ANTIFUNGAL PROPERTIES OF ANTIBIOTIC SUBSTANCES[1]

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INTRODUCTORY

Fungi, especially the filamentous types, have become definitely established as the most important group of organisms that is capable of producing antibacterial substances under proper conditions of culture. No less significant, even if less well known, is the fact that many microorganisms, including fungi on the one hand, and bacteria and actinomycetes on the other, are capable of producing agents which possess antifungal properties. Some of the antibiotic substances which are active against bacteria are also characterized by fungistatic and fungicidal activities; others, however, may be active upon bacteria but not upon fungi.

These facts have long been known to the plant pathologists, who were impressed by the ability of fungi to live in close association with other microorganisms in natural substrates, especially in the soil. Many of the organisms antagonistic to fungi were known to have a specific capacity of inhibiting the growth of various plant-pathogenic fungi and even of causing their destruction. This effect is selective and varies for different fungi, some being affected greatly and others slightly or not at all. The practical utilization of this phenomenon for the control of plant diseases has, therefore, been suggested (Chudiakov, 1935; Novogrudsky, 1936) and has actually been utilized on a limited scale. However, the ability of antagonistic microorganisms to attack fungi causing animal diseases (Chambers and Weidman, 1928) and the utilization of the antibiotic substances produced by these organisms for the purpose of combating such pathogens have received only cursory consideration (Waksman, 1945).

The favorable effects that resulted from the practical utilization of certain antibiotic substances, such as tyrothricin, penicillin, and streptomycin, for the control of various human and animal diseases caused by bacteria suggested the possibility that some of these agents may also be utilized for the control of human and animal diseases caused by fungi.

No attempt will be made to review here the very extensive literature on the antagonistic effects of various microorganisms upon fungi (Waksman, 1941). Studies of these reactions have resulted in the isolation of crystalline gliotoxin (Weindling, 1934) and in the demonstration that various bacteria produce antifungal agents (Chudiakov, 1935; Cordon and Haenseler, 1939).

The effect of antibiotic substances upon fungi resulted in a modification of the morphology of the organisms, a change in some physiological mechanism such

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as pigment production or growth, or in a complete destruction of the organism. Fungi treated with certain yeasts, for example, produced thick gnarled mycelia, without any conidia or pigment (Cook *et al.*, 1941). The mechanism of disintegration of the hyphae of the plant-pathogenic fungus *Rhizoctonia* by the antagonistic fungus *Trichoderma*, as well as by its specific agent gliotoxin, was studied in detail by Weindling (1934). The hyphae of the *Rhizoctonia* were killed in less than 10 hours, as was shown by a loss of the homogeneous appearance of the protoplasm and the vacuolate structure of the hyphae.

Stokes *et al.* (1942) reported that the antibacterial agents, pyocyanine, hemipyocyanine, and tyrothricin, also possess marked fungistatic properties. The fungi affected included the pathogenic forms *Achorion schoenleinii*, *Trichophyton gypseum*, *Microsporum gypseum*, and *Candida albicans*, the first of these being the most sensitive, and the last the most resistant. Tyrothricin inhibited the growth of all four fungi in dilutions of 1:5,000 to 1:20,000. Pyocyanine was the least active of the three compounds, and hemipyocyanine the most active. Among the other antibiotic agents found to inhibit the growth of fungi, it is sufficient to mention actinomycin and clavacin. The ability of actinomycin to affect the growth of *Ceratostomella ulmi* could be partly inhibited or neutralized by the addition of pyridoxine, thus pointing to the possible mechanism of the fungistatic action of actinomycin (Waksman and Bugie, 1943). The marked fungistatic properties of clavacin have also been indicated (Waksman, Horning, and Spencer, 1942, 1943).

In a study of the antifungal properties of streptothricin and streptomycin, two closely related antibiotic substances produced by actinomycetes, Robinson, Smith, and Graessle (1944) demonstrated that whereas streptothricin has considerable activity against both pathogenic and saprophytic fungi, streptomycin has very little effect against these organisms.

A comparison has been made (Geiger and Conn, 1945) of the bacteriostatic and fungistatic properties of certain antibiotic substances, notably penicillic acid and clavacin, that possess chemical properties similar to ketones. The first of these had only slight antifungal activity, but the second was very active. Certain synthetic unsaturated ketones were found to have even greater antifungal properties than clavacin.

EXPERIMENTAL

Different fungi are known to vary greatly in their sensitivity to the same antibiotic substance; it has also been established that there is a marked variation in the activity of different substances upon the same organism. The purpose of this investigation was to compare the action of various antibiotic substances upon several fungi, in order to justify certain broad generalizations. In an attempt to evaluate an antibiotic substance from the point of view of its possible use as a chemotherapeutic agent, special consideration is also given to the toxicity of the substance to animals. A more toxic substance, even if it is characterized by greater antifungal activities, may offer less promise as a chemotherapeutic agent than another substance which is less toxic and also less active.

Methods. Two methods were used for the evaluation of the fungistatic effect and the antifungal spectrum of an antibiotic substance: (1) the agar streak or agar dilution method, and (2) the agar diffusion or cup method.

In the agar streak method the antibiotic substance is added, in different concentrations, to the agar medium placed in ordinary sterile petri dishes. Several, usually 2 to 5, test organisms are streaked on each plate. The plates are incubated, at 28 C, or 37 C, for 24 to 48 hours and records made. The

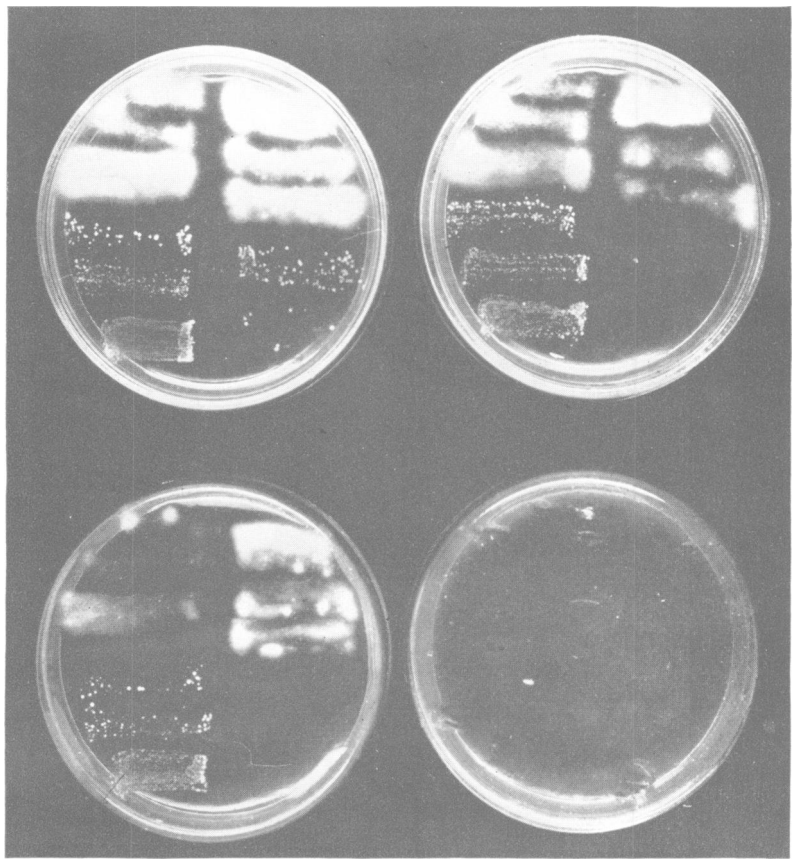

FIG. 1. SENSITIVITY OF DIFFERENT FUNGI TO AN ANTIBIOTIC SUBSTANCE, AS ILLUSTRATED BY THE AGAR STREAK METHOD

amount of the substances required to inhibit completely the growth of each test organism is taken as the end point; the activity of the preparation is calculated on a unit per milliliter basis for one gram of the material. The ratio of the volume of agar to the amount of the substance required to inhibit a given test organism gives the number of antifungal units of a substance. Figure 1 shows that fungi vary in their sensitivity to the same substance, since they are inhibited by different concentrations of the substance.

In the cup method the agar medium is inoculated with a single test organism,

and different dilutions of the substance are placed in several cups, partly inserted into the agar. The zone of inhibition of growth of the test fungus is taken as a measure of the activity of the substance as compared with that of a standard preparation. Figure 2 shows that there is a direct proportion between the concentration of the active material and the zone of the diffusion. Attention may be directed here to the two zones in each ring. The outer zone is the one that

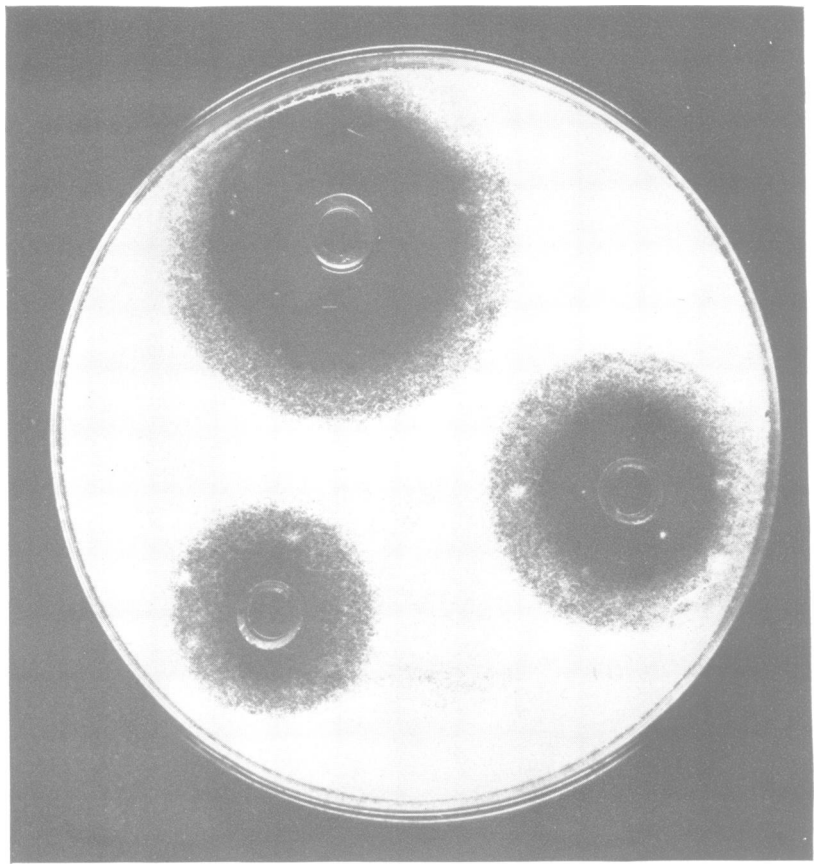

FIG. 2. CUP METHOD FOR TESTING THE FUNGISTATIC ACTION OF AN ANTIBIOTIC SUBSTANCE, AS SHOWN BY THE EFFECT OF STREPTOTHRICIN UPON TRICHOPHYTON MENTAGROPHYTES
The dilutions used are 1, 1:5, and 1:25

is measured. The inner zone is due to the overgrowth by the surface mycelium of some portion of the inhibited zone. Certain fungi, such as the pathogen *Trichophyton mentagrophytes*, can be readily utilized for assaying the fungistatic potency of various substances (Emmons, 1944) in a manner similar to the use of *Staphylococcus aureus* or *Bacillus subtilis* for testing the antibacterial potency of antibiotic substances. The results can also be expressed in dilution units by standardizing the results of the cup method against the agar streak method with the same organism.

Test fungi. Four fungi pathogenic to animals and to man and four saprophytes were used in this study. The first group included *Candida albicans*, *Trichophyton mentagrophytes* 589, *Trichophyton mentagrophytes* 640, and *Cryptococcus neoformans*; the second group comprised a strain of *Penicillium luteum-purpurogenum*, *Dematium*, *Fusarium*, and *Aspergillus clavatus*.

In carrying out the tests, two different media were used, namely, fungous (glucose peptone salts) agar and ordinary nutrient (meat-extract peptone salt) agar.

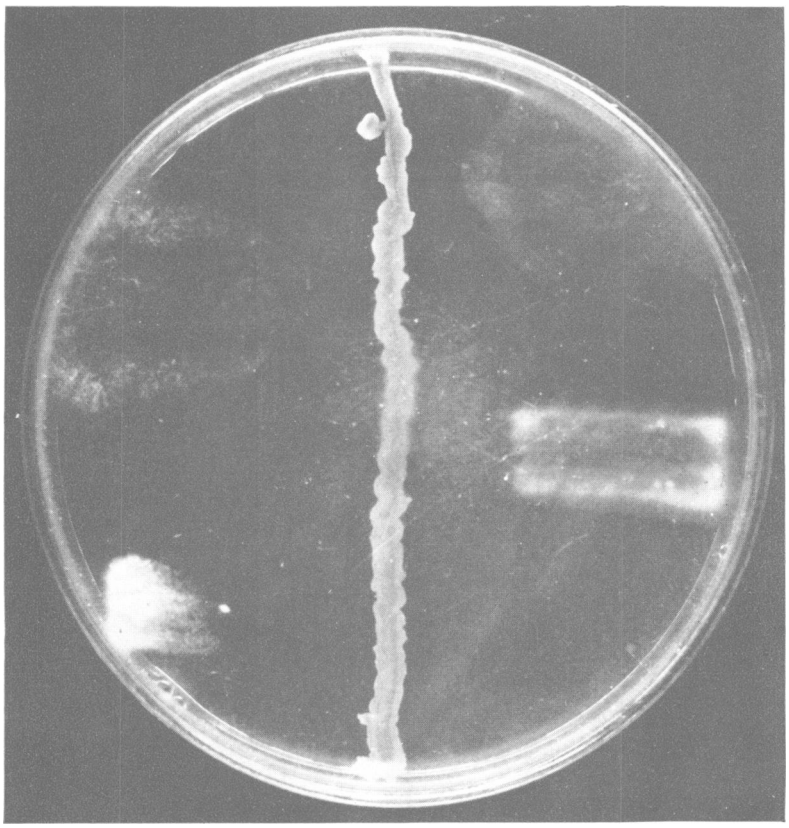

FIG. 3. METHOD OF TESTING THE PRODUCTION OF AN ANTIFUNGAL SUBSTANCE BY A FRESHLY ISOLATED CULTURE

In the isolation from natural substrates of organisms that have the capacity of producing fungistatic substances, the agar streak method, similar to the one used in the testing of organisms producing substances active against bacteria (Waksman and Horning, 1943) was used. The method is illustrated in figure 3.

Antibiotic substances. Several antibiotic substances were used in this study. These substances varied greatly in chemical nature, in biological activity, and in the degree of their purification. Some, like actinomycin, fumigacin, gliotoxin, and clavacin, were crystalline preparations; others, like chaetomin, strepto-

thricin, and streptomycin, were highly purified but not as yet crystallized. The antibacterial activity of each substance can serve as a relative measure of its respective antibiotic potency.

Fungistatic action of antibiotic substances. In the following studies, known antibiotic substances have been used. The results obtained are presented in tables 1 and 2. The various substances used in these studies can be arranged, on the basis of their relative fungistatic activity, in the following order, beginning with the most potent: gliotoxin, actinomycin, clavacin, fumigacin, streptothricin, chaetomin, and streptomycin.

Gliotoxin is fairly toxic to animals, but because of its extremely high fungistatic properties it deserves more detailed consideration. The antifungal

TABLE 1

Fungistatic action of antibiotic substances—saprophytic fungi

Activity, units per gram*

ANTIBIOTIC SUBSTANCE	ANTIBACTERIAL ACTIVITY		PENICILLIUM LUTEUM-PURPUROGENUM	DEMATIUM SP.	FUSARIUM SP.	ASPERGILLUS CLAVATUS 14a
	E. coli	*B. subtilis*				
Fungistatic activity, using fungus agar						
Actinomycin		>20,000,000	<500,000	<500,000	<500,000	<500,000
Chaetomin		750,000,000	<4,000	<4,000	<4,000	<4,000
Clavacin	100,000	200,000	25,000	15,000	25,000	3,000
Fumigacin	7,200	600,000	8,900	22,000	22,000	3,000
Gliotoxin	15,000	2,000,000	600,000	6,000,000	600,000	200,000
Streptomycin	125,000	625,000	<45	<45	<45	<45
Streptothricin	100,000	500,000	3,000	6,000	9,000	<60
Fungistatic activity, using nutrient agar						
Streptomycin	125,000	625,000	<120	<120	<120	<120
Streptothricin	100,000	500,000	7,100	29,000	4,800	2,900

* 24 hours' incubation at 28 C.

activities of this substance vary greatly, quantitatively, with respect to the pathogenic as well as the saprophytic fungi.

Actinomycin and clavacin are highly toxic to animals, and the second is not characterized by a very high activity as compared with gliotoxin. Both of these substances can, therefore, be eliminated from further consideration as fungistatic agents offering promise for chemotherapeutic purposes.

The fumigacin used in these experiments was a crude preparation, having a slight admixture of gliotoxin, which may account for its limited antifungal properties. Therefore, fumigacin can hardly be considered as offering much promise for practical utilization.

Chaetomin and streptomycin were completely inactive against fungi and can, therefore, also be eliminated from further consideration.

Streptothricin was found to possess definite fungistatic properties, and, since

this is not a very toxic compound, it should be considered further. It is of particular interest to note that the two basic preparations used in this study, streptothricin and streptomycin, both of which possess similar chemical and antibacterial properties, differ greatly in their antifungal activities: the second is completely inactive, whereas the first has some definite activity against fungi. These observations thus tend to confirm those obtained by Robinson, Smith, and Graessle (1944).

The results of a survey of seven different antibiotic substances brought out the fact that only two of these, gliotoxin and streptothricin, merit consideration for possible practical application. They were therefore selected for further study.

TABLE 2

Fungistatic action of antibiotic substances—pathogenic fungi

Activity, units per gram*

ANTIBIOTIC SUBSTANCE†	CANDIDA ALBICANS	TRICHOPHYTON MENTAGROPHYTES 598	TRICHOPHYTON MENTAGROPHYTES 640	CRYPTOCOCCUS NEOFORMANS
Fungistatic activity, using fungus agar				
Actinomycin	<500,000	5,000,000	1,500,000	1,500,000
Chaetomin	<4,000	<4,000	<4,000	<4,000
Clavacin	3,300	33,000	25,000	10,000
Fumigacin	8,900	14,800	8,900	30,000
Gliotoxin	4,000,000	6,000,000	2,000,000	>20,000,000
Streptomycin	<45	<45	<45	<45
Streptothricin	<60	4,500	4,500	12,000
Fungistatic activity, using nutrient agar				
Streptomycin	<120	<120	<120	<120
Streptothricin	1,900	2,900	2,900	19,000

* 2 to 3 days' incubation at 28 C.

† The antibacterial activity of these preparations is given in table 1.

Fungicidal action of antibiotic substances. The fungicidal action of antibiotic substances was determined as follows: 5-ml portions of nutrient broth were placed in 50-ml Erlenmeyer flasks and inoculated with 2 drops of a sterile water suspension of the spores of the test fungus. The cultures were incubated, with frequent shaking, at 37 C for 1 to 3 days. The antibiotic agent was added in different concentrations. The cultures were further incubated and streaked on nutrient agar plates after 1, 3, and 7 days. The plates were then incubated at 37 C for 48 hours, and readings taken.

In the first experiment, 3 antibiotic agents were used: fumigacin, 33.8 mg per ml; gliotoxin, 5 mg per ml; and streptothricin (100 units per mg), 100 mg per ml. The results obtained are presented in table 3.

Gliotoxin proved to be the most active fungicidal agent; when too low con-

centrations were used, there appeared to be a definite adaptation of the organism to the antibiotic substance, and an early destructive effect was later overcome. In the case of streptothricin, however, the fungicidal action appeared to be greater on continued contact of the agent with the fungus.

The results of an experiment on the antifungal action of clavacin and synthetic ketones are presented in table 4. Acrylophenone was the most active agent. The antifungal properties of this phenone were compared with those of the two antibiotic substances, gliotoxin and streptothricin. The test fungi were grown in nutrient broth for 1 and 2 days, and the active substances added (table 5). Acrylophenone proved to be most actively fungicidal, followed by gliotoxin. Streptothricin was the least active of the three.

TABLE 3

Fungicidal action of antibiotic substances

ANTIBIOTIC AGENT	MG PER 5 ML CULTURE	*C. albicans* 3147			*T. mentagrophytes* 598		
		1 day	3 days	7 days	1 day	3 days	7 days
Fumigacin	3.4	0+	0	0	0	0	0
Fumigacin	0.7	trace	+++	+++	trace	0	0
Gliotoxin	2.5	0	0	0	0	0	0
Gliotoxin	0.5	0	+	+++	0	0	0
Gliotoxin	0.1	+	+++	+++	0	0	trace
Streptothricin	50.0	+++	++	trace	++	+	+
Streptothricin	10.0	+++	+++	+	+++	+++	++
Alcohol 95%*	0.5 ml	+++	+	+++	+++	0	+
Alcohol 95%*	0.1 ml	+++	+++	+++	+++	+++	+++
Control		+++	+++	+++	+++	+++	+++

+0 = no growth; + = limited growth; ++ = medium growth; +++ = maximum growth.

* The fungicidal activity of 95% alcohol was investigated because the fumigacin and gliotoxin were in 95% alcoholic solution.

The antifungal properties of antagonistic organisms and of antibiotic substances involve three distinct types of effect: (1) a lytic action, whereby the hyphae and the spores of the fungus are dissolved, the organism losing completely its ability to reproduce; (2) a fungicidal action, which is not necessarily accompanied by lysis; (3) a fungistatic effect, the fungus not being killed, but being prevented from growing. The latter effect may be accompanied by an abnormal development of the hyphae, which do not, however, lose the power of growth and reproduction when placed under favorable conditions of culture. A strong fungistatic effect, combined with limited injury to the tissues of the host, appears to be most significant in any consideration of the chemotherapeutic potentialities of a substance.

TABLE 4

Fungistatic action of unsaturated ketones on pathogenic fungi

SUBSTANCE	DILUTION UNITS PER GRAM OF SUBSTANCE			
	Cryptococcus neoformans	*Trichophyton mentagrophytes* 640	*Trichophyton mentagrophytes* 598	*Candida albicans*
Acrylophenone	30,000	>3,000,000	>3,000,000	30,000
Benzalacetone	16,000	160,000	160,000	20,000
Benzalacetophenone	60,000	>200,000	>200,000	20,000
Furfuralacetophenone	6,000	16,000	20,000	2,000
Clavacin	30,000	30,000	50,000	<10,000

TABLE 5

Fungicidal action of antibiotic substances and of a synthetic phenone

ANTIBIOTIC SUBSTANCE	MG PER 5 ML BROTH	1-DAY-OLD CULTURES VIABILITY AFTER TREATMENT FOR		2-DAY-OLD CULTURES VIABILITY AFTER TREATMENT FOR	
		2 days	5 days	2 days	5 days
Candida albicans 3147					
Gliotoxin	0.5	tr	+++	tr	tr
Gliotoxin	0.1	+++	+++	+++	+++
Acrylophenone	1.0	0	0	0	0
Acrylophenone	0.2	+++	+++	+++	+++
Acrylophenone	0.04	+++	+++	+++	+++
Control	0	+++	+++	+++	+++
Trichophyton mentagrophytes 598					
Gliotoxin	0.5	0	0	0	0
Gliotoxin	0.1	tr	tr	tr	tr
Acrylophenone	1.0	0	0	0	0
Acrylophenone	0.2	0	0	0	0
Acrylophenone	0.04	+	tr	+++	+++
Streptothricin	50.0	tr	+	+	+
Streptothricin	10.0	tr	+	+++	+++
Control	0	+++	+++	+++	+++

SUMMARY

From the results of a study of the antifungal action of antibiotic substances produced by microorganisms and of certain synthetic compounds, the following conclusions may be drawn:

(1) Antibiotic substances vary greatly in their antifungal effect. Some, like gliotoxin and actinomycin, are very active, and others, like chaetomin and streptomycin, have very little activity.

(2) The selection of an antibiotic substance for chemotherapeutic purposes depends not only upon the relative activity of the substance but also upon its toxicity to animals. Hence, a substance like actinomycin, which is highly active but is also highly toxic, is eliminated from practical consideration, whereas a substance like streptothricin, which is not so active but also is not very toxic, deserves consideration.

(3) Of seven antibiotic substances tested for their antifungal properties, only two were found worthy of consideration for practical utilization, gliotoxin and streptothricin.

(4) The antifungal action of an antibiotic substance comprises both fungistatic and fungicidal effects.

(5) Certain unsaturated ketones, to which some of the antibiotic substances belong, were found to possess very strong antifungal properties.

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