SPECIFICATION

Lactobacillus casei YIT 9018 for use as an antitumour agent

This invention relates to an antitumour agent.

The invention provides an antitumour agent containing the strain *Lactobacillus casei* YIT 9018 (Deposit No. FERM-P 4751) as an effective ingredient. This strain is deposited at the Fermentation Research Institute, Government Industrial Research, Ministry of International Trade and Industry, Japan and is available to the public.

While the antitumour activity of an alive streptococcal preparation (Japan Tokkyo Koho, 6690, 1968) or the extract from lactobacilli (Japan Tokkyo Koho, 28558, 1970) has been reported, it has not previously been known that the heat-killed whole cell preparation of the strain YIT 9018 has an antitumour activity.

This invention is concerned not only with the antitumour activity of alive but also heat-killed *Lactobacillus casei* YIT 9018 whole cells. This provides the following benefits: the process of the preparation is simpler and the possible side effects due to the presence of additives for maintaining the survival of the bacteria can be prevented. It also deserves a special emphasis that *Lactobacillus casei* YIT 9018 is non-toxic and used in practice in a fermented milk product.

More specifically, it has been found that *Lactobacillus casei* YIT 9018 (Deposit No. FERM-P4751) (hereinafter referred to as "LC 9018") has a higher antitumour activity compared to other lactobacilli. The bacterial strain exhibits antitumour activity against solid and ascites tumour of Sarcoma 180 upon intravenous, subcutaneous, oral or intraperitoneal administration in mice. Furthermore, it shows an antitumour activity against mouse lueukemia L1210 or P-388 on which commercial antitumour streptococcal preparations have no effect. In addition, the toxicity of LC 9018 is significantly lower than those of other lactobacilli and streptococcal antitumour agents.

LC 9018 has the following characteristics:

Cell shape	short rod
Gram's stain	positive
Optimal pH for growth	6.6–7.0 (at 35–39°C)
Range of temperature for growth	15–42°C
Methyl red test	negative
Voges-Proskauer reaction	negative
Production of indole	negative
Production of H_2S	negative
Ammonia from arginine	negative
Reduction of nitrate	negative
Production of catalase	negative
Liquefaction of gelatin and casein	negative
Citrate utilization	negative
Coagulation of milk	positive
Reduction of litmus milk	positive
Utilization of ammonium and urea	negative
Gas from glucose	negative
Fermentation	
arabinose	negative
xylose	negative
rhamnose	negative
glucose	positive
mannose	positive
galactose	positive
sucrose	positive
maltose	positive
lactose	positive
cellobiose	positive
trehalose	positive
melibiose	negative
raffinose	negative
melezitose	positive
mannitol	positive
sorbitol	positive
salicin	positive
amygdalin	positive

LC 9018 can be cultured according to ordinal methods. For example, it can be cultivated on a semisynthetic broth containing lactose, glucose peptone, yeast extract, KH_2PO_4, K_2HPO_4, $MgSO_4$ etc. The culvitation is suitably performed at 37°C for 18 to 24 hours. After cultivation, bacterial cells can be collected by centrifugation and washed with distilled water and lyophilized. While the bacterial cells require no additives for survival, drugs or other substances might be added for maintaining quality, if desired.

LC 9018 is preferably cultivated using the broth shown in Table A. The amounts are per 1000 ml of distilled water.

TABLE A

Trypticase	10 g
Yeast extract	5 g
Tryptose	3 g
K_2HPO_4	3 g
KH_2PO_4	3 g
Ammonium citrate	2 g
Sodium acetate	1 g
Tween 80	80 g
Glucose	20 g
Cystein	0.2 g
$MgSO_4-7H_2O$	0.5 g
$FeSO_4-7H_2O$	0.04 g
$MnSO_4-2H_2O$	0.12 g

After cultivation, at 37°C for 20 to 40 hours, the cells can be washed with distilled water and lyophilized.

LC 9018 is preferably suspended in physiological saline for use as an injection. The daily effective doses are normally 30 to 100 mg/kg (ideally 50 mg/kg) for intravenous injection, 5 to 20 mg/kg (ideally 10 mg/kg) for intraperitoneal injection and 1000 to 2000 mg/kg for oral administration. The frequency and duration of the administration should be varied according to the condition of a patient.

As mentioned above, not only live but also heat-killed LC 9018 has a significant antitumour activity and is not pathogenic. The use of 9018 may bring less restrictions on the preparative process and its clinical usage and preparation can be effected econimically.

The present invention is illustrated by the following Experiments and Examples.

EXPERIMENT 1: ANTITUMOR ACTIVITY OF LC9018

Example (1)

Sarcoma 180 ($1-2 \times 10^6$ cells/mouse) was implanted into ICR main mice subcutaneously on day 0. The suspension of lactobacilli in saline (0.25 mg/mouse) was injected intravenously daily on days + 1– + 5. Mice were dissected 3 weeks after the tumor implantation and the weight of tumor was measured. The inhibition rate was culculated according to a formula :

$$\text{Inhibition rate} = \left(1 - \frac{\text{Average tumor weight of tested mice}}{\text{Average tumor weight of control mice}}\right) \times 100$$

As shown in Table 1, lactobacilli had the inhibition rates of 40–70% as well as the streptococcal preparation. LC 9018 showed the inhibition rate of more than 80%.

Example (2)

Sarcoma 180 ($1-2 \times 10^6$ cells/mouse) was implanted subcutaneously into male ICR mice after admixed with 1 mg of LC 9018. The inhibition rate with LC 9018 was 97.3% which determined as described in Example (1).

Example (3)

Sarcoma 180 ($1-2 \times 10^6$ cells/mouse) was inoculated subcutaneously followed by the subcutaneous injection of LC 9018 24 hr after the tumor implantation. As shown in Table 2, LC 9018 significantly inhibited the growth of Sarcoma 180 at the dose of 40 mg/kg.

Example (4)

Sarcoma 180 ($1-2 \times 10^6$ cells/mouse) was inoculated subcutaneously into male ICR mice on day 0. LC 9018 was given orally daily on days − 10, + 1, and + 10. It was found LC 9018 showed antitumor activity even by oral administration (Table 3).

Example (5)

Sarcoma 180 ($1-2 \times 10^6$ cells/mouse) was inoculated intraperitoneally on day 0. LC 9018, suspended in saline, was injected intraperitoneally daily on days − 5– − 1. The relative survival

rate (T/C %) was calculated as follows:

$$T/C\ \% = \frac{\text{Average survival time of tested mice}}{\text{Average survival time of control mice}} \times 100$$

As shown in Table 4, LC 9018 increased the survival time of mice implanted tumor cells.

Example (6)

Mouse leukemia L1210 or P-388 (1×10^5 cells/mouse) was inoculated intraperitoneally into male BDF_1 mice on day 0. LC 9018, suspended in saline, was given intraperitoneally daily on days + 1 – + 5. As shown in Table 5, LC 9018 also inhibited the growth of leukemia cells. This should be emphasized because the commercial streptococcal preparation has no antitumor activity against leukemia.

EXPERIMENT 2: TOXICITY OF LC9018

(1) LD_{50}

The LD_{50} of lactobacilli which showed the inhibition rate (Table I) of more than 40% was determined using ICR mice according to the method of Lithfiel-Wilcoxon. LC 9018 was less toxic compared to streptococcal preparation as well as other lactobacilli.

(2) Antigenicity

LC 9018 was injected subcutaneously into white guinea pig for three times every 3 days at the total does of 50 mg/kg. The anaphylactic reaction test and agglutination test were carried out 10 days and 12 days after the final injection of LC 9018, respectively. All the guinea pigs given LC 9018 showed negative reactions in both tests.

EXPERIMENT 3: ANTITUMOUR ACTIVITY AND TOXICITY OF HEAT-KILLED CELLS LC 9018

The antitumour activity and the toxicity of heat-killed LC 9018 were same as those alive cells.

EXAMPLE OF PREPARATION

(1) LC 9018 (1×10^7 cells) was inoculated into 1000 ml of the broth of which composition was mentioned above and cultivated at 37°C for 20 hr. The final alive cell number reached at maximum (2.5×10^9/ml). The cells were collected by centrifugation and washed with distilled water. The suspension of LC 9018 in distilled water was divided in ampoules and lyophilized. After leaved at 5°C for 30 days, LC 9018 in ampoules was used for the antitumor experiment. The inhibition rate was 83.2%.

(2) LC 9018 was obtained as in (1). It was autoclaved at 121°C for 20 min and dryed at 80°C. The powder was divided into ampoules and stored at 5°C for 2 months. The inhibition rate against Sarcoma 180 with this preparation was 78.5% and the LD_{50} was 620 mg/kg or 720 mg/kg in male or female ICR mice, respectively.

(3) The culture broth of 300 ml containing 2.5×10^9 cells/ml of LC 9018 was inoculated into 10 l of Rogosa's medium. The cell number was 2.3×10^9/ml after the cultivation at 37°C for 20 hr. The wet cells (165 g) were dryed at 80°C for 3 hr and 40 g of powder was obtained. The powder was added to 40 ml of hydroxypropyl cellulose solution (10%) in ethanol and granulated followed by dryness. The granules were given orally at the dose of 0.3 g/kg/day for 10 days to ICR mice which had been implanted subcutaneously Sarcoma 180 ($1–2 \times 10^6$ cells/mouse) 10 days before the oral administration of the granules. The inhibition rate at 3 weeks after the tumor implantation was 68.2%.

Table 1. Antitumor activity of lactobacilli

Strain or preparation	Inhibition rate*
L. casei YIT 9018 (LC 9018)	82.7
,, N VIII-1	65.5
,, TK IV-2	66.6
L. acidophilus B-3208	23.0
,, YIT 0163	43.5
L. salivarius YIT 0089	21.5
,, YIT 0104	40.9
L. fermentum YIT 0082	14.9
,, YIT 0159	10.2
L. plantarum YIT 0102	17.2
,, YIT 0158	40.4
L. bulgaricus YIT 0046	64.7
L. jugurti YIT 0085	17.7
L. helveticus YIT 0083	29.0
L. lactis YIT 0086	12.8
L. leichmannii YIT 0087	28.4
L. delbrueckii YIT 0080	47.9
L. brevis YIT 0076	5.3
L. jensenii YIT 0084	38.4
Streptococcal preparation	50.8

Sarcoma 180 ($1-2 \times 10^6$ cells/mouse) was implanted into ICR mice subcutaneously on day 0. The suspension of lactobacilli or streptococcal preparation in saline (0.25 mg/mouse) was injected intravenously daily on days + 1 – + 5.

$$\text{*Inhibition rate} = \left(1 - \frac{\text{Average tumor weight of tested mice}}{\text{Average tumor weight of control mice}}\right) \times 100.$$

Table 2. Antitumor activity of subcutaneously administered LC 9018

Dose (mg/kg)	Inhibition rate*
0 (Control)	0
4	39.3
40	84.4

Sarcoma 180 ($1-2 \times 10^6$ cells/mouse) was inoculated subcutaneously into ICR mice. LC 9018, suspended in saline, was injected subcutaneously 24 hr after the tumor inoculation. Animals were dissected 3 weeks after the inoculation and the weight of tumor was measured.
*Inhibition rate was calculated as Table 1.

Table 3. Antitumor activity of orally administered LC 9018

Total dose (mg/kg)	Inhibition rate*
0 (Control)	0
1200	70.5
2000	64.2

Sarcoma 180 ($1-2 \times 10^6$ cells/mouse) was inoculated subcutaneously into ICR mice on day 0.

LC 9018 was given orally daily on days − 10, + 1, and + 10. Animals were dissected 3 weeks after the inoculation of tumor cells and the weight of tomur was measured.
*Inhibition rate was calculated as Table 1.

Table 4. Antitumor activity of intraperitoneally administered LC 9018 against intraperitoneally inoculated Sarcoma 180

Total dose (mg/kg)	T/C %*
0 (Control)	100
1	104
10	367
40	243

Sarcoma 180 ($1-2 \times 10^6$ cells/mouse) was inoculated intraperitoneally on day 0. LC 9018, suspended in saline, was injected intraperitoneally on daily on days − 5 − − 1.

$$*T/C\ \% = \frac{\text{Average survival time of tested mice}}{\text{Average survival time of control mice}} \times 100.$$

Table 5. Antitumor activity of LC 9018 against mouse leukemia

Leukemia cell	Total dose (mg/kg)	T/C %*
L1210	0 (Control)	100
	6	119
	12	128**
	60	138***
	120	127**
P-388	0 (Control)	100
	5	120**
	50	118**
	250	108

Mouse leukemia L1210 or P-388 (1×10^5 cells/mouse) was inoculated intraperitoneally into BDF_1 mice on day 0. LC 9018, suspended in saline, was given intraperitoneally daily on days + 1 − + 5.
*T/C % was calculated as Table 4.
**$P<0.005$, *** $P<0.0001$.

Table 6. Acute toxicity of lactobacilli and streptococcal preparation

Route	Strain or preparation	LD_{50}(mg/kg) male	female
i.p			
	L. casei YIT 9018 (LC 9018)	650	730
	,, N VIII–1	515	581
	,, TK IV–2	350	466
	L. acidophilus YIT 0163	550	612
	L. salivarius YIT 0153	504	566
	L. plantarum YIT 0158	340	361
	L. bulgaricus YIT 0046	310	358
	L. delbrueckii YIT 0080	256	283
	Streptococcal preparation	125	140
i.v.			
	L. casei YIT 9018 (LC 9018)	156	240
	Streptococcal preparation	24.5	25.5
s.c.			
	L. casei YIT 9018 (LC 9018)	2500	2500
	Streptococcal preparation	238	197
p.o.			
	L. casei YIT 9018 (LC 9018)	5000	5000
	Streptococcal preparation	500	500

Lactobacilli or streptococcal preparation was given to ICR mice (body weight of about 25 g) intraperitoneally (i.p.), intravenously (i.v.), subcutaneously (s.c.) or orally (p.o.).

CLAIMS

1. *Lactobacillus casei* YIT 9018, for use as an antitumour agent.

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