Original scientific paper
UDC 615.356:615.451

EVALUATION OF MICROBIAL PRESENCE IN LIQUID VITAMIN SUPPLEMENTS

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Abstract

Vitamin supplements are more and more present in our nowadays market. Among a lot of products, the liquid formulations are preferred due to an easy dosage and utilization. They are used for children because of the ease of ingestion. They are more effective for people whose gastrointestinal system have been compromised and cannot effectively use compressed material. Consumers are well informed about their benefits and are also very sensitive toward their safety, quality, characteristics, conditions of storage, shelf-life and their purity. They want to be informed if there are contaminations which may damage the effectiveness or that can possibly be transmitted to the human beings. Microbial contamination presence is possible as it may be related with the ingredients of these liquid forms, overall related with the presence of carbohydrates and other substances that stimulate the growth of microorganisms. Types of ingredients as well as physicochemical parameters as pH are important factors that induce or prevent the development or growth of microorganisms.

The research work was done having as target the selection, isolation and identification of the most common microorganisms present in 20 liquid food supplements. The presence of special strains of yeasts and molds was evidenced as depended by carbon sources present in the analyzed liquid forms. Microbiological evaluation was carried not only to determine the microbial purity of products but to perform also taxonomic studies of the selected strains as mirror effect of some strains, sporulation forms, presence of pseudomicelium etc. The methods used included classical methods of isolation and identification and microbiological techniques to study the morphological characteristics of the isolated strains.

There were identified some important strains as *Rhodotorula* spp., *Aureobazidium* spp., *Aspergillus* and *Penicillium* genera, and others.

As a conclusion the observed cases of contamination were related with the presence of specific strains as osmophiles, and other contaminant strains developed in specific liquid phases. The microbial contamination was related also with the storage conditions of the samples and the way they were used.

***Key words**: Food supplements, Microorganisms, Mirror effect, Pseudomicelium, Sporulation, Rhodotorula spp.*

1. Introduction

Microbiological study of drugs is a very interesting research topic. It includes the discovery of usual strains, osmophyles and others, also contaminant strains derived by the conditions of pharmaceutical preservation. Microbiologists are strongly focused in food microbiology and industrial microbiology because of enormous sources of food contaminations. Recent innovative techniques, successfully applied to packaging, distribution and preservation of drugs have reduced their potential microbiological contamination. So the pharmaceutical world for nowadays microbiologists is protected by microbial contaminations, mostly due to the contribution of specific active substances of drugs to inhibit the destructive action of microorganisms. In spite of a full protection performed by the active ingredients, additives, packaging and also environmental measures, there are microbiological contaminants profiting by the drug structure and the conditions of use. These change the microbial purity of some pharmaceutical products, causing a microbial contamination often problematic for the drug itself and their consumers.

Some pharmaceuticals are constantly subject to microbiological contamination. This is related with the structure of the final product, the drug nature and the nature of the microorganisms, a poor circulation in the market of some drugs, the applied manufacturing practices, poor employee hygiene, etc. Hosted microorganisms can change the structure, the membrane

color of solid forms, color and limpidity of liquid forms can affect the physicochemical parameters, reinforce the damages with time, during the preservation period. Some observed off flavors, discolorations, changes of aromas in daily practices and research work is reported as having microbial origin ([5], [8], [10], [11], and [16]).

Reducing of microbial contamination of pharmaceuticals is related with the measures of Companies to produce and preserve them in the appropriate conditions, with the responsibility of the pharmacies to preserve them well, with a good information and education of consumers for proper handling, good conservation and use.

The object of this research work was not to focus and achieve results related with manufacturing practices of Pharmaceutical Companies from which samples were originated. The aim was to observe and study the presence of microorganisms in liquid pharmaceuticals having as target isolation, identification and preservation of selected strains in order to use them for further experiments.

Twenty food supplements were microbiologically analyzed. Liquid pharmaceuticals were selected because of the specific behavior and development of microorganisms in liquid forms rich with different C-sources, vitamins and mineral salts. The selection of liquid substrates was based on their own ingredients and different uses. Environmental bacteria as *Bacilli* and *Cocci* were isolated and identified ([1], [5], [7] and [8]). Taxonomic studies were performed to identify some important yeasts and molds classified as normal microflora and problematic strains also. *Basidomycetes* with specific characteristics as *Aureobasidium* species, called as polyextremotolerants because of their adaptation skills in hipper concentration solutions were isolated. *Sporobolomycess* spp. and others were taxonomically studied. A database was designed related with the most important microorganisms hosted in liquid pharmaceuticals during their use and conservation.

2. Materials and Methods

The study was carried out in the Laboratory of Industrial Microbiology in the Faculty of Natural Sciences in Tirana University. Some pharmaceuticals in liquid forms overall vitamin supplements were the objects of the study. Syrups were selected because of their ingredients, sugars and additives with an important effect in the microbial growth. Previous experiments in the same research field suggested us to be focused in vitamin supplements because of their wide use and microbial affinity towards their ingredients. In some cases, there is not always a proper handling of them when they are used periodically in homes for children and adults. A general survey of microbial contamination of drugs was realized in a former research work in order to determine the methodology of experiments. Based on the obtained experience, twenty samples were selected by the lots in sale in pharmacies and by usual consumers in their homes. Samples not handled properly and/or preserved in homes after the expiration date, were objects of the study, in order to make possible the comparison of the results. The selected samples were in general representative products well-known by consumers and commonly used by them. All information taken by labeling as manufacturing and expiry dates, ingredients, indications and the mode of use were taken into consideration and registered in order to be used in the discussion of results. Samples and indications are detailed in Table 1.

Table 1. The samples selected for the microbiological survey

Nr.	Sample	Expiry date	C-source for microbial development
1	D-1	January 2013*	Sorbitol
2	D-2	March 2014*	Sorbitol
3	FL-1	April 2014*	Lactose
4	FL-2	April 2015	Lactose
5	RIN-1	August 2012*	Lactose
6	P-1	January 2012*	Sucrose, Vit. A, B-group, Vit. C, Vit. D
7	P-2	June 2012*	Sucrose, Vit. A, B-group, Vit. C, Vit. D
8	D-3	March 2016	Sorbitol
9	D-4	March 2016	Sorbitol
10	AL-1	December 2015	Mg-gluconate
11	AL-2	December 2015	Mg-gluconate
12	AS-G	June 2016	Vit. C
13	Hydra-M	June 2016	Sucrose, sodium citrate, potassium phosphate, sucralose, etc.
14	TR	June 2016	$NaHCO_3$, NaCl, KCl
15	PH-S	March 2016	Glucose, maltodextrin, Na, K, Cl, etc
16	MV-S1	June 2016	Folic acid, pantotenic acid, Vit. A, Vit. K, Vit. C, Vit. D, starch , sorbitol, maltodextrin, etc
17	MV-S2	June 2014	Folic acid, pantotenic acid, Vit. A, Vit. K, Vit. C, Vit. D, starch, sorbitol, maltodextrin, etc
18	VitC-S	June 2016	Vit. C
19	AL-1plus	December 2015	Mg-gluconate
20	AL-2plus	December 2015	Mg-gluconate

*All the samples, resulting now after the expiry date, were analyzed before and after expiration.

Standard culture media were selected to isolate, purify and identify the strains. Plate Count Agar - PCA, Potato Dextrose Agar - PDA and Sabouroad's Dextrose Agar - SDA were the most important selected media for microbiological tests. Serial dilutions in sterile water and sterile saline in some cases were prepared in order to obtain microbial solutions from selected samples. Final products and respective dilutions were analyzed at first in parallel in order to have an idea about the total number of microorganisms. In cases with a poor microbial growth the samples were treated directly, in other cases serial dilutions were successfully applied. Taxonomic studies, morphological and physiological tests were performed for each sample based on Barnett, Frasheri, Lodder & Kreger van Rij and Pelczar ([4], [7], [9], and [13]). Pseudomicelium forming by some isolated yeasts was studied in PDA culture media in order to use in the identification steps. Morphological tests of sporulation were also performed according to Barnett and Lodder ([4], [9]). Vitamin supplements and liquid pharmaceuticals were sampled from the bottles in sterile environments, respecting the rules of sampling. 1mL aliquots were pureed in Petri dishes covering in the meantime with sterile selected media in order to determine all microorganisms present.

Other experiments were performed inoculating 0.1 and 0.5 mL of aliquots in solidified sterile media in order to determine aerobic strains. Petri dishes were incubated in 28 ^{0}C. The enumeration of colonies was made using a colony counter. Tests were performed twice and the average results are presented ([4], [7], and [9]).

3. Results and Discussion

It was taken into consideration during the experimental work that by the regulatory systems, in non sterile pharmaceuticals as tablets, oral liquids, creams and others there are allowed limited microbial contaminations related with production and transfer conditions. So the experiments were carried out evaluating the possible contaminations related with all factors mentioned above, responsible to increase microbiological contamination during conservation and use.

The evaluation of bacterial charge

The contamination was not observed in all the samples. The total number of bacteria is detailed in Table 2 and in the chart of Figure 1.

Table 2. Determination of bacterial charge in selected samples

Nr.	Sample	Total charge in PCA (CFU/mL) *before the expiry date*	Total charge in PCA (CFU/mL) *after the expiry date*
1	D-1	3 (bacterial colonies)	75 bacterial colonies (20 pigmented in orange)
2	D-2	5 (bacterial colonies)	20 bacterial colonies pigmented in yellow and orange
3	FL-1	a -180 rod-shaped Gr+ bacilli b* - 236 in total 200 rod shaped bacilli and 36 bacterial colonies pigmented in orange	-
4	FL-2	a - 7 bacilli colonies b* - 5 bacilli colonies	-
5	RIN-1	0	0
6	P-1	4 bacterial colonies	41 different bacterial colonies
7	P-2	1 bacterial colony	12 bacterial colonies (Gr+ cocci and Gr+ bacilli)
8	D-3	0	-
9	D-4	0	-
10	AL-1	0	-
11	AL-2	0	-
12	AS-G	24 bacterial colonies	-
13	Hydra-M	0	-
14	TR	92 bacterial colonies	-
15	PH-S	16 bacterial colonies	-
16	MV-S1	21 bacterial colonies	-
17	MV-S2	85 bacterial colonies	-
18	VitC-S	0	-
19	AL-1plus	0	-
20	AL-2plus	2 Gr+ bacilli	-

*b - Second microbial contamination after 1 year for the samples tested since 2012

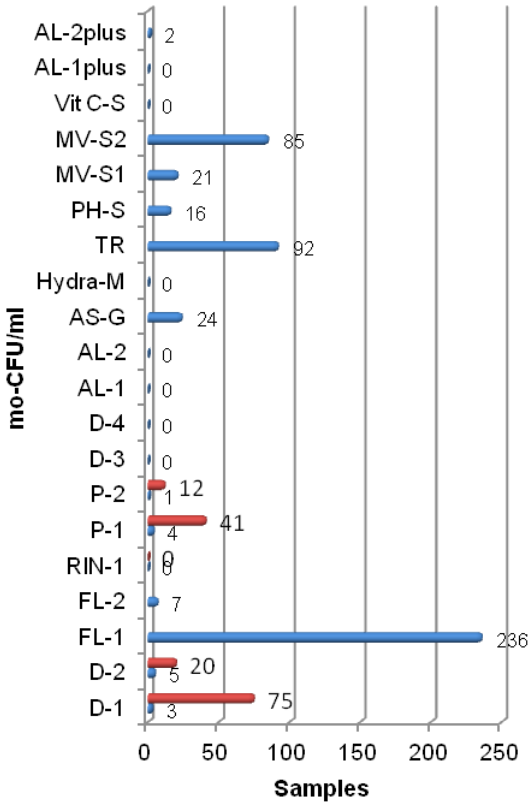


Figure 1. Evaluation of total bacterial charge (microorganisms-CFU/ml) in sterile PCA culture media
(*blue - samples before expiration date, red - samples after expiration date*)

The obtained results show that a small total bacterial charge was observed in all cases before expiry date and a clear increase after it. Antimicrobials added in liquid pharmaceuticals conserve well the selected samples. The cases of contaminations as FL-1, MV-S2 and others are related with favorable conditions of bacterial growth (C-source as polysaccharides, mineral salts some vitamins, etc.). Serial analysis of FL-1 offer the same morphological forms potentially induced by the active ingredient. The cases of a small bacterial contamination are potentially related with strain resistance in liquids with a high osmotic pressure and in the potential inhibition effect of vitamin C. The most likely bacterial types of traceability to air and surrounding environments are discovered as bacterial populations of the analyzed pharmaceuticals - *Bacillus* spp. *and Micrococcus*. Sometimes it was observed a survival of *Staphylococcus* spp. Some bacterial strains mentioned above as *Staphylococcus* spp. are microorganisms of sputum culture, so an careless use can transmit them from sputum to liquid pharmaceutical forms, finding there the best conditions to growth. The isolation of bacterial normal microflora shows a contamination derived from inappropriate conditions of use (careless periodical use, not hermetically closed during the long time conservation and use, preservation in warm temperatures in inappropriate places, etc.). Surrounding environments and surrounding air are sources of the above contamination as well ([2], [8], [11], and [12]).

Referring to the determination of total bacterial charge the percentages of them for each sample are detailed in the chart of Figure 2. It is obvious that 48% of discovered microorganisms are hosts of FL-1 and 17% of MV-S2. In all cases there were identified only Gr+ bacteria. Some *Leuconostoc* strains forming polysaccharide capsules were isolated from D-1. *Lactobacilluses* were isolated from RIN-1. *Bacillus megatherium* was isolated, purified and identified from FL-1 and FL-2. Similar strains were hosted in liquid forms with the same active ingredient, for example pigmented bacterial strains were characteristics for D-1 and D-2, rod shaped bacilli were present in FL-1 and FL-2. MV-S1 and MV-S2 had bacteria with the same morphological characteristics ([6], [7], [13]).

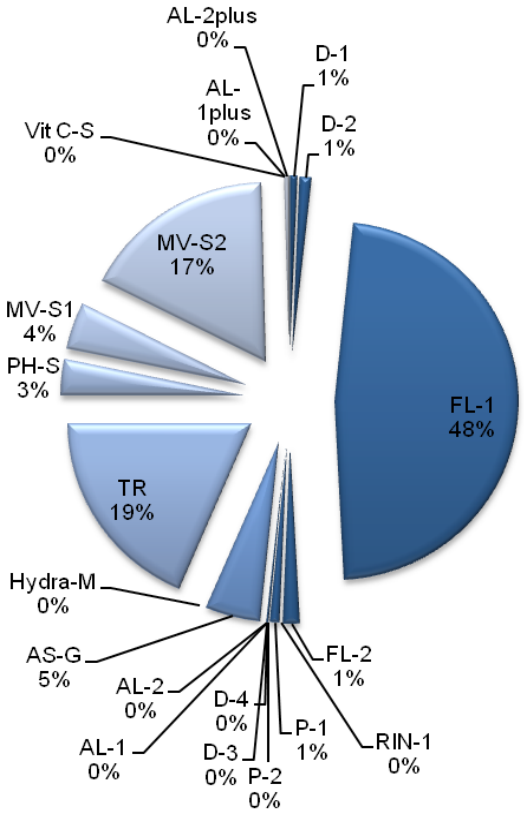


Figure 2. Percentage of total bacterial charge in all the selected samples

The evaluation of yeasts and molds

Microbiological analysis to evaluate the presence of yeasts and molds was done using PDA as standard culture media and PDA and SDA for purification processes. The following results of the observation of inoculated samples after 7 day incubation in 28 ^{0}C were given

for all the selected samples. P-1 and P-2 samples were free from yeast and molds. This was verified in two year analysis observing only a mold contamination after the expiry date. The high percentage of sucrose in above samples is an inhibitor for their development. D-1 and D-2 were sources of *Penicillium crysogenum*, *Aspergillus niger*, *Rhisopus nigricans*, *Aspergillus flavus*, *Penicillium versicolor* and some other identified molds. *Penicillium species* were normal contaminations but *Aspergillus* strains were classified as problematic because of toxin production. MV-S1 and MV-S2 were sources of *Sporobolomyces* spp. and *Rhodotorula glutinis*, their presence potentially related with the vitamin A concentrations in both samples ([3], [4], [7], and [9]). The inoculation of molds is given in Figure 3. A *Geotricum candidum* was observed in D-2 in all serial microbial tests performed in a two year research work; microscopic image of it is in Figure 4.

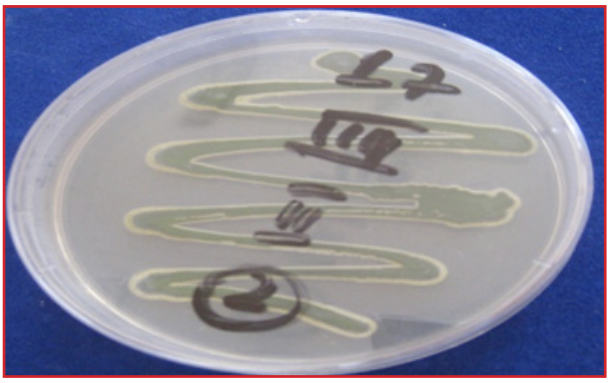
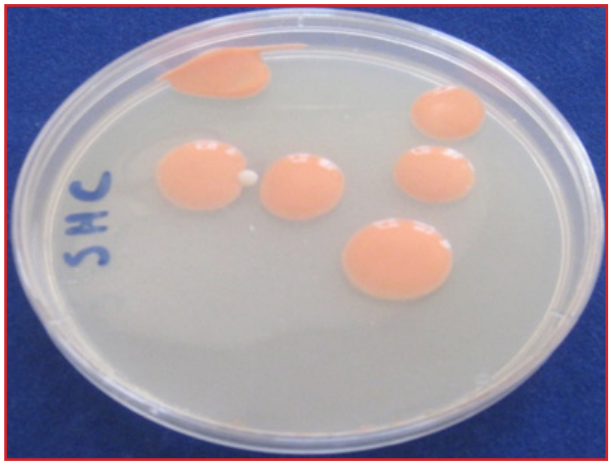


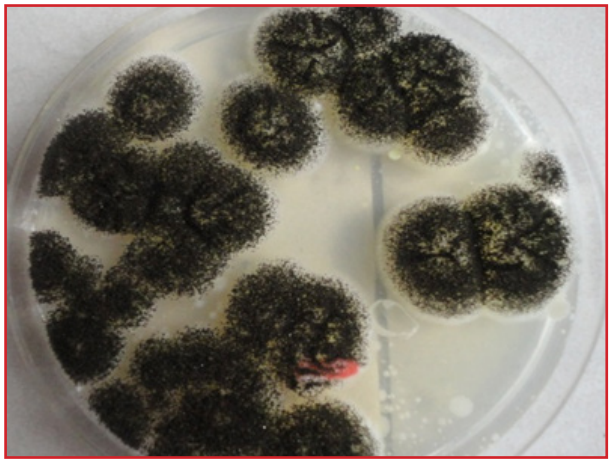
Figure 3. Isolated *Aureobasidium* spp. *during* taxonomic tests (before changing color to black)

Figure 4. *Geotrichum* spp. originated from D-2 in an identification process

A typical mirror effect was observed during isolation and purification of both two strains of *Sporobolomyces* spp. and *Rhodotorula* spp., identified with taxonomic tests as *Rhodotorula glutinis*. The image in *Figure 5* was taken during the purification of *Rhodotorula* spp., separating it from *Saccharomyces* spp. and *Aspergillus niger* [3], [4], and [9].



(a)



(b)

Figure 5. (a, b) *Rhodotorula* spp. in serial purification processes from *Saccharomyces* spp. and *Aspergillus niger* *(identified from D1)*

"Mirror yeast" of *Sporobolomyces* spp. during identification is shown in Figure 6.

Figure 6. "Mirror yeast" of *Sporobolomyces* spp. identified from MV-S1

Predominant isolated molds were identified after a long purification process ([3], [4], [9], [14], [15]). Images of Figure 7 are those of serial inoculations to obtain pure cultures before identification.

Based on all the experimental work done for mold isolation and identification, their presence in liquid phases was limited in some specific strains mentioned above, verified during all the microbiological tests in a two year experimental work. Some xerofilic molds were guests of the analyzed concentrated liquid phases. Liquid pharmaceuticals and/or medication syrups theoretically are protected by antimicrobials permitted by law in the production and conservation period, but household molds often use them as appropriate substrates to be developed and various practices show a typical development of them in periodically used syrups. Hazard molds to human health as mycotoxins producers were identified in some liquids that resulted free of them and free of other microorganisms just after buying them. Microbiological analysis showed that a prolonged conservation in steamy or warm areas, humid environments and prolonged use, although before the expiry date, may be harmful. More sensitive are children because of symptoms caused by molds, as watery eyes, tiredness, sinus problems, nasal blockage and others.

4. Conclusions

- Microbiological contamination was observed during microbiological control of pharmaceuticals in liquid phases.

- Predominant bacteria were Gr+ *Bacilli,* all the samples were free from Gr- bacterial species.

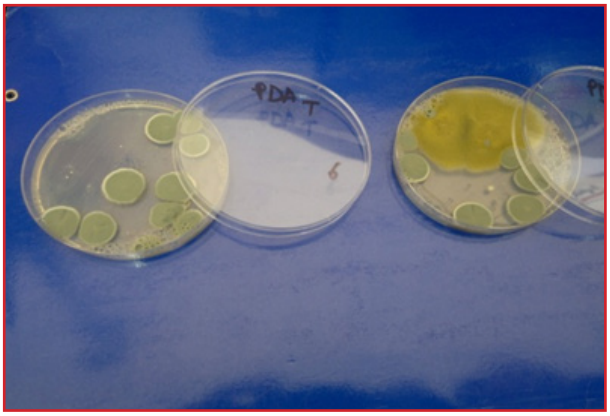
- *Bacillus megatherium* was identified as a normal contaminant.

- All the samples rich with vitamin D were sources of the development of *Sporobolomyces* spp., the so-called "mirror yeasts".

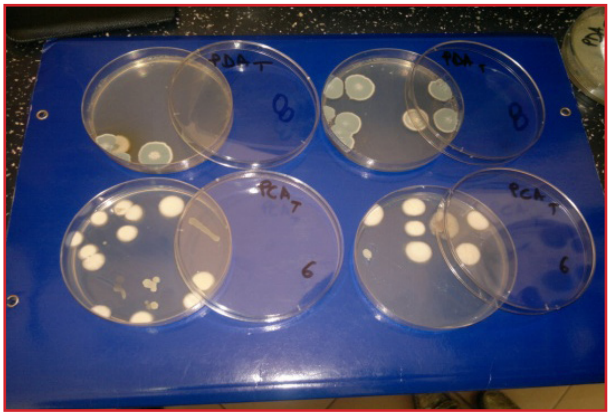
- Identification was successfully performed for *Rhodotorula glutinis* from the same liquid sources rich with vitamin A and others.

- *Aureobazidum pullulans* was identified from liquid phases rich with ingredients originating from plants.

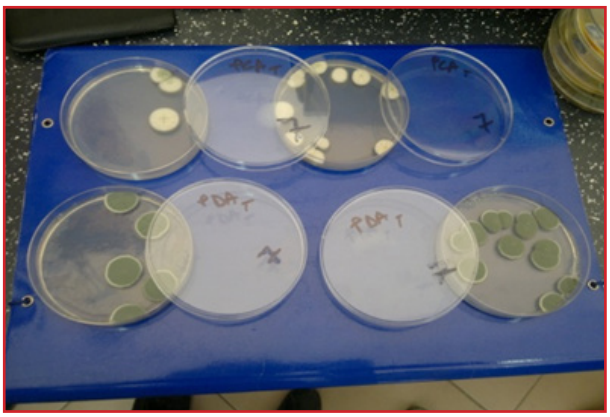
- Taxonomic studies were performed to identify mold colonies. The most widely spread were *Penicillium* species as *Penicillium crysogenum*, *Penicillium versicolor* considered as environmental contaminants derived from conservation places. *Aspergillus* species as *Aspergillus niger* and *Aspergillus flavus* were isolated and identified from liquid phases with polysaccharides



(a)



(b)



(c)

d)

Figure 7. Serial inoculations before the identification of mold species

as C sources. They were considered as toxin producer contaminants. Their presence, derived from environments or from drugs by themselves was observed in previous studies, was confirmed by the work here presented and will be an important part of future research work.

- Identified microorganisms are recently in conservation, in order to examine the potential of some antibacterial substances to reduce their growth.

5. References

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