PULLULAN

First draft prepared by

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1.   EXPLANATION

Pullulan is a naturally occurring, fungal polysaccharide produced by fermentation of liquefied corn starch by *Aureobasidium pullulans*, a ubiquitous yeast-like fungus. It has a linear structure consisting predominantly of repeating maltotriose units, which are made up of three α-1,4-linked glucose molecules (Wallenfels et al., 1965; Catley, 1971; Carolan et al., 1983), linked by α-1,6-glycosidic bonds. The maltotriose units are interspersed with about 6% maltotetraose units consisting of four α-1,4-linked glucose molecules; rarely, branch points occur, at which poly-maltotriosyl side-chains are attached to the main chain by a 1,3-glycosidic bond (Figure 1; Sowa et al., 1963; Catley et al., 1986).

Pullulan is used as a glazing agent, as a film-forming agent, as a thickener or as a carrier in the production of capsules for dietary supplements as a substitute for gelatin, coatings for coated tablets containing dietary supplements, for production of edible flavoured films used as breath fresheners, and in the production of jams and jellies, confectionery and some meat and fruit products. It is also used as a texturizer in chewing-gum and as a foaming agent in milk-based desserts (Sugimoto, 1978; Wiley et al., 1993; Gibbs & Seviour, 1996; Madi et al., 1997; Lazaridou et al., 2002).

Figure 1. Structure of pullulan

Maltotriose   Maltotriose
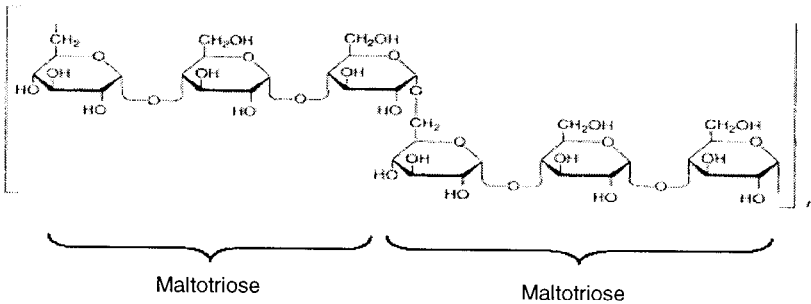
        The Codex Committee on Food Additives and Contaminants at its Thirty-sixth Session (Alinorm 04/27/12) asked the Committee to review pullulan. This substance has not been evaluated previously by the Committee.
        Pullulan is produced by fermentation from a food-grade hydrolysed starch with a non-toxin-producing strain of *Aureobasidium pullulans*. After fermentation, the fungal biomass is removed by microfiltration, the filtrate is heat-sterilized, and pigments and other impurities are removed by adsorption and ion-exchange chromatography. The product contains not less than 90% glucan on a dried basis. The main impurities are mono-, di- and oligosaccharides from the starting material. The average relative molecular mass of pullulan varies considerably, depending on culture conditions. A commercially available product has an average relative molecular mass of 200 000 Da.
        Pullulan is stable in aqueous solution over a wide pH range (pH 3–8). Pullulan decomposes upon dry heating and carbonizes at 250–280 °C. It dissolves readily in water but is insoluble in organic solvents. Aqueous solutions of pullulan are viscous but do not form gels. Upon drying, pullulan forms transparent, water-soluble, fat-resistant, odourless, anti-static, flavourless films.

2.   BIOLOGICAL DATA

2.1   Biochemical aspects

2.1.1   Absorption, distribution, biotransformation and excretion

        Early experiments on the digestibility of pullulan by human salivary amylase and hog pancreatic amylase in vitro demonstrated that pullulan is either hydrolysed either slowly or not at all by these enzymes (Ueda et al., 1963; Wallenfels et al., 1965).
        In another study on the digestibility of pullulan, two samples of relative molecular mass of 50 000 and 200 000 Da were treated sequentially with human salivary amylase, porcine pancreatic amylase, artificial gastric juice and an enzyme preparation from the small intestinal mucosa of rats. The 50 000-Da pullulan was

hydrolysed by the intestinal enzymes to produce 2.7% glucose but was not affected by the other treatments. The 200 000-Da pullulan was sequentially converted to substances of lower relative molecular mass (average, 70 000 Da) after treatment with the intestinal enzyme preparation; no glucose was released after salivary or pancreatic amylase treatment, but a small increase in reducing sugar content was observed (0.6% and 0.7%, respectively). A 6.6% increase in glucose concentration was observed after digestion with the intestinal enzyme preparation. The authors suggested that glucose is formed as a result of hydrolysis of the α-1,4-glycosidic bond from the non-reducing end of the molecule, but that the hydrolysis stops at the α-1,6-glycosidic bond. The amount of glucose released from pullulan standards (relative molecular mass, 990–380 000 Da) digested in vitro in the small intestinal enzyme preparation ranged from 1.5% (relative molecular mass, > 100 000 Da) to 36.3% for the smallest sample (990 Da) (Okada et al., 1990).

In a study to determine the digestibility of pullulan film (relative molecular mass unspecified), a 1% pullulan solution was treated with an enzyme mix containing α-amylase, amyloglucosidase, peptidase, protease, invertase and lipase for up to 2 h. About 9% pullulan, < 5% levan powder and cellulose film and > 90% starch powder and starch film were hydrolysed (Kunkel & Seo, 1994).

In another study of the digestion of pullulan (relative molecular mass, 10 000 Da), 37% raw pullulan and 42% cooked substance were hydrolysed in vitro by a-amylase and amyloglucosidase within 30 min. Control samples of raw and cooked maltodextrin were completely hydrolysed during this time. Hydrolysis of the remaining pullulan proceeded more slowly, reaching 95% completion after 5 h (Wolf et al., 2003).

In a further study on the digestion of orally administered pullulan, five fasted male Wistar rats were given 2 ml of a 10% pullulan solution in 0.9% saline by gavage. The pullulan used in this study had a relative molecular mass of 49 000 Da and consisted of 302 glucose molecules; 93% of the glucose units were in the form of maltotriose and 7% in the form of maltotetraose. The animals were killed 1 h after gavage, and the contents of their stomachs and small intestines were collected, homogenized and analysed for glucose to determine the extent of pullulan hydrolysis. The glucose concentrations in homogenates of pullulan-treated animals suggested that about 3% of the pullulan had been hydrolysed; however, it was not known if the hydrolysis products of pullulan were absorbed by the small intestine. The finding that about 3% pullulan was hydrolysed was close to the estimate that 2.5% would be hydrolysed, on the basis of 7% maltotetraose units containing one α-1,4-glycosidic bond susceptible to amylase. Nevertheless, low glucoamylase activity was present in the intestinal tract, which can slowly hydrolyse α-1,4- and α-1,6-glycosidic bonds from the non-reducing end (Oku et al., 1979).

In a study to determine the rate and extent of disappearance of starch from the small intestine, groups of seven Sprague-Dawley rats (two groups per treatment) were fed pullulan, cornstarch, maltodextrin, modified maltodextrin or amylomaize and then killed; their small intestines were then removed and clamped to give 15 equal-sized portions. The contents of the small intestines were expressed and precipitated in ethanol. Starch disappearance, expressed as total starch, was measured in each of the 15 intestinal segments. Pullulan (average relative molecular mass, 10 000 Da) disappeared gradually, reaching a maximum disappearance of 81.4 g/100 g pullulan at segment 13. The authors noted that, with this method, all

pullulan and its products that are soluble in ethanol would be considered to be digested and that the estimate of pullulan digestion might be exaggerated (Bauer et al., 2003).

In a study on the effects of caecal microflora on pullulan, the concentration of short-chain fatty acids was significantly greater in rats fed diets containing 10% pullulan for 4 weeks than in control rats fed diets containing 5% cornstarch (Sugawa-Katayama et al., 1994).

Another study showed that pullulan (average relative molecular mass, 50 000 Da) is fully digested in human faecal cultures within 4–8 h, yielding a maximum of 52.7 g short-chain fatty acids/100 g pullulan (mainly acetic, propionic and butyric acids). The energy value for pullulan was estimated to be 2.05 kcal/g, assuming absorption of 100% short-chain fatty acids; however, with increasing pullulan intake, it is unlikely that all short-chain fatty acids produced will be absorbed (Okada et al., 1990).

In a study of the digestion of pullulan by intestinal bacteria, the compound was not detected in the faeces of six volunteers who had consumed 10 g pullulan (relative molecular mass, 50 000 Da) daily for 14 days. The short-chain fatty acid concentration in the faeces increased from 6 mg/g to 8.8 mg/g faeces. The authors concluded that pullulan is completely fermented to short-chain fatty acids by intestinal bacteria (Yoneyama et al., 1990).

The effect of pullulan on the postprandial glycaemic response of healthy non-diabetic adults was compared with that of maltodextrin, a rapidly absorbed starch that normally elicits a high glycaemic response. In a randomzed, double-blind, two-period, two-treatment, cross-over meal tolerance test, 28 volunteers (19 men and 9 women) were asked to eat a high-carbohydrate diet for 3 days and to avoid exercise for 24 h before testing. After an overnight fast, the volunteers were given a sterilized flavoured drink containing either 50 g pullulan (relative molecular mass, 100 000 Da) or 50 g maltodextrin. Blood glucose was measured in finger-prick blood before treatment, every 15 min for the first hour and then every 30 min up to 180 min. Carbohydrate absorption was measured by analysing breath hydrogen every hour for 8 h. The persons were asked to report any symptoms of nausea, abdominal cramping, distension or flatulence for 48 h after treatment. The cross-over treatment was carried out 5–13 days after the first treatment. The postprandial blood glucose concentration (1.97 mmol/l) was significantly lower in persons who ate pullulan than in those who ate maltodextrin (4.24 mmol/l), and the time to peak glucose concentration was delayed in the pullulan-treated group. Carbohydrate malabsorption was greater in persons taking pullulan. Flatulence was the main side-effect and was commonest during the first 24 h after treatment. The authors concluded that pullulan is slowly digested in the human gut (Wolf et al., 2003).

In a study to determine the glycaemic index of pullulan (average relative molecular mass, 200 000 Da) after a 12-h fast, five volunteers (one with non-insulin-dependant diabetes) were given a bolus dose of 25 g pullulan. A 25-g dose of maltose was taken 6 days later as a positive control. Blood glucose concentrations were determined before dosing and 15, 30, 45, 60, 90 and 180 min after dosing. The glycaemic response to pullulan relative to that to maltose was 12.8% when all persons were considered, but the one diabetic person, who had an exaggerated response to maltose, influenced this response. When this person was excluded, the relative glycaemic index for pullulan was 18.6%. Statistical comparisons at each sampling

time showed that the blood glucose values for the maltose control at 30 and 45 min were significantly greater than those for pullulan ($p < 0.01$). The mean area under the curve of concentration:time value for the control (103 ± 37) was significantly greater ($p < 0.02$) than that for pullulan (19 ± 13) (Richards & Higashiyama, 2004).

In 1990, a United States patent indicated that pullulan reduces peak blood glucose concentrations when taken with food products containing starch or sucrose at a ratio of pullulan:starch or pullulan:sucrose of 1:400 to 1:20; however, the effect was not consistent and varied with dose, the relative molecular mass of the pullulan and the age and health of the persons. In the absence of a mechanistic explanation for this finding, the conclusions are questionable (Hiji, 1990).

Another study showed that pullulan had no effect on the blood glucose concentrations (measured every 30 min from 0 to 180 min) of a 39-year-old volunteer given a solution of 50 g glucose containing 0, 5 or 10 g pullulan in 200 ml water (Oku et al., 1983).

Overall, the studies indicate that pullulan is hydrolysed only very slowly by gastrointestinal enzymes but is fermented by intestinal microorganisms to short-chain fatty acids. In humans, the glycaemic response for pullulan relative to maltose was 18.6%.

2.1.2 Effects on enzymes and other biochemical parameters

The possibility that large amounts of indigestible carbohydrate in the diet can decrease vitamin and mineral absorption has been addressed in several review articles. It has generally been accepted that the presence of dietary fibre at recommended levels in the diet does not adversely affect vitamin and mineral status (Kelsay, 1990; Rossander et al., 1992; Gorman & Bowman, 1993). Even when dietary fibre was consumed in large amounts (50 g per day), no adverse effects on mineral absorption or nutrition were observed (Gordon et al., 1995).

One study on the inhibitory effect of pullulan on intestinal calcium absorption was conducted in groups of six male Wistar rats fed diets containing 20% pullulan (relative molecular mass unspecified), another unavailable carbohydrate (cellulose or glucomannin) or a control diet containing cornstarch for 8 weeks. Rats were fasted for 16 h before sacrifice, and then a homogenate of duodenal mucosa was prepared. The calcium-binding activity of the duodenal supernatant was measured, as were serum calcium concentrations. Calcium-binding activity was significantly reduced in animals fed diets containing 20% pullulan; however, there was no significant difference in serum calcium. Alkaline phosphatase and sucrase activity in the duodenum were also reduced (by approximately one-half and one-third, respectively) from that of the control group. Nevertheless, the parameters were similar between groups receiving unavailable carbohydrate-containing diets. The authors suggested that the inhibitory effect of unavailable carbohydrate on intestinal calcium absorption is due partly to loss of calcium-binding proteins by gastrointestinal transit of large amounts of undigested substances (Oku et al., 1982).

2.2 Toxicological studies

2.2.1 Acute toxicity

The acute toxicity of pullulan (quality and relative molecular mass unspecified) was examined in one study in male mice (number per group unspecified). No

information was given on GLP or other guidelines used. The LD_{50} was > 14 g/kg bw (Department of Public Hygiene, 1974a).

2.2.2 Short-term studies of toxicity

Rats

In a study (no information on GLP supplied) on the effects of pullulan on the digestive tract, groups of eight male Wistar rats were fed diets containing 0, 5%, 10%, 20% or 40% pullulan, equivalent to 0, 2500, 5000, 10 000 and 20 000 mg/kg bw per day, for 4 or 9 weeks. Another group of rats were fed diets containing 20% and 40% cellulose as a comparison. Body-weight gains were reduced by day 10 in the rats given 20% or 40% pullulan and by day 20 in that fed 40% cellulose. The weight differences increased throughout the remainder of the study. The weight gain of animals at 5% or 10% and that of rats given 20% cellulose were also lower than those of controls after 7 weeks, although the difference was not statistically significant. Diarrhoea was observed at 40% pullulan, but the number of rats affected and the frequency were not reported. Unlike maltitol-induced diarrhoea, it did not resolve after a period of adaptation and occurred only occasionally throughout the study. The relative weight of the caecum was increased in a dose-related manner in rats given pullulan. In general, the relative weights of the stomach, small intestine and large intestine were increased in treated animals (Oku et al., 1979).

In a study on the toxicity of an unspecified polysaccharide produced by A. pullulans, groups of 10 male and female Wistar rats received an oral dose of 2.5 or 25 mg/kg bw per day of the substance daily for 7 weeks. No adverse effects were reported (Fujii & Shinohara, 1986).

In a study on changes in the colon mucosa of rats fed pullulan, groups of eight 6-week-old male Sprague-Dawley rats were fed diets containing 1% or 10% pullulan (relative molecular mass not specified), equivalent to 500 and 5000 mg/kg bw per day, for 4 weeks. A control group of rats was fed a diet containing 5% cellulose (equivalent to 2500 mg/kg bw per day). Changes in the colon mucosa were analysed by scanning electron micrography and by comparing colon cell sizes (protein:DNA ratios). Scanning electron micrographs of the colons suggested that the haustra coli (pouches formed in the colon by muscle contractions of the colon walls) were broader in the pullulan-fed rats than in the control group. Faecal weight was significantly decreased in a dose-related manner in rats given pullulan. The wet weight of the colon mucosa was significantly increased in rats given 10% pullulan. The mucosal protein content (reported in mg/cm of colon) was decreased in rats at either concentration of pullulan but more markedly in those given 1%, and the DNA content was significantly increased in rats given 10% pullulan. The authors concluded that pullulan decreased the size of colon mucosa cells (Sugawa-Katayama et al., 1993).

In a study conducted according to GLP, groups of 10 SPF Wistar rats of each sex were fed diets containing pullulan (relative molecular mass, 200 000 Da) at a concentration of 0, 2.5%, 5% or 10%, equal to 0, 1960, 4100 and 7900 mg/kg bw per day. The controldiet and those containing the two lower doses of pullulan were supplemented with potato starch (10%, 7.5% and 5%, respectively) to achieve 10% in the diet. The animals were examined daily for clinical signs of toxicity, and body weights and food consumption were recorded at regular intervals. Grip strength

and locomotor activity were measured in week 13. At the end of the 13-week treatment, urine and blood were collected from fasting animals for urinalysis, haematology and clinical chemistry. Animals were killed and organs and tissues examined macroscopically and weighed. Histological examinations were performed on the liver, kidney, caecum, duodenum, colon, ileum, jejunum, rectum, lungs, spleen and mesenteric and mandibular lymph nodes. No deaths occurred, and no treatment-related clinical signs were observed. Food consumption, food use and body weight were similar in all groups. There were some minor changes in grip strength in males, but this was not dose-related. Significantly reduced motor activity ($p < 0.05$) was observed in females at the medium dose (after 45 and 60 min) and in the group at the highest dose (after 60 min). This was reflected in total motor activity and appeared to be related to treatment; however, the changes seemed to reflect physiological phenomena due to unused carbohydrate in the diets, rather than to any toxic effects. Haematological analyses revealed no treatment-related effects. Males and females at the lowest dose showed slightly lower prothrombin activity. Males at the two lower doses showed increased activated partial thromboplastin time, and females at these doses had lower relative reticulocyte counts.

Clinical chemistry parameters showed a number of significant differences, males at the lowest dose having significantly decreased uric acid and sodium, males at the two lower doses significantly decreased cholesterol, phospholipids and potassium, males at the two higher doses significantly decreased calcium, males at the highest dose significantly increased plasma glucose and all treated males having significantly reduced globulin. Females at the two higher doses had significantly increased sodium, and females at the highest dose had decreased triglycerides. The authors reported that these values were within or marginally outside the reference range and therefore not biologically significant. Furthermore, the observed differences were not dose-dependent, nor were they observed in both sexes. There were no changes in urinary parameters in male rats at any dose; however, urine volume was significantly increased in females at the two higher doses (308% and 285% of control, respectively). As there were no concurrent changes in relative densities or in pH values, the urine volume changes were considered by the authors to be artificial rather than treatment-related effects. Dose-dependent increases in absolute and relative caecal weights were found in males and females, which was statistically significant in males at the two higher doses for empty caecum weight and for males at the intermediate dose for full caecum weight. In females, the increase was statistically significant at the highest dose for empty caecal weight, and at the two higher doses for the relative weight of the empty caecum. One male at the lowest, two at the intermediate and one at the highest dose had distended caecums. Other macroscopic findings consisted of renal pelvic dilatation (one male each at the two lower doses, one female at the intermediate dose and two females at the highest dose), diaphragmatic herniation of the liver (one male at the intermediate dose), uterus dilatation (one female each at the two higher doses), and a dark-red, discoloured focus in the thymus of one male at the lowest dose. No test-related microscopic changes were observed on histopathological examination. As caecal hypertrophy is considered to be a physiological response to poorly digested carbohydrates, a dietary level of 10% pullulan (equal to 7900 mg/kg bw per day) was tolerated by male and female rats without toxicological effects (Sommer et al., 2003).

2.2.3 Long-term studies of toxicity and carcinogenicity

In a test for toxicity not conducted under GLP, groups of 15 4-week-old Sprague-Dawley (SD-JCL) rats of each sex were fed diets containing pullulan (relative molecular mass not specified) at a concentration of 0, 1%, 5% or 10%, equivalent to 0, 500, 2500 and 5000 mg/kg bw per day, for 62 weeks. The study was intended to be conducted over 24 months but was terminated at 14 months (62 weeks) owing to high mortality in all groups as a result of infection. Animals were observed daily; body weights and food consumption were recorded weekly. At the end of the study, the animals were killed and blood and urine were taken for analysis. The blood was tested to determine the red blood cell count, haemoglobin concentration, haematocyte count, white blood cell count and percentage, serum aspartate and alanine aminotransferase and alkaline phosphatase activity, serum total cholesterol, serum cholinesterase activity, serum protein, albumin:globulin ratio and blood sugar. Urine was analysed for protein, sugar, ketones, pH and blood. Internal organs were weighed and examined macro- and microscopically.

The high mortality observed in all groups was attributed to pneumonia. Survival to the end of the study appeared to be dose-related in females (87%, 67%, 67% and 40% at the four doses of pullulan, respectively) but not in males (47%, 60%, 27% and 47%, respectively). No significant differences were observed between groups in terms of food intake or body-weight gain. The terminal body weights of males at 1% and 10% were significantly lower than those of the controls; however, this effect was not dose-related (89%, 96% and 91% of control at 1%, 5% and 10% pullulan, respectively). Some significant differences were observed in absolute organ weights. The absolute weight of the brain was decreased in females at the two lower doses and in males at the lowest dose. In females, the absolute weights of the heart, liver, spleen and caecum were increased at some doses; in males, the absolute weights of the heart, liver, kidneys, stomach and submandibular gland were decreased at some doses. No significant differences were reported in relative organ weights. The 46% increase in absolute caecal weight in females at the highest dose was attributed to a physiological response to undigested pullulan. No data were provided on male caecal weights. Post-mortem macroscopic examination of tissues revealed pneumonia and pulmonary abscesses in animals in all groups. Histopatho-logical observations confirmed bronchitis in animals in all groups but did not indicate a dose-related effect. A few statistically significant differences were observed in haematological and clinical chemistry parameters, which were not dose-related. Urine analysis showed no significant differences in any group.

The changes seen between the control and test groups were not consistent or treatment-related; therefore, the NOEL was the highest dose tested, 10% pullulan in the diet (equal to 5000 mg/kg bw per day) (Kotani et al., 1976; Kimoto et al., 1997). The value of this study is limited because of the high mortality in all groups.

2.2.4 Genotoxicity

The results of studies on the genotoxicity of pullulan in vitro and in vivo are shown in Table 1.

Table 1. Results of assays for genotoxicity with pullulan

Test system	Test object	Concentration	Results	Reference
In vitro				
Reverse mutationa	*S. typhimurium* TA1535, TA100, TA1537, TA98	10–10 000 µg/plate	Negative[a]	Hatano Research Institute (1978); Kimoto et al. (1997)
DNA damage	*Bacillus subtilus*	20 mg/plate	Weakly positive[b]	Kuroda et al. (1989)
Chromosomal aberrations	Chinese hamster lung fibroblasts	12 mg/ml	Negative (after 48 h)	Ishidate et al. (1985)
In vivo				
Micronucleus	Mouse bone marrow (ddy mice, killed at 24 h)	1800 mg/kg bw once (intra-peritoneally)	Negative	Ishidate et al. (1988)[b]
		1000 mg/kg bw four times over 24 h (intraperito-neally)	Negative	

[a] In presence and absence of microsomal enzymes from Aroclor-induced rat liver (S9 mix)
[b] Negative and positive control results were not shown in the English summary.

2.2.5   Special studies

(a)   Effects on gastrointestinal microflora

In a study on the effects of pullulan on caecal microflora, 3-week-old male Sprague-Dawley rats were fed a diet containing 10% pullulan, equivalent to 5000 mg/kg bw per day, for 4 weeks. A control group received a diet containing 5% cellulose (equivalent to 2500 mg/kg bw per day). Food intake and body-weight gain were similar for the two groups, but faecal weight was significantly reduced in the group fed pullulan. When the caecal microflora were examined, the relative numbers of *Bifidobacteria* and *Streptococcus* were found to be increased and those of Bacteriodaceae decreased in comparison with controls (Sugawa-Katayama et al., 1994).

When six volunteers were given 10 g of pullulan (relative molecular mass, 50 000 Da) daily for 14 days, the substance was not detected in stool samples, and it was concluded that pullulan is completely fermented by intestinal bacteria. The faecal populations of *Bifidobacteria* increased in five of the six volunteers, from 11.9% to 21.9% (Yoneyama et al., 1990.

(b)   Aurobasidium pullulans

A. pullulans is a ubiquitous, yeast-like fungus. It has been found in soil, on leaves, in lake water, on weathered wood, on latex paint films and synthetic plastic materials, as well as in used cosmetics and on foods such as fruits, cereals, tomatoes and cheese (Cooke, 1961; Durrell, 1967; Zabel & Terracina, 1980; Domsch et al., 1993; Mislivec et al., 1993; Vadkertiová & Sláviková, 1994; Weidenbörner et al., 1997; Webb et al., 1999; Cronin et al., 2000).

(i)   Pathogenicity

In a study in rabbits, intramuscular injection of *A. pullulans* spores produced a nodule at the site of injection. No spread to other sites in the body was observed (Bulman & Stretton, 1974).

Intravenous injection of *A. pullulans* caused infection in the visceral organs of both healthy and immune-suppressed rats (Vishnoi et al., 2002).

A. pullulans has been isolated from humans but appears to occur as an opportunistic infection. *A. pullulans* was associated with fungal peritonitis in five patients receiving continuous ambulatory dialysis. It was also found in blood samples from a small number of immuno-compromised persons (Ajello, 1978; Kaczmarski et al., 1986; Salkin et al., 1986; Pritchard & Muir, 1987; Girardi et al., 1993).

(ii)   Toxicity

Some strains of *A. pullulans* produce aureobasidin A, a cyclic depsipeptide that is toxic to fungi and yeast at low concentrations (0.1–0.5 μg/ml) but has low acute toxicity in mice (LD_{50} > 200 mg/kg bw) (Takesako et al., 1992). When the strain used for production of pullulan was analysed for aureobasidin A activity in *Saccharomyces cerevisiae*, none was detected (limit of detection, 2 ppm) (Hashimoto & Fukuda, 2002).

Two batches of pullulan were examined for the presence of the mycotoxins aflatoxin B_1, B_2, G_1 and G_2, zearalenone, sterigmatocystin and ochratoxin. None was found (Institut Europeen de l'Environment de Bordeaux, 2002).

The acute toxicity of *A. pullulans* in mice and rats is shown in Table 2.

In a study designed to assess the efficiency ratio of different microbial proteins, six male Long-Evans rats were fed a diet containing 27% *A. pullulans* cells (providing 12% crude protein in the diet) for 2 weeks. Their body-weight gain did not differ from that of a group fed a diet containing approximately 27% brewers' yeast. No signs of toxicity were observed (Han et al., 1976).

No signs of toxicity were observed in meadow voles (*Microtus canicaudus*) fed acid-hydrolysed straw that was subsequently fermented by *A. pullulans* for 10 days (Israilides et al., 1979).

(iii)   Allergenicity

A. pullulans spores have been implicated in reactions such as allergic alveolitis and hypersensitivity pneumonia (Woodard et al., 1988; Karlsson-Borga et al., 1989; Kurup et al., 2000; Apostolakos et al., 2001); however, these allergic reactions have not been associated with ingestion of the vegetative form of the fungus. Moreover, there have been no reports over the past 25 years of allergic reactions in persons

Table 2. Acute toxicity of **Aurobasidium pullulans** *administered orally*

Species	Sex	No. animals/ group	LD$_{50}$ (g/kg bw)	Reference
Mouse	Male	Not specified	> 24	Department of Public Hygiene (1974a)[a]
Rat	Male and female	5 per sex	> 20 (66.7% w/v *A. pullulans* lysate)	Ohnishi & Tsukamoto (1996)

[a] No GLP or other guideline specified

exposed occupationally during fermentation of *A. pullulans* or production of pullulan (R. Asakura, personal communication, 2002).

2.3 Observations in humans

In a study of tolerance, 13 volunteers consumed 10 g of pullulan (relative molecular mass, 50 000 Da) daily for 14 days. Before and after pullulan intake, blood pressure and blood components (total, high-density and low-density lipoprotein cholesterol, β-lipoprotein, total fat, phospholipid, neutral fat, Ca, Na, K, Cl, aspartate and alanine aminotransferase activity and blood glucose) were measured in all volunteers. Faecal weight, pH, composition of faecal microflora and short-chain fatty acid concentration were examined in the faeces of six persons. No pullulan was detected in the stool samples, but daily stool weight was increased by 33%, and mean faecal pH was decreased in response to treatment (pH 6.5 before and pH 6.0 after pullulan intake). The faecal populations of *Bifidobacteria* increased in five of the six persons (11.9% total microflora before and 21.9% after treatment), and the short-chain fatty acids concentration increased from 6 to 8.8 mg/g faeces. No significant differences were observed in blood components. Abdominal fullness was the only symptom reported (Yoneyama et al., 1990).

In a study that addressed the effects of pullulan, dextran and soluble starch on bacterial flora, eight male volunteers received 10 g/day pullalan (relative molecular mass unspecified), dextran and soluble starch sequentially for 14 days with a 14-day wash-out period between each treatment. Before and after each 14-day treatment period, the men's faeces were tested for wet weight, relative change in wet weight, pH, total cell count per gram of fresh faeces, bifid bacteria ratio and relative change in bifid bacteria count. There was little difference in faecal bacterial count according to treatment. Pullulan and dextran resulted in increased faecal weight (from 129 g/day to 188 g/day with pullulan and 127 g/day to 144 g/day with dextran), and an increased percentage of bifid bacteria (12% to 25% with pullulan and 13% to 19% with dextran). No adverse effects were reported (Mitsuhashi et al., 1990).

3. DIETARY EXPOSURE

Pullulan is used as a substitute for gelatin in the production of capsule shells, as an ingredient of coated tablets and in edible flavoured films (breath fresheners). In Japan, it is used in a variety of foods, including savoury snacks, nuts and instant fried noodles, as a coating and glazing agent with oxygen barrier properties. Pullulan

is also widely used as an excipient in pharmaceutical tablets (Ministry of Health and Welfare, 1993).

No national assessments of exposure to pullulan were submitted. An estimated daily exposure based on data on the consumption of food supplements in the United Kingdom was submitted by the sponsor (Bär, 2004). The predicted exposures were based on the maximum use of pullulan in three products: capsule shells, tablets and flavoured films (Table 3).

Surveys in the United Kingdom on the consumption of food supplements indicated that 24% of 1724 adults, 14% of 1701 young persons (4–18 years) and 17% of 1675 toddlers (1.5–4.5 years) consumed food supplements (Gregory et al., 1995, 2000; Henderson et al., 2002). As no distinction was made between tablets and capsules, exposure was calculated conservatively, assuming that all supplements were in capsule form except those for toddlers. The 97.5th percentile of estimated exposure was seven capsules per day by adult consumers and two capsules per day by young people. Dietary supplements for children are usually formulated as tablets. If it is assumed that toddlers consume only tablets, with a consumption of seven tablets per day, the estimated 97.5th percentile exposure was 210 mg of pullulan per day. This hypothesis would result in ingestion of $\leq 135 \times 7 = 945$ mg/day pullulan for adults and $135 \times 2 = 270$ mg/day pullulan for young persons. The substitution of some or all of the capsules by tablets would result in lower exposure. The consumption of pullulan by children would be lower than that of adults and would typically not exceed 90 mg/day on the basis of three tablets per day (Bär, 2004).

Similar results were found for 259 adults in France during a survey of consumers of vitamin and mineral supplements (Touvier et al., 2004). Table 4 summarizes the number of capsules consumed by all persons and by consumers of capsules only.

A 'worst-case scenario' was proposed by the European Food Safety Authority (2004) Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food, which assumed that individuals would not normally take more than six food supplement capsules per day and that extreme consumers would not take more than double this amount ($135 \times 12 = 1620$ mg/day pullulan). In addition, the sponsor and the Panel assumed that persons would not consume more than one standard packet of breath-freshening films per day (700 mg pullulan). Therefore, the maximum daily exposure to pullulan for adults was estimated to be about 2.3 g for a person who ingested 12 supplements as capsules and a packet of pullulan strips per day. The actual exposure is likely to be lower. It was also assumed that small children would not consume this product.

Table 3. Estimated maximum levels of use of pullulan

Product	Amount of pullulan	Estimated maximum level of pullulan
Capsule shell (100–150 mg)	15–90%	135 mg per capsule
Tablet (1.2–1.5 g)	2% in tablet	30 mg per tablet
Pullulan-based flavoured film (32 mg per film)	≤ 90%	29 mg per film / 0.7 g per packet (12 films)

Table 4. Numbers of capsules taken per day at different percentiles of intake

Persons	Mean	Standard deviation	5th percentile	25th percentile	50th percentile	75th percentile	95th percentile
All	0.6	1.5	0.0	0.0	0.0	0.0	4.0
Consumers of capsules (21.7%)	2.6	2.3	0.4	0.9	2.0	4.0	7.0

Pullulan is used in Japan in various foodstuffs, at levels ranging from 2 g/kg in ham and sausages to 30 g/kg in processed products; a concentration of 50 g/kg was reported in hard sweets. A conservative estimate of dietary exposure from various foods was made with the budget method, assuming the presence of pullulan at the maximum reported level in a limited fraction of the diet (30 g/kg in 1/16 of the diet, corresponding to 187 g/day). This calculation resulted in a dietary exposure of about 6 g/day. Consumption of sweets by children was considered separately, with consumption figures available in France and the USA, resulting in an estimate of about 2.5 g/day.

5.   COMMENTS

Toxicological data

Pullulan is largely resistant to digestion in the gastrointestinal tract as a result of the occasional presence of 1,3-glycosidic linkages and the high percentage of α-1,6-glycosidic linkages, which are resistant to hydrolysis by salivary and pancreatic amylases. The degree of digestion appears to depend on the relative molecular mass. A commercially available pullulan (relative molecular mass, 200 000 Da) releases only a small amount of reducing sugar after salivary amylase treatment but is converted to a substance with a lower relative molecular mass (about 70 000 Da) after treatment with an intestinal enzyme preparation.

Pullulan is fermented in the colon in vitro and in vivo by intestinal microflora, to produce short-chain fatty acids, although the degree of fermentation depends on the degree of polymerization of the pullulan. In humans, pullulan (relative molecular mass, 50 000 Da) could not be detected in faeces after daily consumption of 10 g for 14 days, suggesting that it was completely fermented. In contrast to maltodextrin, pullulan reduced the glycaemic response in healthy non-diabetic persons.

Although no studies were conducted to examine the effect of pullulan on the bioavailability of vitamins and minerals, there is no evidence from the published literature that similar polysaccharides of high relative molecular mass have adverse effects on vitamin or mineral bioavailability. When fed to rats at 20% in the diet, pullulan reduced intestinal calcium absorption but did not affect serum calcium levels.

The oral LD_{50} of A. pullulans was reported to be > 24 g/kg bw. In rats, a single oral dose of *A. pullulans* lysate at 10 or 20 g/kg bw caused no signs of toxicity. Other studies indicate that *A. pullulans* does not produce toxins and is not toxic when fed to rats.

The oral LD_{50} of pullulan was reported to be > 14 g/kg bw in mice. Short-term studies in rats showed that pullulan has little toxicity. In a 13-week study in rats given diets containing up to 10% pullulan (relative molecular mass, 200 000 Da), no evidence of treatment-related toxicity was found. The study showed a dose-dependent increase in caecum weight (full and empty) as a result of an increased level of poorly digested polysaccharide in the diet. This effect is considered to be a physiological response common to indigestible polysaccharides and of no toxicological significance. The NOEL was 10% in the diet, equal to 7900 mg/kg bw per day, on the basis of the highest dose used in this study. The results of other short-term studies in rats (9 and 62 weeks) support these conclusions. No long-term studies of toxicity or of reproductive toxicity were available on pullulan. Assays for genotoxicity with pullulan in vitro and in vivo assays gave negative results.

In a 14-day study in humans, daily consumption of 10 g of pullulan (relative molecular mass, 50 000 Da) had no adverse effects. The faecal *Bifidobacteria* population and short-chain fatty acid concentration increased, but no other clinical changes were observed. Abdominal fullness was the only clinical symptom reported. After a single dose of 50 g pullulan (relative molecular mass, 100 000 Da), the frequency of flatulence was increased for 24 h.

Assessment of dietary exposure

Pullulan is used as a substitute for gelatin in the production of capsule shells, as an ingredient of coated tablets and in edible, flavoured films (breath fresheners). The amount of pullulan ingested from one unit of each of these products is, respectively, 135 mg per capsule, 30 mg per tablet and 29 mg per film.

Specific data on consumption of food supplements were available from both France and the United Kingdom. For consumers at the 97.5th percentile, the intake of seven capsules per day was reported to correspond to a dietary exposure to pullulan of 950 mg/day. As dietary supplements for children are usually formulated as tablets, the consumption of pullulan by children was estimated to be lower than that of adults and typically not to exceed 90 mg/day on the basis of intake of three tablets per day, as reported in the United Kingdom. If a maximum daily consumption on a regular basis of seven capsules (950 mg/day of pullulan) and of one standard packet of breath-freshening films (700 mg/day of pullulan) is assumed, the maximum daily exposure to pullulan would be 1.65 g.

Pullulan is used in Japan in various foodstuffs, at levels ranging from 2 g/kg in ham and sausages to 30 g/kg in various processed products; use of 50 g/kg was reported in hard sweets. A conservative estimate of dietary exposure from various food by the budget method, assuming the presence of pullulan at the maximum reported level in a limited fraction of the diet (30 g/kg in 1/16 of the diet, corresponding to 187 g/day), resulted in a value of about 6 g/day. Consumption of sweets by children was considered separately, with consumption figures for France and the USA, resulting in an estimate of about 2.5 g/day.

The Committee recognized that the conservative estimates should not be summed.

6.   EVALUATION

The Committee concluded that the current uses of pullulan as a food additive and the studies on its safety provided sufficient information to allocate an ADI 'not specified'.

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