli S.[22], Bottona-Parisi-Zilli [23]).

3 – MATERIALS AND METHODS

As reported by the producing company (Turval Laboratories srl of Udine) the strain utilized is *Kluyveromyces fragiilis marxianus B0399,* deposited at the BCCM-Belgium Coordinated Collections of Microrganism, Culture Collection Mycoteque de l'Université Catholique de Lovain (Belgium) with the trade-mark B0399, (Attachments A, A2)

3.3.4 – Product object of the study and dosage

The product in capsule form, which is normally found on the market in pharmacies and has been notarized by the Italian Ministry of Health, was utilized.

The active ingredient Kluyveromyces marxianus fragilis B0399 was administered at a dosage of one capsule a day ($20*10^6$ cfu per caps.), for a total daily dosage of $20*10^6$ CFU of *Kluyveromyces B0399*, for 14 days.

3.1 – Subjects enrolled

23 healthy subjects were enrolled in the same period and in different geographic areas, all over the age of 18 (ages 23 to 72, with average age of 49), 8 males and 15 females.

17 of the 23 subjects were from the areas of Udine and Trieste and were overseen by the team of Prof. Giorgio Mustacchi (Oncology Center for Health Services #1 – Triestina, *Università degli Studi di Trieste)* and were responsible for the recruiting and diagnostic phases.

The remaining 6 subjects were soldiers in the military at the barracks of Venzone (Udine), overseen by Dr. Teresa De Monte.

The evaluation of the state of health was made through medical consultation for the collection of all the necessary case-history data. In particular, the subjects were asked which, if any, drugs they had taken during the six months prior to testing; which, if any, recent illnesses they had had; which, if any, symptoms they experienced, and what was the typology of their routine, everyday diet.

Criteria for exclusion

Subjects were excluded who had: taken antibiotics or probiotics in the three months prior to testing, a severe chronic illness and/or other disorder of the colon which causes fragility of the mucosa, celiac disease, an intestinal occlusion and sub-occlusion, previous abdominal surgery with the exception of hernia and appendectomy, taken antipsychotic drugs in the previous three months or steroids in the preceding month, an intolerance to lactose or immunodeficiency, scare compliance.

During the period of treatment the assumption of drugs which could alter the motor function

or intestinal absorption were not allowed, including laxatives and anti-diarrhea, products capable of altering the intestinal bacterial flora (antibiotics and commercial products containing probiotcs.)

Examination of the fecal samples

The feces of the people selected were analyzed for the verification of the presence of *Candida albicans.*

Before the beginning of the treatment with *Kluyveromyces marxianus fragilis B0399,* samples were collected at time T0 (T with 0) to determine the initial state of the fecal composition.

To determine the evolution in time and the influence of the treatment with *Kluyveromyces marxianus fragilis B0399*, the analysis of the fecal samples was repeated after 14 days of administration (time T14).

Initial criteria of evaluation

To determine the presence of Candida albicans in the fecal sample, a screening of the colonies (of yeast) positive at the Sabouraud agar was conducted through the identification of the colonies of Candida (Medium Chromoalbicans agar) and through their examination at the microscope (contrast of phase 12,5 x 40).

Collection of samples:

The fecal material was collected from the individual subjects in sterile containers (BIO-BOX , container for feces samples, for.me.sa.) and delivered to the doctor in charge. All containers with fecal material were carefully preserved in refrigerators at a constant temperature of 4°C.

Homogenization of the samples for analyses

All original samples were prepared with the aim of obtaining the most homogeneous material possible to execute the analyses and the count of the yeasts quantitatively.
The laboratory prepared the suspension of known concentration beginning with the initial solid sample.
The dilution of the fecal material was done with sterile physiological solution.
The dates of the collection and arrival of the samples are indicated in Tab 1.

Tab 1 Summary chart of collection and arrival dates of samples, both at Time zero (T/0) and Time 14 (T/14)

Sample No.	Date of sample collection	Date of sample arrival	Sample #	Date of sample collection	Data of sample arrival
1 T/0	23/06/09	24/06/09	**1 T/14**	06/07/09	07/07/09
2 T/0	23/06/09	24/06/09	**2 T/14**	06/07/09	07/07/09
3 T/0	23/06/09	24/06/09	**3 T/14**	06/07/09	07/07/09
4 T/0	‘23/06/09	24/06/09	**4 T/14**	06/07/09	07/07/09
5 T/0	23/06/09	24/06/09	**5 T/14**	06/07/09	07/07/09
6 T/0	23/06/09	24/06/09	**6 T/14**	06/07/09	07/07/09
7 T/0	23/06/09	24/06/09	**7 T/14**	06/07/09	07/07/09
8 T/0	23/06/09	24/06/09	**8 T/14**	06/07/09	07/07/09
9 T/0	23/06/09	24/06/09	**9 T/14**	06/07/09	07/07/09
10 T/0	23/06/09	24/06/09	**10 T/14**	06/07/09	07/07/09
11 T/0	23/06/09	24/06/09	**11 T/14**	06/07/09	07/07/09
12 T/0	23/06/09	24/06/09	**12/ T/14**	06/07/09	07/07/09
13 T/0	23/06/09	24/06/09	**13 T/14**	06/07/09	07/07/09
14 T/0	23/06/09	24/06/09	**14 T/14**	06/07/09	07/07/09
15 T/0	23/06/09	24/06/09	**15 T/14**	06/07/09	07/07/09
18 T/0	23/06/09	24/06/09	**18 T/14**	**S. not collected**	**S. not collected**
19 T/0	23/06/09	24/06/09	**19 T/14**	06/07/09	07/07/09
2 VT/0	06/07/09	07/07/09	**2VT/14**	20/07/09	21/07/09
7VT/0	06/07/09	07/07/09	**7VT/14**	20/07/09	21/07/09
8VT/0	06/07/09	07/07/09	**8VT/14**	20/07/09	21/07/09
10VT/0	06/07/09	07/07/09	**10VT/14**	20/07/09	21/07/09
13VT/0	06/07/09	07/07/09	**13VT/14**	20/07/09	21/07/09
14VT/0	06/07/09	07/07/09	**14VT/14**	20/07/09	21/07/09

The principal characteristics of the subjects and the dates of collection are indicated in Tab2.

Tab 2 : Characteristics of the subjects and collection times

Subjects	Sex	Zone	Age	Collection T/0		Prelievo T/14	
				# on label	Date	# on label	date
A. S.	F	Trieste	39	1 T/0	23.06.09	1 T/14	06.07.09
G. D.	M	Trieste	50	2 T/0	23.06.09	2 T/14	06.07.09
R. C.	F	Trieste	53	3 T/0	23.06.09	3 T/14	06.07.09
F. S.	F	Trieste	49	4 T/0	23.06.09	4 T/14	06.07.09
D. A.	F	Udine	38	5 T/0	23.06.09	5 T/14	06.07.09
D. A.	F	Trieste	38	6 T/0	23.06.09	6 T/14	06.07.09
S. L.	F	Trieste	57	7 T/0	23.06.09	7 T/14	06.07.09
M. N.	F	Trieste	66	8 T/0	23.06.09	8 T/14	06.07.09
C. M.	F	Udine	59	9 T/0	23.06.09	9 T/14	06.07.09
M. M.	M	Trieste	72	10 T/0	23.06.09	10 T/14	06.07.09
G. G.	F	Trieste	64	11 T/0	23.06.09	11 T/14	06.07.09
B. G.	F	Trieste	70	12 T/0	23.06.09	12 T/14	06.07.09
C. L.	F	Trieste	49	13 T/0	23.06.09	13 T/14	06.07.09
B. C.	F	Trieste	55	14 T/0	23.06.09	14 T/14	06.07.09
M. L.	F	Trieste	44	15 T/0	23.06.09	15 T/14	06.07.09
C. M.	F	Trieste	35	18 T/0	23.06.09	18 T/14	06.07.09
C. A.	M	Trieste	57	19 T/0	23.06.09	19 T/14	06.07.09
V.Z.	M	Venzone	44	2 VT/0	06.07.09	2VT/14	20.07.09
F.D.	M	Venzone	30	7VT/0	06.07.09	7VT/14	20.07.09
G.D.	M	Venzone	29	8VT/0	06.07.09	8VT/14	20.07.09
C.D.	F	Venzone	23	10VT/0	06.07.09	10VT/14	20.07.09
A.P.	M	Venzone	31	13VT/0	06.07.09	13VT/14	20.07.09
G.S.	M	Venzone	39	14VT/0	06.07.09	14VT/14	20.07.09

Analyses conducted on samples obtained at time T 0

Count of total yeasts at candida identification.

1) Presence and quantification of microorganisms (yeasts) positive at Sabouraud agar.
2. To evaluate the presence of candida, a screening of the colonies (of yeast) positive at Sabouraud agar was conducted through the identification of the colonies of Candida (Medium Chromoalbicans agar) and through their examination at the microscope (contrast of phase 12,5 x 40).

Analyses conducted on samples obtained at time T 14

Count of total yeasts at *candida* identification.

1) Presence and quantification of microorganisms (yeasts) positive at Sabouraud agar.
2) To evaluate the presence of candida, a screening of the colonies (of yeast) positive at Sabouraud agar was conducted (like above) through the identification of the colonies of Candida (Medium Chromoalbicans agar) and an examination on all the plates were

conducted at the microscope (contrast of phase 12,5 x 40).

4 – STATISTICAL ANALYSIS:

For the analysis of the information obtained from the answers in the pre-treatment and post-treatment questionnaires, the Chi-square test of Yates and the Chi-square test of Pearson (with Vassar Stats: Statistical Computation Web Site) was selected, with the aim of verifying if their differences were purely random or not. If the difference is not random, then it is considered "statistically significant."

Chi-square: test Initially, the existing difference between the two series of data to compare is considered "hypothesis zero". Hypothesis zero simply asserts that the difference observed – whatever the entity- is purely random. This hypothesis can be either accepted or rejected on the basis of the result of the statistical test.
If on the basis of this hypothesis the calculated value of χ^2 is greater than a certain critical value, we will have to conclude that the frequencies observed significantly differ from the frequencies anticipated and we will have to refute HO at the corresponding level of significance. Otherwise, we will have to accept it, or at least not refute it. This procedure is called chi-square test of the hypothesis.

5 - RESULTS:

The results are evaluated in 22/23 of enrolled subjects, with the withdrawal of one patient on itinerary.

Tab 5.1: Results obtained at time T0.

Data of sample collection	Sample	Total yeasts CFU/g of feces	Candida albicans
23/06/09	1 T/O	$2X10^3$cfu/g	absent
23/06/09	2 T/O	Absent	absent
23/06/09	3 T/O	$4X10^3$cfu/g	absent
23/06/09	4 T/O	$1X10^3$cfu/g	present
23/06/09	5 T/O	$6X10^3$ cfu/g	present
23/06/09	6 T/O	80 cfu/g	present
23/06/09	7 T/O	Absent	absent
23/06/09	8 T/O	$6,5X10^3$cfu/g	absent
23/06/09	9 T/O	$7X10^3$cfu/g	absent
23/06/09	10 T/O	20 cfu/g	absent
23/06/09	11 T/O	$8X10^2$cfu/g	present
23/06/09	12 T/O	$2X10^5$cfu/g	present
23/06/09	13 T/O	$4X10^2$cfu/g	present
23/06/09	14 T/O	$1X10^4$cfu/g	present
23/06/09	15 T/O	$1,6X10^2$cfu/g	present
23/06/09	18 T/O	80 cfu/g	absent
23/06/09	19 T/O	absent	absent
06/07/09	2 V T/O	$2,4\ X10^4$cfu/g	absent
06/07/09	7 V T/O	absent	absent
06/07/09	8 V T/O	$2,4X10^4$cfu/g	absent
06/07/09	10 V T/O	$2,4X10^4$cfu/g	present
06/07/09	13 V T/O	$2,4X10^2$cfu/g	absent
06/07/09	14 V T/O	$1,7X10^4$cfu/g	present

Tab 5.2: Results obtained at time T14

Date of sample collection	Sample	Lactic yeasts (kluyveromyces B0399) CFU/g di feci	Candida albicans
06/07/09	1 T/14	$7X10^{4}$cfu/g	absent
06/07/09	2 T/14	$5X10^{4}$cfu/g	absent
06/07/09	3 T/14	$5,7X10^{4}$cfu/g	absent
06/07/09	4 T/14	$7X10^{4}$cfu/g	absent
06/07/09	5 T/14	$2,5X10^{4}$cfu/g	absent
06/07/09	6 T/14	$2,5X10^{4}$cfu/g	absent
06/07/09	7 T/14	$1,8X10^{5}$cfu/g	absent
06/07/09	8 T/14	$3X10^{4}$cfu/g	absent
06/07/09	9 T/14	Non determinabile	absent
06/07/09	10 T/14	$3,2X10^{5}$cfu/g	absent
06/07/09	11 T/14	$2,5X10^{4}$cfu/g	absent
06/07/09	12 T/14	$4,4X10^{5}$cfu/g	present
06/07/09	13 T/14	$1,7X10^{3}$cfu/g	present
06/07/09	14 T/14	$2,5X10^{4}$cfu/g	absent
06/07/09	15 T/14	$3X10^{4}$cfu/g	absent
06/07/09	18 T/O	withdrawn	withdrawn
06/07/09	19 T/14	$1,5X10^{5}$cfu/g	absent
20/07/09	2 V T/14	$2,8X10^{6}$cfu/g	absent
20/07/09	7 V T/14	$4,6X10^{3}$cfu/g	absent
20/07/09	8 V T/14	$2,2X10^{6}$cfu/g	absent
20/07/09	10 V T/14	$4X10^{6}$cfu/g	absent
20/07/09	13 V T/14	$2,4X10^{6}$cfu/g	absent
20/07/09	14 V T/14	$3,9X10^{5}$cfu/g	present

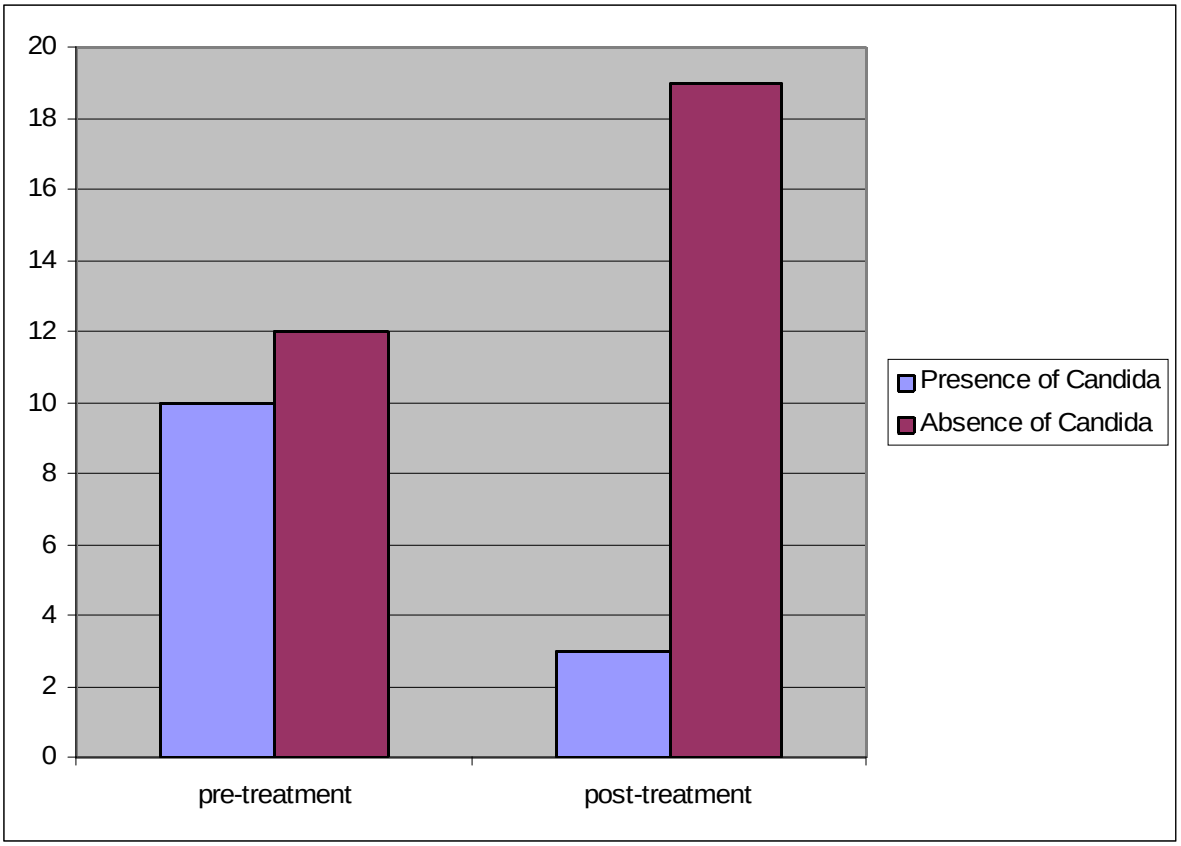
Tab 5.1: frequencies of presence and absence of Candida albicans before and after treatment with Kluyveromyces marxianus fragilis.

	Pre-administration	Post-administration
Presenza di candida	10	3
Assenza di candida	12	19

Tab 5.2: risults of the statistical analysis of the data shown in Tab 2.

	Chi-square	
Phi	Yates	Pearson
-0,35	3,93	5,35
P	0,047432	0,020722

Fig 5.1: Representation of the cases of presence or absence of Candida albicans before and after treatment with Kluyveromyces marxianus fragilis BO399.



The statistical analysis shows that there is a significant difference (with $p < 0,05$) between the presence of Candida albicans found in the pre-treatment analysis and in those post-treatment (Chi-square Yates = 3,93 con p = 0,04743; Chi-square Pearson = 5,35 con p = 0,020722).

Profile of toxicity

None of the subjects treated complained of any side effects.

7 - CONCLUSIONS:

The analysis in the fecal samples of *Candida albicans* shows a reduction of the presence of the fungus after treatment with *Kluyveromyces marxianus fragilis BO399.*
The statistical analysis shows that there is a significant difference (with p< 0,05) between the presence of *Candida albicans* found in the pre-treatment analysis and in those post-treatment (Chi-square Yates = 3,93 with p = 0,04743; Chi-square Pearson = 5,35 with p = 0,020722). It was therefore demonstrated that *Kluyveromyces marxianus B0399* has the capacity of influencing the development of colonies of *C.albicans* at the intestinal level.
We know that the intestine constitutes the natural receptacle of this type of fungus and therefore the importance of this observation *in vivo* is evident.
Since this probiotic may also be taken with antibiotics (thanks to its proven resistance) the phenomenon of re-colonization of *Candida albicans* (spore-forming) after the treatment with fungicides and antibiotics would be inhibited since an impoverishment of the intestinal flora would be avoided.

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