The effect of goat milk yoghurt casein antioxidant activity on histopathology of lung in male *Rattus norvegicus* exposed by 2,3,7,8-tetrachlorodibenzop-dioxin (TCDD)

A E P Haskito\(^1\), C Mahdi\(^2\), M C Padaga\(^1\) and A Roosdiana\(^2\)

\(^1\) Veterinary Public Health Laboratory, Faculty of Veterinary Medicine, Brawijaya University, Malang 65151, Indonesia
\(^2\) Biochemistry Department, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang 65145, Indonesia

*Corresponding author: drherika1989@gmail.com

**Abstract.** Dioxins are highly toxic and carcinogenic compounds. The most dangerous dioxin is 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD). TCDD dioxin can cause health problems that accumulate over time. Goat milk yoghurt casein can reduce free radicals caused by TCDD. Goat milk yoghurt casein contains bioactive peptides that act as antioxidants. The aim of the current research was to determine the effect of goat milk yoghurt casein towards lung histopathology after TCDD exposure. This research was an experimental study using a completely randomized design (CRD). Male *Rattus norvegicus* were divided into 5 groups: K- (negative control), K+ (positive control induced by TCDD 100 mg/kgBW), K1 (given TCDD 100 mg/kgBW and goat milk yoghurt casein 300 mg/kgBW), K2 (given TCDD 100 mg/kgBW and goat milk yoghurt casein 600 mg/kgBW), and K3 (given TCDD 100 mg/kgBW and goat milk yoghurt casein 900 mg/kgBW). Lung were collected for histopathological examination with scoring methods such as vascular congestion, thickening of septa alveoli, and focal necrosis around blood vessels. Quantitative analysis with Kruskal Wallis and Mann-Whitney test showed that goat milk yoghurt casein could prevent lung damage due to TCDD exposure. It can be concluded that goat milk yoghurt casein 900 mg/kgBW can prevent lung damage such as vascular congestion, thickening of septa alveoli, and focal necrosis around blood vessels. Thus, goat milk yoghurt casein could be used as an antioxidant source against TCDD exposure.

1. **Introduction**

Recently, industry undergoes rapid development in Indonesia for both quality and quantity. This is a part of the effort in improving economic growth in Indonesia. The impact of developing industry is the rising environmental damage caused by the produced waste. Relatively serious environmental pollution known caused by dioxin compound contained in wastes originating from chemical industry, uncontrolled garbage burning, and so on. The largest source of dioxin in the environment are from the burning process of products containing hydrocarbon chlorin in the incinerator which contributed 95% of dioxin [1].

Dioxin may enter the body through food chain, skin contact, inhalation, and transplacenta [2,3]. Dioxin poisoning can cause neural disorder, liver cancer, chloracne, functional disorder on kidney and liver, anemia, and immunosuppression. Dioxin poisoning is accumulative, in which toxic effect may
appear years after. Dioxin retention could be detected in liver, heart, spleen, kidney, lung, fat, and muscle tissues as well as in egg, meat carcass, and milk [2].

Dioxin is the common name of a group of prominent pollutant chemicals which belongs to persistent organic pollutants (POPs). Dioxin consists of 210 types of chemicals; 75 types of polychlorinated dibenzo-p-dioxin (PCDD) and 135 types of polychlorinated dibenzofuran (PCDF) [4]. The highest toxic in the dioxin is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [5]. The effects of TCDD’s toxicity for human are in the form of disorders in the nervous system, reproductive system, endocrine system, and degradation of immune system. In addition, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) can cause chloracne (a skin problem symptomized by hyperkeratosis and black spots or blackheads), kidneys problems, liver problems, and anemia. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) also influences oxidative stress [6-8].

Goat milk has long been used as cow milk replacement, especially for those who are at risk to cow milk allergy. Goat milk has various benefits for it contains several bioactive peptides. These peptides are usually still bonded to its natural protein and can be isolated through hydrolysis process. Hydrolysis can occur by the help of lactic acid bacteria (LAB) [9]. Milk fermentation process with the help of LABs produces milk processed product known as yoghurt. The process caused casein to be hydrolyzed by LABs and thus produced bioactive peptides with various biological functions [10]. Biological functions of bioactive peptides have been identified to include as anti-bacteria [11], antioxidant [12], and inhibitor of Angiotensin Convertin Enzyme (ACE) [13].

Intake of antioxidant is among the ways to reduce the negative effect of dioxin. Researches have proven that antioxidant consumption such as Vitamin A, Vitamin E, and Omega 3 fatty acid can prevent toxicity of TCDD exposure [14]. With the information of goat milk yoghurt containing bioactive peptides as antioxidant, there is a need for a research on the benefits of goat milk yoghurt bioactive peptides in inhibiting TCDD toxicity.

2. Materials and Methods

2.1. Material
Yoghurt was made by yoghurt starter containing 3 LAB strains: \textit{L. bulgaricus}, \textit{S. thermophilus}, and \textit{L. acidophilus} (Yogourmet®, Lyo-SAN INC: 500 Aeroparc, C.P. 589, and Lachute, QC. Canada, J8H, 464). Chemical used was TCDD with more than 99% purity (Supelco Analytical Bellefonte, PA, Cat No: 48599). Stock solution of 10 µg/mL TCDD was diluted into 100 ml of corn syrup as solution dosage. Animal model used were 20 male \textit{Rattus norvegicus} of Wistar strain, aged 8-12 weeks, weighing 150-200 grams obtained from animal house D’wistar Bandung. \textit{Rattus norvegicus} were kept in Bioscience Institute, Brawijaya University. Laboratory condition was kept in below normal temperature of 24±2°C and given 12 hours light/dark lighting durations. Acclimatization was conducted for 14 days before the experiment with animal general condition observation occurred every day. Feed and drink were given \textit{ad libitum}.

2.2. Making of goat milk yoghurt and goat milk yoghurt casein preparation
Initially mother working culture was made by adding 0,35 grams of starter powder (Yogourmet® containing \textit{L. bulgaricus}, \textit{S. thermophilus}, and \textit{L. acidophilus} ) into 70 ml of pasteurized goat milk and incubated in 45°C for 4 hours until it reached pH 4,4-4,5. Yoghurt making followed with the addition of working mother culture in 3% concentration into 480 ml into pasteurized goat milk and incubated in 45°C for 4 hours until it reached pH 4,5-5. Goat milk yoghurt was centrifuged in 5°C with 1200 rpm for 5 minutes and then meshed to separate casein and whey. Afterwards, casein was freeze dried to stabilize casein pH. Goat milk yoghurt casein was stored in -20°C until used.

2.3. Goat milk yoghurt casein antioxidant activity test
Goat milk yoghurt casein with 50 µg/mL, 75 µg/mL, 100 µg/mL, 125 µg/mL, and 150 µg/mL concentration were added into 2 mL DPPH 0,1 mM respectively. The solutions were mixed and
incubated in room temperature for 30 minutes in dark room. These solutions were then measured for absorbance with spectrophotometer in λ max 516 nm. The same treatment was done to blank solution containing 2 mL DPPH 0.1 mM and 1 mL methanol p.a.

### 2.4. Research design

This research has been ethically approved with certificate from Research Ethical Committee of Brawijaya University (Komisi Etik Penelitian Universitas Brawijaya—KEP). *Rattus norvegicus* were randomly divided into 5 groups. Each group contained 4 *Rattus norvegicus* as repetition for every treatment. Negative control group (K-) was given standard feed and drink without treatment. Positive control group (K+) was exposed to TCDD 100 ng/kg BW/day. Treatment group 1 (P1) was given goat milk yoghurt casein 300 mg/kg BW/day and exposed to TCDD 100 ng/kg BW/day. Treatment group 2 (P2) was given goat milk yoghurt casein 600 mg/kg BW/day and exposed to TCDD 100 ng/kg BW/day. Treatment group 3 (P3) was given goat milk yoghurt casein 900 mg/kg BW/day and exposed to TCDD 100 ng/kg BW/day. Goat milk yoghurt casein was given orally by being diluted in reverse osmosis water, while TCDD exposure was given orally diluted with corn syrup. The volume of casein solution and TCDD given was 1 ml for each. Treatment was conducted for 21 days and ended with *Rattus norvegicus* euthanized with cervical dislocation.

### 2.5. Lung histopathology analysis

*Rattus norvegicus* was dissected and their lung taken and put into 10% formalin. Lung samples were made into histopathological preparations with Hematoxylen Eosin (HE) and followed by preparations reading under light microscope with 100x magnification. Scoring method used was a modification of Klopfleisch method [15]. The scale and lesions of each variables were measured as presented:

**Table 1. Lung Scoring Method**

| Lesions                  | Score | Information                      |
|--------------------------|-------|----------------------------------|
| Focal necrosis around blood vessels | 0     | There is no necrosis around blood vessels |
|                          | 1     | Fair focal necrosis (2 blood vessels) |
|                          | 2     | Moderate focal necrosis (3 blood vessels) |
|                          | 3     | Multifocal necrosis (>3 blood vessels) |
|                          | 0     | There is no thickening of alveoli septum |
| Thickening of alveoli septum | 1     | Fair thickening of alveoli septum 25% |
|                          | 2     | Moderate thickening of alveoli septum 26-50% |
|                          | 3     | Severe thickening of alveoli septum >50% |
|                          | 0     | There is no vascular congestion |
| Congestion               | 1     | Fair vascular congestion (1/3 blood vessels) |
|                          | 2     | Moderate vascular congestion (½-2/3 blood vessels) |
|                          | 3     | Severe vascular congestion (>2/3 blood vessels) |

### 3. Results

#### 3.1. Goat milk yoghurt casein antioxidant activity

Results obtained from antioxidant activity of goat milk yoghurt showed 4.52 µg/ml. A compound is considered to be very strong antioxidant if its IC\textsubscript{50} value < 50 µg/ml; strong if IC\textsubscript{50} value 50-100 µg/ml; moderate if IC\textsubscript{50} value 250-500 µg/ml; weak if IC\textsubscript{50} value > 500 µg/ml [16].
3.2. Lung histopathology
Features of Rattus norvegicus TCDD caused lung damage prevention by goat milk casein were indicated by lesions in the form of focal necrosis around blood vessels, thickenings of alveoli septum, and the presence of vascular congestion (figure 1).

Figure 1. Lung histopathology of all treatment group (100x magnification; red arrow is congestion; blue arrow is focal necrosis around blood vessels; yellow arrows are thickening of alveoli septa).

Note: K- : Negative control group; K+ : exposed to TCDD 100 ng/kg BW/day; P1 : Treatment with goat milk yoghurt casein 300 mg/kg BW/day and exposed to TCDD 100 ng/kg BW/day; P2 : Treatment with goat milk yoghurt casein 600 mg/kg BW/day and exposed to TCDD 100 ng/kg BW/day; P3 : Treatment with goat milk yoghurt casein 900 mg/kg BW/day and exposed to TCDD 100 ng/kg BW/day.

3.3. Median value of lung histopathology injury analysis result
Based on statistic measurements in scoring data of histopathology preparates from group K-, K+, KP, P1, P2, and P3 based on Kruskal Wallis test followed by Mann-Whitney test, median value of each lesions was obtained (focal necrosis around blood vessels, thickening of alveoli septum, and congestion), as presented:

| Table 2. Median Value of Focal Necrosis around Blood Vessels |
|-------------------------------------------------------------|
| Treatment Group   | Median |
| K-                | 0.2<sup>a</sup> |
| K+                | 0.4<sup>b</sup> |
| P1                | 0.6<sup>a</sup> |
| P2                | 0.4<sup>a</sup> |
| P3                | 0.2<sup>a</sup> |

Note: Different notation showed significant difference between treatment groups (p<0.05)

Table 2 showed that from P1, P2, and P3 group did not significantly differ with K+ group. This may happen because goat milk yoghurt casein administration in the given dosage was able to prevent
pulmonary damage in the form of congestion and thickening of alveoli septum, and thus the more severe damage of focal necrosis around blood vessels did not form. The results showed that P3 group did not significantly differ with K- group (table 3). P1 and P2 groups showed significant difference with P3. This is based on the finding of thickening alveoli septa with leucocyte accumulation.

### Table 3. Median value of thickening alveoli septa

| Treatment Group | Median |
|-----------------|--------|
| K-              | 0,2\(^a\) |
| K+              | 0,4\(^b\) |
| P1              | 0,6\(^{ab}\) |
| P2              | 0,4\(^{ab}\) |
| P3              | 0,2\(^a\) |

Note: Different notation showed significant difference between treatment groups (p<0.05)

Table 4 showed that P2 and P3 group did not significantly differ with K- while P1 group did not significantly differ with K+. This showed that P3 can prevent congestion around pulmonary blood vessels. According to Greaves [17] congestion is an increase of fluid volume in a region which is caused by passive process due to the failure in fluid flow out of the tissue.

TCDD toxicity is mediated by **Arylhydrocarbon Receptor** (AhR). Upon TCDD exposure, activated AhR would move towards nucleus and form dimer compound with Ah Receptor Nuclear Translocator (ARNT). TCDD-AhR-ARNT will bind certain DNA elements, which is DRE. This bonding will increase P450 cytochrome expression, especially CYP1AI and CYPIBI [18]. Cytochrome P450 is involved in the chain of free radical creation. Free radicals which induce oxidative stress can damage several important cellular components; lipid, protein, and DNA. Upon experiencing stress, free radicals trigger several biochemical reactions which cause changes on cellular components. Free radical is an atom or molecules which have one or more unpaired electrons in its orbital [19]. It can damage some vital cellular components; those are fat, protein, and DNA [20].

Basically, the body already has antioxidant system to inhibit free radicals. However, during high oxidative stress condition, the supply of body antioxidant is inadequate and may further cause damage on cell components. Therefore, extra antioxidant from outside of the body is needed, obtained from foods which contain antioxidant or by consuming antioxidant vitamins [21,22]. Peptides from goat milk showed free radical-scavenging activity (capturing free radicals) and electron donor Bioactive peptides contained in goat milk that has been fermented will stabilize superoxide by donating H atom thus preventing cell DNA damage process [23].

### 4. Conclusion

Goat milk yoghurt casein may prevent the rise of proinflammatory cytokine TNF-α and IL-1β level in animal model *Rattus norvegicus* exposed to TCDD and thus can be used as alternative nutrition with antioxidant that can prevent cell damage caused by toxic environmental pollutants.
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