

MICROORGANISM, PROCESS FOR OBTAINING IT,
AND FEEDSTUFF ADDITIVE

The present invention relates to a microorganism
5 of the genus Eubacterium, which is suitable in pure
culture or mixed culture for the detoxification of
trichothecenes, and to a process for the isolation
thereof, its production and formulation and its use and
a feedstuff additive comprising the microorganism.

10 Trichothecenes which belong to the mycotoxins
class are contained in numerous animal feedstuffs,
where they are customarily introduced into the feed-
stuffs via mould fungi found on cereals or grasses. As
a result of the undesired administration of mycotoxins,
15 in particular trichothecenes, to animals, both their
productivity and, for example, the growth of the ani-
mals is inhibited, an increased consumption of feed-
stuff together with a simultaneously poorer feedstuff
utilization rate occurring in addition to damage to the
20 health of the animals. To eliminate the adverse effects
of mycotoxins, numerous processes for binding or
adsorbing these toxins have already been disclosed.

Thus, in WO 91/13555, for example, a feedstuff
additive and a process for the inactivation of myco-
25 toxins is described, where particles of a phyllosili-
cate mineral are added to the feed in order to inac-
tivate the mycotoxins. To increase the effect of these
phyllosilicates, the particles are coated with a
sequestering agent in order to accelerate the effect. A
30 feedstuff is furthermore known, for example, from
WO 92/05706 in which montmorillonite clay is contained
as a feedstuff additive. These natural clay minerals
having large internal surface areas should bind the
mycotoxins to the surface on account of their porosity
35 and immobilize them in this manner.

Furthermore, a feedstuff additive has been dis-
closed in the Austrian Utility Model AT-U 504 in which



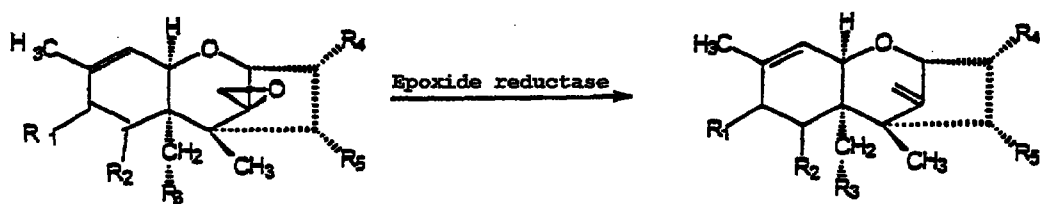
an enzyme preparation is used which is capable of forming epoxidases and lactonases and degrading mycotoxins chemically both in the feedstuff and in the gastrointestinal tract of animals. According to AT-U 504, the
5 action of this enzyme preparation can be increased by the addition of zeolites and the like.

The present invention now aims at making available a specific microorganism or a defined mixed culture isolated from a natural habitat, with which it is
10 possible to convert mycotoxins, in particular trichothecenes, in a controlled manner into substances which are physiologically harmless and which are harmless, in particular in animal breeding, by biochemical degradation.

15 To solve this object, a microorganism of the genus Eubacterium was isolated which is suitable for the detoxification of trichothecenes in pure culture, DSM 11798, or mixed culture with the strain Enterococcus casseliflavus, DSM 11799, or other anaerobic
20 microorganisms. According to a novel refinement of the invention, the microorganism is suitable for the detoxification of trichothecenes in mixed culture with other anaerobic microorganisms, in particular from the genus Enterococcus, Streptococcus, Lactococcus, Bacillus or Lactobacillus.
25

The microorganism of the genus Eubacterium, which is also called Eubacterium sp. on account of its association with the genus Eubacterium, and which was deposited in pure culture in the Collection of German
30 Microorganisms under the number DSM 11798, or in mixed culture with the strain Enterococcus casseliflavus, which was deposited in the Collection of German Microorganisms under the number DSM 11799, is in particular suitable according to the invention for the detoxi-
35 fication of, in particular, deoxynivalenol (DON), T-2 toxin, HT-2 toxin, nivalenol, monoacetoxyscirpenol, diacetoxyscirpenol, trichodermol, verrucaridin, rorodin,

acetyldeoxynivalenol, isotrichodermin, hydroxyisotrichodermin, calonectrin, T-2 tetraol, T-2 triol, deacetylneosolaniol, neosolaniol, acetylneosolaniol, sporotrichiol, trichotriol, sambucinol and culmorin. The
 5 microorganism according to the invention detoxifies the trichothecenes by reductive biotransformation of the epoxide group contained in the molecule, which epoxide group is responsible for the toxicity of the mycotoxins, in particular trichothecenes. In the trichothecenes corresponding to the following formula, the
 10 degradation of the epoxide group is carried out by reductive cleavage of the toxic 12,13-epoxy ring:



Trichothecene

reduced non-toxic form

The morphology of the microorganism according to
 15 the invention shows preferably that it is an anaerobic gram-positive, rod-like, non-spore-forming bacterium, in particular 0.1 to 3 μm long, which occurs both individually, in pairs or in long chains, in particular
 up to approximately 150 μm . Phylogenetic analysis of
 20 the microorganism according to the invention has in particular shown a 16S RNA sequence, namely

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      1  CCTGGCTCAG  GATGAACGCT  GGCGGCGTGC  TTAACACATG
CAAGTCGAAC GGATAACCCG
25      61  CCTCCGGGCG  GTTATAGAGT  GGCGAACGGG  TGAGTAACAC
GTGACCAACC TACCTCCCAC
      121  TCCGGGATAA  CCCAGGGAAA  CCTGCGCTAA  TACCGGATAC
TCCGGGGCCC CCGCATGGGG
      181  GCGCCGGGAA  AGCCCCGACG  GTGGGAGATG  GGGTCGCGGC
30  CTATTAGGTA GTCGGCGGGG
  
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	241	TAACGGCCCA	CCGAGCCCGC	GATAGGTAGC	CGGGTTGAGA
		GACCGATCGG	CCACATTGGG		
	301	ACTGAGATAC	GGCCCAGACT	CCTACGGGAG	GCAGCAGTGG
		GGAATTTTGC	GCAATGGGGG		
5	361	AAACCCTGAC	GCAGCAACGC	CGCGTGCGGG	ACGAAGGCCT
		TCGGGTTGTA	AACCGCTTTC		
	421	AGCAGGGAAG	AAGTTGACGG	TACCTGCAGA	AGAAGCTCCG
		GCTAACTACG	TGCCAGCAGC		
	481	CGCGGTAATA	CGTAGGGAGC	GAGCGTTATC	CGGATTTATT
10		GGGCGTAAAG	CGCGCGTAGG		
	541	CGGGCGCTTA	AGCGGAATCT	CTAATCTGAG	GGCTCAACCC
		CCAGCCGGAT	TCCGA ACTGG		
	601	GCGCCTCGAG	TTCGGTAGAG	GAAGACGGAA	TTCCCAGTGT
		AGCGGTGAAA	TGCGCAGATA		
15	661	TTGGGAAGAA	CACCGATGGC	GAAGGCAGTC	TTCTGGGCCG
		TAAGTGACGC	TGAGGTGCGA		
	721	AAGCTAGGGG	AGCGAACAGG	ATTAGATACC	CTGGTAGTCC
		TAGCCGTAAA	CGATGGGCAC		
	781	TAGGTGTGGG	GGGGAATGCC	CCTCCGTGCC	GCAGCTAACG
20		CATTAAGTGC	CCCGCCTGGG		
	841	GAGTACGGCC	GCAAGGCTAA	AACTCAAAGG	AATTGACGGG
		GGCCCGCACA	AGCAGCGGAG		
	901	CATGTGGCTT	AATTCGAAGC	AACGCGAAGA	ACCTTACCAG
		GGCTTGACAT	GCAGGTGAAG		
25	961	CGGCGGAAAC	GCCGTGGCCG	AGAGGAGCCT	GCACAGGTGG
		TGCATGGCTG	TCGTCAGCTC		
	1021	GTGTCGTGAG	ATGTTGGGTT	AAGTCCCGCA	ACGAGCGCAA
		CCCCTGTCGT	ATGTTGCCAT		
	1081	CATTCAGTTG	GGGACTCGTA	CGAGACTGCC	GGCGTCAAGC
30		CGGAGGAAGG	TGGGGACGAC		
	1141	GTCAAGTCAT	CATGCCCTTT	ATGCCCTGGG	CTGCACACGT
		GCTACAATGG	CCGGTACAAC		
	1201	GGGCTGCGAG	CCAGCGATGG	CGAGCGAATC	CCTCAAAACC
		GGTCCCAGTT	CGGATCGGAG		
35	1261	GCTGCAACCC	GCCTCCGTGA	AGTCGGAGTT	GCTAGTAATC
		GCGGATCAGC	ATGCCGCGGT		

1321 GAATACGTTC CCGGGCCTTG TACACACCGC CCGTCACACC
ACCCGAGTTG TCTGCACCCG

1381 AAGTCGACGG CCCAACCCGC GAGGGGGGAG TCGCCGAAGG
TGTGGGGAGT AAGGGGGGTG

5 1441 AAGTCGTAAC AAGGTAGCCG TACCGGAAGG TGCGGCT,

The sequence data of the microorganism being compared with known 16S RNA gene sequences of representative microorganisms which are part of the domain of
10 bacteria. This comparison analysis showed the greatest correspondence to bacteria of the genus Eubacterium. However, it was not possible to find any gene sequence corresponding sufficiently to a known microorganism, from which it results that the microorganism according
15 to the invention is a microorganism within the genus Eubacterium which has still not been isolated and classified to date. Physiological investigations, such as, for example, fermentation spectra, reduction of nitrate to nitrite, also clearly showed the association
20 with the genus Eubacterium.

A further object of the present invention is to make available a process for obtaining both a pure culture of the microorganism DSM 11798 and its mixed culture with the strain Enterococcus casseliflavus,
25 DSM 11799, and other anaerobic microorganisms, an optimization of yield both in the economical and in the quantitative respect being aimed at in particular.

To achieve this object, the process according to the invention is carried out in such a way that a mixed
30 culture DSM 11799 is obtained from the microorganism and Enterococcus casseliflavus from bovine rumen by culturing or fermenting at least twice in dilution series and anaerobic culturing conditions. To obtain both the mixed culture and the pure culture of the
35 microorganism according to the invention, culturing and/or fermenting in dilution series at least twice has proved favourable, since in this manner an ensured

purity of the desired products and, in particular, a removal of interfering by-products or contaminations with undesired microorganisms can be achieved. To maintain the anaerobic conditions, the culturing and/or fermentation according to the invention was preferably performed in a gas atmosphere of H_2 and CO_2 , the gas atmosphere having a ratio of $H_2:CO_2$ of 10:90 to 90:10, in particular approximately 80:20, being particularly preferably selected. For the growth of the micro-
10 organism according to the invention, anaerobic conditions with a low redox potential are important, it surprisingly only being possible to achieve a sufficiently rapid growth in the presence of H_2 .

An even more rapid growth of the microorganism according to the invention can be achieved by carrying
15 out the culturing and/or fermentation at an overpressure of 0.2 to 3 bar, in particular 0.5 to 1 bar, as this corresponds to a further preferred embodiment. It was possible to achieve further improved growth of the microorganism DSM 11798 according to the invention
20 by preferably carrying out the culturing and/or fermentation at a temperature of 35 to 42°C, in particular approximately 37°C. The pH optimum for the culturing or fermentation in the process according to the invention was preferably a pH of between 6 and 8 and in parti-
25 cular between 7 and 7.5. Under these conditions, it is possible to obtain both a pure culture of the microorganism DSM 11798 and its mixed culture (DSM 11799) described above in as short a time as possible and
30 using relatively few dilution series. Optimal results can be achieved with the process according to the invention by preferably carrying out the culturing and/or fermentation in a media preparation comprising components selected from: arginine, citrulline, pep-
35 tone, yeast extract, fatty acid mixture(s), mineral solution(s), glucose, haemin solution, menadione, vitamin solution, trace elements and reducing agents.

The components contained in a media preparation are in this case partially exchangeable, it being possible, for example, by addition of glucose to achieve a shift of the equilibrium in the mixed culture in the direction of *Enterococcus casseliflavus* or corresponding other anaerobic microorganisms, it being possible to control the process specifically depending on the amount of glucose added. According to a particularly preferred aspect of the invention, at the start of the culturing and/or fermentation 0.1 to 0.5% by weight, in particular 0.2% by weight, of glucose is added. By addition of 0.1 to 0.5% by weight of glucose, the growth of *Enterococcus casseliflavus* is promoted at the start of the culturing and/or fermentation, which leads to a fall in the redox potential. By lowering the redox potential, optimum growth conditions for the microorganism according to the invention were created, so that, for example, chemicals, such as cysteine, in the media preparation can be dispensed with by means of a controlled addition of glucose.

In order to achieve the detoxification of mycotoxins, in particular trichothecenes, to other advantageous effect with the microorganisms and/or mixed culture according to the invention, enzyme preparation of the active, trichothecene-detoxifying microorganism and/or other anaerobic microorganisms can preferably also be added according to the invention.

To obtain a pure culture of the microorganism DSM 11798, the process according to the invention is preferably carried out such that the pure culture of the microorganism DMS 11798 is obtained from the culture or fermentation solution DSM 11799 by at least two further dilution series in the media preparation, in particular with addition of L-arginine as a growth stimulator. By carrying out two further dilution series in the media preparation from the culture or ferment-

tation solution, the microorganism DMS 11798 can be obtained completely pure from the mixed culture with *Enterococcus casseliflavus*, the addition of the growth stimulator L-arginine advantageously shifting the equilibrium in the direction of the pure culture of the microorganism. In this connection, the growth of the bacterium according to the invention is promoted the higher the concentration of L-arginine.

In order to lower the redox potential of the media preparation further, a procedure is preferably used according to the invention in which, for the fermentation of the pure culture of the media preparation, 1 to 4% by weight of a reducing agent, in particular of a mixture of cysteine/sodium sulphide/sodium carbonate solution, is added. Particularly for reasons of economy, according to the invention the addition of the reducing agent is kept as low as possible, it having been shown in the course of comparison experiments that an increase in the concentration of the reducing agent beyond 4% by weight causes no further acceleration of the growth of the microorganism.

To obtain a storable finished product, the process according to the invention is preferably continued by working up the culture or fermentation solution by concentrating, in particular centrifuging or filtering and/or stabilization, in particular by freeze- or spray-drying or encapsulating. In this connection, for example, the culture or fermentation solution is concentrated in a first step by removing liquid by centrifuging or filtering, and/or carrying out the stabilization directly from the fermentation solution, preferably with addition of a filler or carrier material, such as aluminium silicates, kieselguhrs, carbohydrates, sugar alcohols, starches, milk and whey powder, protein hydrolysates, yeasts and PVPP. By addition of these carriers or fillers, it is possible in the following stabilization step, in particular the

freeze-drying, spray-drying, encapsulation of pelleti-
zation step, to obtain a solid product in which the
pure culture of the microorganism DSM 11798 or its
mixed culture with the strain *Enterococcus casseli-*
5 *flavus*, DSM 11799, or other anaerobic microorganisms,
in particular from the genus *Enterococcus*, *Strepto-*
coccus, *Lactococcus*, *Bacillus* or *Lactobacillus*, which
are suitable for the detoxification of trichothecenes,
is deposited directly on a carrier, as a result of
10 which a particularly easily manageable and storable as
well as metabolically favourable product is obtained.
By depositing the microorganism or its mixed culture on
a substance having a large internal surface area, such
as argillaceous earths, aluminium silicates, zeolites
15 and the like, the intended degradation according to the
invention of trichothecenes is further facilitated,
since these are bound physically to the substance
having a large internal surface area, whereupon the
biochemical attack with the microorganism according to
20 the invention is distinctly facilitated.

According to the invention, the microorganism is
further used in pure and/or mixed culture (DSM 11798,
DSM 11799) for the production of a feedstuff additive.
A particularly preferred use according to the invention
25 essentially results in that the pure and/or mixed
culture (DSM 11798 and DSM 11799) is employed as a
freeze- or spray-dried and/or encapsulated or pelleted
immobilizate, if appropriate with addition of a carrier
material. Both the pure and/or mixed culture of the
30 microorganism according to the invention and the spray-
dried immobilizate can be used directly as a feedstuff
additive, it even being possible to admix the feedstuff
additive to the feedstuff directly during preparation
and/or to mix it into the feedstuff either in solid or
35 in liquid form during feeding to the animals.

In order to achieve an as complete as possible
degradation or chemical conversion of the tricho-

thecenes into physiologically acceptable substances, a feedstuff additive according to the invention for the inactivation of trichothecenes in feedstuffs or in the digestive tract of animals is essentially characterized in that the feedstuff additive contains a pure and/or mixed culture of a microorganism according to the invention or of a microorganism prepared according to the invention in an amount from 10^5 to 10^{12} cells/kg, in particular 10^7 to 10^9 cells/kg, per 1000 kg of feedstuff. By use of 10^5 to 10^{12} cells/kg, in particular 10^7 to 10^9 cells/kg, of the microorganism according to the invention or of the mixed culture according to the invention of the microorganism and *Enterococcus casseliflavus* or other anaerobic microorganisms, in particular of the genus *Enterococcus*, *Streptococcus*, *Lactococcus*, *Bacillus* or *Lactobacillus*, which are suitable for the detoxification of trichothecenes, it is possible to convert high concentrations of trichothecenes, in particular of deoxynivalenol, T-2 toxin, HT-2 toxin, nivalenol, monoacetoxyscirpenol, diacetoxyscirpenol, trichodermol, verrucarol, rorodin, acetyldeoxy-nivalenol, isotrichodermin, hydroxyisotrichodermin, calonectrin, T-2 tetraol, T-2 triol, deacetyl-neosolaniol, neosolaniol, acetylneosolaniol, sporo-trichiol, trichotriol, sambucinol and culmorin in feedstuffs into chemically harmless substances, such as the deepoxy metabolite of deoxynivalenol (DOM-1), so that, using the feedstuff additive according to the invention, both an increase in productivity of the animals and, on account of the reduced toxicity, an improved feedstuff conversion rate can be achieved.

In order to further facilitate the biochemical degradation of the mycotoxins, in particular trichothecenes, according to the invention a carrier material and/or filler can preferably be additionally contained in the feedstuff additive in an amount of 0.5 to 8 kg/1000 kg, in particular 0.7 to 4 kg/1000 kg, of the

feedstuff. By means of the addition of carrier materials and/or fillers, it is possible, if desired, to bind the mycotoxins and also other harmful substances to be degraded which can be contained in the feedstuff, physically to the substances, as a result of which they are no longer available for metabolism.

In this case, in particular, aluminium silicates, kieselguhrs, carbohydrates, sugar alcohols, starch, milk and whey powder, protein hydrolysates, yeasts and/or PVPP are employed as a carrier material and/or filler, these carrier materials and/or fillers having proved to be particularly advantageous for the binding of toxins, in particular trichotoxins.

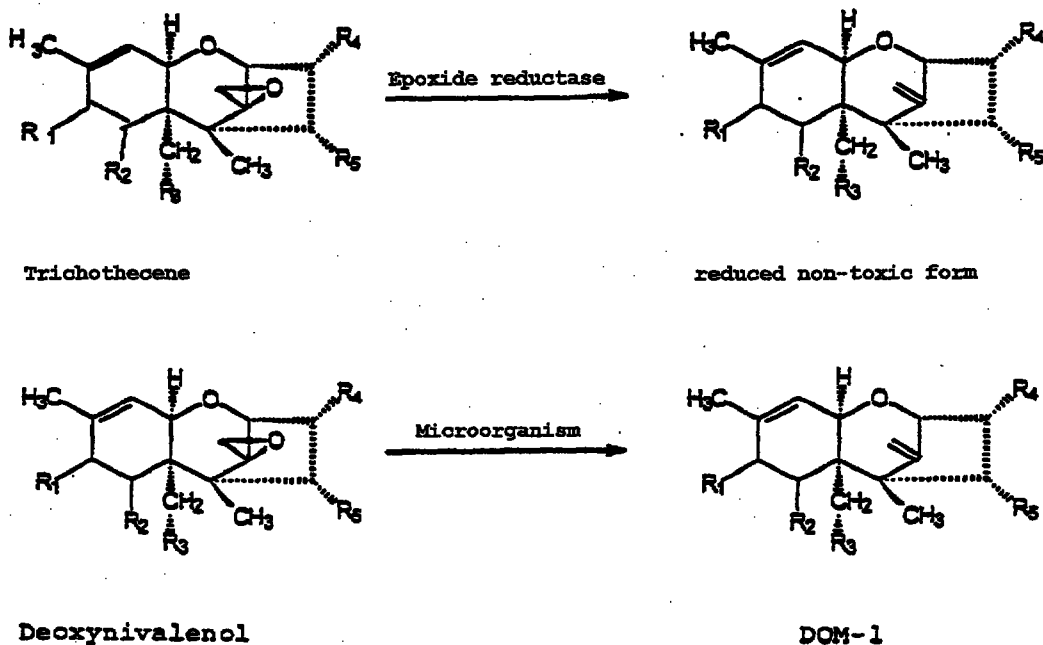
A particularly preferred feedstuff additive is characterized in that the feedstuff additive consists of a mixture of 1 to 65% by weight, in particular 5 to 50% by weight, of the spray- or freeze-dried immobilize of the microorganism and 99 to 35% by weight, in particular 95 to 50% by weight, of carrier material and/or filler. Feedstuff additives of this type are suitable, in particular, for the inactivation of deoxynivalenol (DON), T-2 toxin, HT-2 toxin, nivalenol, monoacetoxyscirpenol, diacetoxyscirpenol, trichodermol, verrucarol, rorodin, acetyldeoxy-nivalenol, isotrichodermin, hydroxyisotrichodermin, calonectrin, T-2 tetraol, T-2 triol, deacetyl-neosolaniol, neosolaniol, acetylneosolaniol, sporo-trichiol, trichotriol, sambucinol and culmorin both in the feedstuff and in the digestive tract of animals.

The invention is further explained below by means of characterization of the microorganism according to the invention, its growth and activity conditions and the formation of metabolic products of trichothecenes with the aid of the microorganism according to the invention and by means of working examples of feeding tests.

The microorganism according to the invention is an active, trichothecene-transforming strain, in particular a deoxynivalenol-transforming strain, and is obtained from bovine rumen by repeated culture in an optimized nutrient medium under anaerobic culturing conditions, namely a CO₂:H₂ gas atmosphere (20/80 v/v) and an overpressure of 0.5 to 1.5 bar. PYG and PY media were used here as a media preparation, which in each case consisted of differing concentrations of two different mineral solutions, menadione stock solution, haemin solution, yeast extract, peptone, glucose being additionally added to the PYG medium. In order to lower the redox potential, 2 to 4% by weight of a reducing solution consisting of cysteine/Na₂S/Na₂CO₃ solution were added both to the PYG and the PY medium and the pH was adjusted to a value of 7 to 7.5 by CO₂ gassing. It is possible with the aid of this media preparation to obtain both a pure culture of the microorganism DSM 11798 by carrying out several dilution series, and also, in particular with the PYG medium, to obtain a mixed culture of the microorganism and *Enterococcus casseliflavus* (DSM 11799). The growth of the microorganism is exclusively achieved under strictly anaerobic conditions with a sufficiently low redox potential and the presence of H₂. The optimum growth of the microorganism can be achieved at approximately 37°C, it also being possible, however, to achieve adequate growth of the microorganism between 35°C and 38°C. For culturing the pure culture of the microorganism DSM 11798, L-arginine in liquid medium has a stimulating effect.

Using the microorganism according to the invention, it is possible, in trichothecenes, to detoxify these by reductive cleavage of the toxic 12,13-epoxy ring.

The reaction scheme is subsequently shown here with the aid of the trichothecenes generally and also the specific trichothecene deoxynivalenol.



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Both the fermentation conditions and useable fermentation processes will subsequently be illustrated by way of example for the fermentation of the micro-organism DSM 11798 according to the invention and in coculture with other facultative and anaerobic micro-organisms and in mixed culture with *Enterococcus casseliflavus* (DSM 11799).

Fermentation conditions:

- 15 Fermentation temperature between 35 and 42°C, in particular approximately 37°C;
 pH range for the fermentation between 6 and 8, in particular 7.0-7.5;
 Redox potential: 0 - -350 mV, depending on how the
 20 process is carried out;
 Gas atmosphere: H₂/CO₂ 10:90 to 90:10, in particular 80:20;
 Fermentation pressure: 0.2 - 3 bar, in particular 0.5 to 1 bar.

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Essential media constituents: arginine, citrulline, yeast extract, peptones, haemin and haemin-containing substances, lower fatty acids, mineral solution, carbonate buffer (sodium carbonate + CO₂), optionally glucose, trace element solution, vitamin solution and reducing agent.

Various ways of carrying out the fermentation process can be selected here

10 1) Batch fermentation of the pure culture DSM 11798:

Procedure: sterilization of the medium at 121°C and 1.5 bar or sterile filtration. Cooling of the medium to fermentation temperature of 35-42°C, in particular 37°C, while gassing with sterilized CO₂ and addition of sodium carbonate and reducing agent. The gassing is continued until a pH of 6-8, in particular of 7-7.5, is achieved. Subsequent addition of 1-10% of inoculum which was precultured for 24-48 h, in particular 5%. Fermentation until the start of the stationary phase - duration approximately 20-50 h, depending on the substrate concentration or until a microorganism count in the range from 10¹³-10¹⁶ is achieved.

The process is essentially controlled by the substrate concentration

2) Fed-batch fermentation of the pure culture DSM 11798:

Procedure: sterilization, buffering, reduction and inoculation of the medium as in 1. Increase in the biomass yield by means of batchwise or continuous addition of substrate, e.g. arginine, citrulline. The culture is kept in the exponential growth phase by keeping the substrate concentration at a relatively high level. A fermentation time of up to 60 h is possible using this method of carrying out the process.

The process is controlled by the substrate addition and fermentation time (accumulation of metabolic final products).

- 5 3) Continuous fermentation of the pure culture DSM 11798:

Procedure: sterilization, buffering, reduction and inoculation of the medium as in 1. Batch fermentation up to the start of the stationary phase then conversion
10 to continuous fermentation by means of addition of sterile nutrient solution. The effluent is collected in a storage tank and worked up batchwise or continuously spray-dried.

- 15 4) Fermentation of the pure culture DSM 11798 in coculture with other facultative and strictly anaerobic microorganisms or fermentation of the coculture DSM 11799

20 Examples of cocultures which can be employed:

H₂ producers DSM 11798 + Butyrivibrio sp.

 DSM 11798 + Ruminococcus sp.

Probiotics DSM 11798 + Enterococcus casseliflavus
 = DSM 11799

25 DSM 11798 + Streptococcus sp.
 (enterococci, lactic acid
 streptococci, anaerobic streptococci)

 DSM 11798 + Leuconostoc sp.

 DSM 11798 + Pediococcus sp.

30 DSM 11798 + Lactobacillus sp.

 DSM 11798 + Bifidobacterium sp.

 DSM 11798 + Bacillus sp.

 DSM 11798 + Megasphaera sp.

Yeasts DSM 11798 + Saccharomyces sp.

35 DSM 11798 + Klyveromyces sp.

 DSM 11798 + Candida sp.

Use of co-organisms in the fermentation serves on the one hand to reduce the redox potential in the fermentation, to produce hydrogen for DSM 11798 and as a protective organism in the work-up and stabilization. A minimization of microorganism count losses of DSM 11798 takes place here and they serve in some cases as additional productivity promoters in animal production.

4a) Batch fermentation in coculture:

I) Preculturing of the co-organism on carbohydrate-containing medium. The medium described above, which, however, contains no reducing agent but additionally carbohydrates for this purpose, is used for reducing the redox potential. Subsequent inactivation of the coorganism and inoculation of DSM 11798.

II) Simultaneous inoculation of the co-organism and DSM 11798 and addition of 0.1-1% carbohydrate to the medium. The medium described above, which, however, contains no reducing agent but additionally carbohydrates for this purpose, is used. The growth of the co-organism is promoted - rapid fall in the redox potential - DSM 11798 begins to grow on account of the ideal growth conditions.

III) In combination with I + II: at the end of the fermentation addition of carbohydrates for refermentation of the co-organism. This leads to a protective effect (oxygen) in the work-up and stabilization on account of the increased biomass yield.

4b) Fed-batch fermentation in coculture:

*) batch phase corresponding to 4aI, subsequently continuous/batchwise addition of substrate (arginine, citrulline) corresponding to 2.

**) batch phase corresponding to 4aI, subsequently continuous/batchwise addition of a substrate combination (arginine/carbohydrates or citrulline/carbo-hydrates).

5 4c) Continuous fermentation of the coculture:

Batch phase corresponding to 4aI, subsequent conversion to continuous fermentation by means of addition of an arginine/carbohydrate- or citrulline/carbohydrate-containing nutrient solution. Work-up as in 3.

10

Work-up of the fermentation products:

1) Concentration by membrane filtration processes (ultrafiltration, microfiltration) or centrifugation. Subsequent spray-drying or lyophilization with or
15 without organic and/or inorganic carrier materials.

2) Direct spray-drying or lyophilization with or without organic and/or inorganic carrier materials.

3) Continuous spray-drying of the fermentation broth with or without organic and/or inorganic carrier
20 materials.

4) Encapsulation or pelletization in combination with 1, 2 or 3.

To check the activity of the DON-biotransforming strain (DSM 11798) in the intestinal medium, an *in vitro* model using pig's intestine was developed.
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In this connection, in a 1st experiment an *in vitro* model with intestinal contents in buffer with addition of lyophilizate was developed.

The small intestine and large intestine of a
30 freshly slaughtered pig were kept under a CO₂ atmosphere. The emptying of the contents of the sections of anterior, median and posterior small intestine and large intestine into individual vessels was carried out under a CO₂ atmosphere.

20 ml of anaerobic buffer + 1 g of intestinal content (CO₂ gassed) + DON were incubated at 37°C under a CO₂ or H₂/CO₂ atmosphere.

5 It was found here that in the anterior section of the small intestine a markedly positive effect can be noted on addition of active lyophilizate. The fact is particularly significant here that the marked activity could also be detected under pure CO₂ gassing.

10 A 2nd *in vitro* experiment was carried out using complete pieces of pig's intestine with addition of active suspension.

Pieces of pig's intestine (anterior, median, posterior, large intestine, each 6-8 cm) were incubated for 24 h with 200 ppm of DON in anaerobic, reduced, 15 preincubated buffer (30 ml) at 37°C.

This modified *in vitro* test variant has the advantage that the physiological condition of the intestine is scarcely affected, since immediately after slaughter the entire intestine is transported into the 20 laboratory and pieces of desired length are tied off there. The pieces are then separated off and incubated in buffer solution with DON and active culture.

The results are clear. The deepoxidation of DON to DOM-1 can be achieved by means of the active culture. 25 It is significant that this activity can be demonstrated in all sections of the intestine, the highest activity being particularly to be found in the anterior intestine. This is important insofar as the major part of the food absorption and thus also the 30 release of the mycotoxins likewise takes place there.

The action of the microorganism according to the invention both in pure culture (DSM 11798) and in mixed culture of the microorganism and *Enterococcus casseliflavus* (DSM 11799) as well as other anaerobic micro- 35 organisms, in particular of the genus *Enterococcus*, *Streptococcus*, *Lactococcus*, *Bacillus* or *Lactobacillus*

will be demonstrated subsequently with the aid of a laboratory protocol concerning chicken cell cultures and in feeding examples on pigs and chickens.

With the aid of a laboratory protocol using
5 chicken cell cultures, it is shown for the microorganism that this is able to chemically degrade mycotoxins, in particular trichothecenes, and especially deoxynivalenol and T-2 toxin, in particular to reduce
10 them and convert them into physiologically acceptable substances, in the case of deoxynivalenol into the deepoxymetabolite thereof, namely DOM-1.

To culture the chicken lymphocytes, the following conditions were adhered to:

15 Cell numbers used: 2×10^6 cells/ml
 Stimulation: ConA 5 μ g/ml
 Mycotoxins: DON, DOM-1 and T2 toxin,
 Concentration range: DON: 10-0.08 μ g/ml
 DOM-1: 232-1.81 μ g/ml
20 T2 toxin: 30-0.234 ng/ml

Total incubation time: 44 hours, of which 16 hours labelling time during culturing in an incubator: 40°C, 5% CO₂, saturated water vapour atmosphere.

25 With the aid of a laboratory protocol using chicken lymphocyte cell cultures, it is shown for the active culture that this is able to biochemically degrade mycotoxins, in particular trichothecenes, in particular to reduce them, and to convert them into
30 physiologically acceptable substances. This is shown in the following by way of example of DON and its deepoxymetabolite DOM-1:

Microscopic checking of the cell culture:

The cell culture containing the chicken lymphocytes was continuously microscopically checked during the culturing.

5 Checking after 20 hours:

The unstimulated cells are thickly and uniformly distributed, the checks with ConA show powerful stimulation and pronounced proliferation foci.

10 DON: proliferation foci are seen in all concentration stages, it being remarkable, however, that in a concentration range of 10 $\mu\text{g/ml}$ -0.625 $\mu\text{g/ml}$ marked reductions of the proliferation foci are to be observed with increasing toxin concentration.

15 DOM: proliferation foci in all concentration stages without an apparent change in comparison with the control up to the highest concentration stage of 58 $\mu\text{g/ml}$.

20 This means that even after 20 hours a marked adverse effect on the cell activity in the toxin batch is present from a concentration of 0.625 $\mu\text{g/ml}$, while with the deepoxymetabolite itself no negative effects on the cell culture were seen even at a concentration of 58 $\mu\text{g/ml}$.

Checking after 28 and after 44 hours:

25 The control batches (unstimulated and ConA) are unchanged.

30 The action of the DON on the cell culture has additionally increased. In those series in which a change was found even after 20 hours, marked damage to the cells was seen.

Even after these incubation times, it was not possible to find any adverse effect on the cell activity even at a concentration of the deepoxymetabolite of 58 $\mu\text{g/ml}$.

35 In a second experiment, the action of a feedstuff additive according to the invention in a feedstuff comprising the mycotoxin deoxynivalenol in an amount of

450 ppb which was fed to weaned piglets, was investigated.

Animals: Piglets of the breed "Large White" and "Landrasse" were divided into negative comparison groups, positive comparison groups and test groups. The experiment started directly after weaning (age of the piglets 20 to 22 days); the productivity parameters of the animals were determined 14 days later.

Feed: A commercially available piglet starter was fed to the pigs 7 days after weaning, after which they received a commercially available piglet growth feed. The mycotoxin deoxynivalenol was mixed with a small aliquot of the feed in a concentration of 450 ppb (dissolved in ethanol) in order to introduce it into the feed of the positive comparison group and the test group. The feed was given ad libitum.

Dose of the feedstuff additive: A feedstuff additive was added to the feed of the test group in a dose of 1 kg/t. The feedstuff additive used was a mixed culture of the microorganism according to the invention with *Enterococcus casseliflavus* (DSM 11799), aluminium silicate being added to the mixed culture as a carrier.

Microorganism count: 4×10^8 cells/kg of finished feedstuff

Results: The results are summarized in Table 1, which shows the average growth in weight and the average feed conversion rate in this test, the negative comparison group being fed with feed comprising neither mycotoxin nor the microorganism according to the invention, the positive comparison group being fed with feed comprising only mycotoxin, and the test group being fed with feed comprising mycotoxin and microorganism according to the invention.

Table 1

	Negative comparison group	Positive comparison group	Test group
Number of animals	45	45	45
Test period (d)	14	14	14
Weight increase/ animal/day (g)	154.0	126.2	153.2
Feedstuff intake/animal/day	247	226.3	246.5
Losses (total)	0	0	0
FCR	1.60	1.79	1.61

Discussion: It is evident from this test that the
5 feedstuff additive was capable of compensating the
adverse effect of the deoxynivalenol contamination on
weaned piglets, and that the piglets which received
both the mycotoxin and the feedstuff additive consumed
essentially the same amounts of feedstuff as the nega-
10 tive comparison group and also showed an identical feed
conversion rate (FCR). These results make it clear that
it was almost completely possible to compensate the
negative effect of deoxynivalenol by the mixed culture
according to the invention (DSM 11799).

15 In a further test, the effect of the feedstuff
additive according to the invention against contamina-
tion with trichothecenes was shown in a chick feed. The
parameters used were the final weight, the feed intake
and the feed conversion rate as well as the losses of
20 chicks. The clinical symptoms were also recorded.

Animals: Chicks of the breed Cobb were investigated
from the first day of life onwards. The test was
carried out using three groups, comprising 10,700,
10,900 and 15,700 chicks. A specific chicken feed was
25 administered ad libitum to the chicken.

Dose of the feedstuff additive: The feedstuff additive was contained in a dose of 1 kg/t of chick feed, only the test group receiving the feedstuff additive. The feedstuff additive employed was a pure culture of the microorganism (DSM 11798).

Microorganism count: 1×10^9 cells/kg of finished feed

Trichothecenes were administered in the feedstuff to the test group and the positive comparison group in a total amount of 750 ppb (500 ppb of DON and 250 ppb of T-2 toxin).

Results: The following table shows the productivity parameters of all three groups.

Table 2

	Positive comparison group	Negative comparison group	Test group
Number of animals	10,700	10,900	15,700
Mean final weight in kg	1.806	1.91	1.92
Mean feedstuff intake in kg	3.161	2.729	2.722
Feed conversion rate (FCR)	1.75	1.43	1.42
Losses	205 (1.92%)	175 (1.61%)	248 (1.58%)

Clinical observations: Marked oral irritation was evident in many animals of the positive comparison group.

Discussion: Even in the present case, the feedstuff additive was capable of completely compensating the adverse effect of the trichothecenes on the poultry in relation to the productivity parameters and the clinical symptoms. Even in the case of the poultry, it is

seen that chicks of the test group, which also received the microorganism DSM 11798 in addition to mycotoxins, even had a slightly higher mean final weight than the negative comparison group, and this with a slightly
5 lower mean feedstuff intake, as a result of which somewhat improved productivity parameters result even in relation to the negative comparison group. On using the feedstuff additive according to the invention comprising the microorganism according to the invention,
10 this shows that not only the adverse effect of the mycotoxins was compensated, but it was possible to achieve a further increase in productivity in the animals which received the microorganism DSM 11798.



C l a i m s (Original)

1. Microorganism of the genus Eubacterium, DSM 11798, which in pure culture, in mixed culture with the strain Enterococcus casseliflavus, DSM 11799, or in mixed culture with other anaerobic microorganisms is suitable for the detoxification of trichothecenes.

2. Microorganism according to claim 1, characterized in that the anaerobic microorganisms are selected from the genus Enterococcus, Streptococcus, Lactococcus, Bacillus or Lactobacillus.

3. Microorganism according to claim 1 or 2, characterized in that it is suitable for the detoxification of deoxynivalenol (DON), T-2 toxin, HT-2 toxin, nivalenol, monoacetoxyscirpenol, diacetoxyscirpenol, trichodermol, verrucarol, rorodin, acetyldeoxynivalenol, isotrichodermin, hydroxyisotrichodermin, calonectrin, T-2 tetraol, T-2 triol, deacetylneosolaniol, neosolaniol, acetylneosolaniol, sporotrichiol, trichotriol, sambucinol and culmorin.

4. Microorganism according to claim 1, 2 or 3, characterized in that the microorganism detoxifies the trichothecenes by reductive biotransformation of the epoxide group which is contained in the molecule.

5. Microorganism according to one of claims 1 to 4, characterized in that it is an anaerobic gram-positive, rod-shaped, non-spore-forming bacterium which occurs individually, in pairs or in long chains.

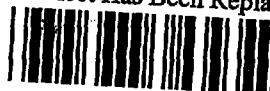
6. Microorganism according to one of Claims 1 to 5, characterized in that it contains a 16S RNA sequence, namely

1 CCTGGCTCAG GATGAACGCT GGCGGCGTGC TTAACACATG
CAAGTCGAAC GGATAACCCG

61 CCTCCGGGCG GTTATAGAGT GGCGAACGGG TGAGTAACAC
GTGACCAACC TACCTCCCAC

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121 TCCGGGATAA CCCAGGGAAA CCTGCGCTAA TACCGGATAC
TCCGGGGCCC CCGCATGGGG
181 GCGCCGGGAA AGCCCCGACG GTGGGAGATG GGGTCGCGGC
CTATTAGGTA GTCGGCGGGG
5 241 TAACGGCCCA CCGAGCCCGC GATAGGTAGC CGGGTTGAGA
GACCGATCGG CCACATTGGG
301 ACTGAGATAC GGCCCAGACT CCTACGGGAG GCAGCAGTGG
GGAATTTTGC GCAATGGGGG
361 AAACCCTGAC GCAGCAACGC CGCGTGCGGG ACGAAGGCCT
10 TCGGGTTGTA AACCGCTTTC
421 AGCAGGGAAG AAGTTGACGG TACCTGCAGA AGAAGCTCCG
GCTAACTACG TGCCAGCAGC
481 CGCGGTAATA CGTAGGGAGC GAGCGTTATC CGGATTTATT
GGGCGTAAAG CGCGCGTAGG
15 541 CGGGCGCTTA AGCGGAATCT CTAATCTGAG GGCTCAACCC
CCAGCCGGAT TCCGAAC TGG
601 GCGCCTCGAG TTCGGTAGAG GAAGACGGAA TTCCCAGTGT
AGCGGTGAAA TGCGCAGATA
661 TTGGGAAGAA CACCGATGGC GAAGGCAGTC TTCTGGGCCG
20 TAACTGACGC TGAGGTGCGA
721 AAGCTAGGGG AGCGAACAGG ATTAGATACC CTGGTAGTCC
TAGCCGTAAA CGATGGGCAC
781 TAGGTGTGGG GGGGAATGCC CCTCCGTGCC GCAGCTAACG
CATTAAGTGC CCCGCCTGGG
25 841 GAGTACGGCC GCAAGGCTAA AACTCAAAGG AATTGACGGG
GGCCCGCACA AGCAGCGGAG
901 CATGTGGCTT AATTCGAAGC AACGCGAAGA ACCTTACCAG
GGCTTGACAT GCAGGTGAAG
961 CGGCGGAAAC GCCGTGGCCG AGAGGAGCCT GCACAGGTGG
30 TGCATGGCTG TCGTCAGCTC
1021 GTGTCGTGAG ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA
CCCCTGTCGT ATGTTGCCAT
1081 CATTCAGTTG GGGACTCGTA CGAGACTGCC GCGTCAAGC
CGGAGGAAGG TGGGGACGAC
35 1141 GTCAAGTCAT CATGCCCTTT ATGCCCTGGG CTGCACACGT
GCTACAATGG CCGGTACAAC

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1201 GGGCTGCGAG CCAGCGATGG CGAGCGAATC CCTCAAAACC
GGTCCCAGTT CGGATCGGAG

1261 GCTGCAACCC GCCTCCGTGA AGTCGGAGTT GCTAGTAATC
GCGGATCAGC ATGCCGCGGT

5 1321 GAATACGTTC CCGGGCCTTG TACACACCGC CCGTCACACC
ACCCGAGTTG TCTGCACCCG

1381 AAGTCGACGG CCCAACCCGC GAGGGGGGAG TCGCCGAAGG
TGTGGGGAGT AAGGGGGGTG

1441 AAGTCGTAAC AAGGTAGCCG TACCGGAAGG TCGGGCT,

10

7. Process for obtaining a culture of the micro-
organism according to one of claims 1 to 6, characteri-
zed in that a mixed culture DSM 11799 is obtained from
the microorganism and Enterococcus casseliflavus from
15 bovine rumen by culturing and/or fermenting at least
twice in dilution series and anaerobic culturing condi-
tions.

8. Process according to claim 7, characterized in
that the culturing and/or fermentation is carried out in
20 a gas atmosphere of H₂ and CO₂.

9. Process according to claim 8, characterized in
that the gas atmosphere is selected with a ratio of
H₂:CO₂ of 10:90 to 90:10, in particular of approxima-
tely 80:20.

25 10. Process according to one of claims 7 to 9,
characterized in that the culturing and/or fermentation
is carried out at an overpressure of 0.2 to 3 bar, in
particular 0.5 to 1 bar.

11. Process according to one of claims 7 to 10,
30 characterized in that the culturing or fermentation is
carried out at a temperature of 35 to 42 °C, in parti-
cular 37 °C.

12. Process according to one of claims 7 to 11,
characterized in that the culturing and/or fermentation
35 is carried out at a pH between 6 and 8, in particular
at a pH between 7 and 7.5.

13. Process according to one of claims 7 to 12,



characterized in that the culturing and/or fermentation is carried out in a media preparation comprising components selected from amongst: arginine, citrulline, peptone, yeast extract, fatty acid mixture(s), mineral solution(s), glucose, haemin solution, menadione, vitamin solution, trace elements, reducing agents.

14. Process according to one of claims 7 to 13, characterized in that 0.1 to 0.5 % by weight, in particular 0,2 % by weight, of glucose is added at the start of the culturing and/or fermentation.

15. Process according to one of claims 7 to 14, characterized in that enzyme preparations of the active, trichothecene-detoxifying microorganism and/or other anaerobic microorganisms are added.

16. Process according to one of claims 7 to 15, characterized in that the pure culture of the microorganism DSM 11798 is obtained from the culturing and/or fermentation solution by at least two further dilution series in the media preparation, in particular adding L arginine as a growth stimulator.

17. Process according to claim 16, characterized in that, for the fermentation of the pure culture of the media preparation, 1 to 4 % by weight of a reducing agent, in particular a mixture of cysteine/sodium sulphide/sodium carbonate in solution, is added.

18. Process according to one of claims 7 to 17, characterized in that the culturing and/or fermentation solution is worked up by concentrating, in particular by centrifuging or filtering and/or stabilizing, in particular by freeze-drying or spray-drying or encapsulating.

19. Process according to claim 18, characterized in that the stabilizing is carried out with addition of a filler or carrier material, such as aluminium silicates, kieselguhrs, carbohydrates, sugar alcohols, starches, milk and whey powder, protein hydrolysates, yeasts, PVPP.

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20. Use of the microorganism in pure and/or mixed culture (DSM 11798, DSM 11799) for the preparation of a feedstuff additive.

21. Use according to claim 20, characterized in that the pure and/or mixed culture (DSM 11798 and DSM 11799) is employed as a freeze- or spray-dried and/or encapsulated immobilizate, potentially by adding a carrier material.

22. Feedstuff additive for the inactivation of trichothecenes in feedstuffs or in the digestive tract of animals, characterized in that the feedstuff additive contains a pure and/or mixed culture of a microorganism according to one of claims 1 to 6 or a microorganism prepared according to one of claims 7 to 19, in an amount from 10^5 to 10^{12} cells/kg, in particular 10^7 to 10^9 cells/kg, per 1000 kg of feedstuff.

23. Feedstuff additive according to claim 22, characterized in that it additionally contains a carrier material and/or filler in an amount from 0.5 to 8 kg/1000 kg, in particular 0.7 to 4 kg/1000 kg, of the feedstuff.

24. Feedstuff additive according to claim 23, characterized in that aluminium silicates, kieselguhrs, carbohydrates, sugar alcohols, starch, milk and whey powder, protein hydrolysates, yeasts and/or PVPP are contained as a carrier material or filler.

25. Feedstuff additive according to claim 22, 23 or 24, characterized in that the feedstuff additive consists of a mixture of 1 to 65 % by weight, in particular 5 to 50 % by weight, of the spray- or freeze-dried immobilizate of the microorganism and 99 to 35% by weight, in particular 95 to 50 % by weight, of carrier material and/or filler.

26. Use of a feedstuff additive according to one of claims 22 to 25 for the inactivation of deoxynivalenol (DON), T-2 toxin, HT-2 toxin, nivalenol, monoacetoxyscirpenol, diacetoxyscirpenol, trichodermol, verru-



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carin, rorodin, acetyldeoxynivalenol, isotrichodermin, hydroxyisotrichodermin, calonectrin, T-2 tetraol, T-2 triol, deacetylneosolaniol, neosolaniol, acetylneosolaniol, sporotrichiol, trichotriol, sambucinol and culmo-
5 rin in a feedstuff or the digestive tract of animals.

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