Extensive Literature Search on the “Effects of Copper intake levels in the gut microbiota profile of target animals, in particular piglets”

Corporate author
Bent Borg Jensen

Abstract
The potential effect of the copper intake on the microbiota profile of pigs, chickens and cows was reviewed through an extensive literature search. A total of 28 (out of 229), 17 (out of 106) and 0 (out of 114) references were considered relevant for pigs, chickens and cows, respectively. The overall conclusion from the studies with piglets and growing pigs is that copper, even at low concentrations (<50 mg/kg feed), may affect the microbiota in the gastrointestinal tract. Especially, the population of clostridia and coliform bacteria seems to be affected by low copper concentrations. At higher concentrations (>170 mg/kg feed) Cu as CuSO₄ reduces the population of lactobacilli in piglets as well as growing pigs. In slaughter pigs, the addition of Cu as CuSO₄ reduces the population of streptococci in colonic and fecal samples, the population of ureolytic bacteria in the colon of which streptococci make up 74%, the urease activity in the colon, and decarboxylation and deamination of amino acids in the small intestine. No effect of Cu as CuSO₄ on the population of streptococci and on urease activity is seen in piglets. Supplementing piglet diets with 100 to 250 mg/kg Cu as CuSO₄ significantly change the community structure of the microbiota in the gastrointestinal tract by reducing the number of bacterial species and reducing the similarity of the microbiota. The overall conclusion from the studies with broilers is that copper, even at low concentrations (<50 mg/kg feed), may affect the microbiota in the gastrointestinal tract. Especially the population of clostridia seems to be affected by low copper concentrations. In particular, copper bound clay minerals seem to have an effect. At higher concentrations (>200 mg/kg feed) inorganic or organic bound copper also seems to affect the population of lactobacilli and coliform bacteria, to reduce the pH in gizzard content and to produce severe gizzard erosion.

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Key words: pigs, piglets, chickens, cows, copper, gastrointestinal tract, gut microbiota

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Correspondence: feedap@efsa.europa.eu
Effect of copper intake on gut microbiota (pigs/piglets, chickens, dairy cows)

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Summary

Piglets and pigs

Of the 229 references found for pigs and piglet by the literature search, 28 were considered appropriate to be included in the ELS. In total 34 different characteristics related to the gastrointestinal microbiota were investigated in the selected studies. The quality of the papers retrieved from the search on piglet/pig was generally judged to be of good quality although some of them were rather old. Five out of 27 papers were written in foreign languages (1 in German and 4 in Chinese) with English Abstracts and Table and Figure legends.

The copper concentrations used in the studies varied from 1.5 to 375 mg/kg feed. Four types of copper sources were used in the studies: inorganic bound copper, organic bound copper, copper bound to clay minerals and copper-loaded nanoparticles.

The overall conclusion from the studies with piglets and growing pig is that copper even at low concentrations (<50 mg/kg feed) may affect the microbiota in the gastrointestinal tract. Especially the population of clostridia and coliform bacteria seems to be affected by low concentrations of copper. In particular, copper bound clay minerals seem to have an effect against coliform bacteria and clostridia. At higher concentrations (>170 mg/kg feed) Cu as CuSO₄ reduces the population of lactobacilli in piglet as well as growing pigs. In slaughter pigs, addition of Cu as CuSO₄ reduces the population of streptococci in colonic and fecal samples, the population of ureolytic bacteria in the colon of which streptococci make up 74%, the urease activity in the colon, and decarboxylation and deamination of amino acids in the small intestine. No effect of Cu as CuSO₄ on the population of streptococci and on urease activity was seen in piglets. Supplementing piglet diets with 100 to 250 mg/kg Cu as CuSO₄ significantly affects the community structure of the microbiota in as well the small intestine, the caecum and the colon.

Chickens

Of the 106 references found for chickens by the literature search, 17 were considered appropriate to be included in the ELS. In total 20 different characteristics related to the gastrointestinal microbiota were investigated in the selected studies. The quality of the papers retrieved from the search on chickens was generally judged to be low. Ten out of 17 papers were written in foreign languages (7 in Korean and 3 in Chinese) with English Abstracts and Table and Figure legends.

The copper concentrations used in the studies varied from 8 to 375 mg/kg feed. Four types of copper sources were used in the studies: inorganic bound copper, organic bound copper, copper bound to clay minerals and copper-loaded nanoparticles.

The overall conclusion from the studies with broilers is that copper even at low concentrations (<50 mg/kg feed) may affect the microbiota in the gastrointestinal tract. Especially the population of clostridia seems to be affected by low concentrations of copper. In particular, copper bound clay minerals seem to have an effect. At higher concentrations (>200 mg/kg feed), inorganic or organic bound copper also seems to affect the population of lactobacilli and coliform bacteria, to reduce the pH in gizzard content and to produce severe gizzard erosion.

Dairy cows

Of the 114 references found for cows by the literature search, 5 were in the first phase considered appropriate to be included in the ELS. However, a closer examination of the five studies revealed the papers not to be appropriate to be included in the ELS. One of the studies was published in Polish, two of the studies were dealing with antibiotic resistance, one was dealing with the effect of copper on parasite control and the last one on the effect of copper on abomasal ulcers.
Effect of copper intake on gut microbiota (pigs/piglets, chickens, dairy cows)

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background

In the context of the development of the Scientific Opinion linked to the mandate related to the revision of maximum levels of copper in feed, the experts of the ad-hoc Working Group (WG) of the FEEDAP Panel have considered relevant to evaluate the effects of copper intake levels in the gut microbiota profile of target animals. Published scientific papers have addressed this issue and it seems there is a relationship between levels of dietary copper in animals and differences in their gastrointestinal microbiota, which in turn may result e.g. in more nutrients becoming available for absorption; the mode of actions have also been recently studied.

A possible outcome of the opinion might be a recommendation of different maximum levels compared to those currently authorised in feed (Table 1). Therefore it is necessary to know the potential effects of a variation in copper intake levels on the gut microbiota profile of the target animals which might be accounted in the FEEDAP Panel opinion. Piglets should be particularly considered in this literature search, as this is animal category for which the maximum copper in feed is allowed by Commission Regulation (EC) No 1334/2003; at least, two other relevant animal species/categories (chickens for fattening, dairy cows, fish, cat, dog) shall be included.

Table 1: Currently authorised maximum copper contents in feed in EU.

| Animal species | mg Cu/kg complete feedingstuff |
|----------------|---------------------------------|
| Pigs           |                                 |
| — piglets up to 12 weeks | 170 (total) |
| — other pigs | 25 (total) |
| Bovine         |                                 |
| 1. Bovine before the start of the rumination: | |
| — milk replacers | 15 (total) |
| — other complete feedingstuffs | 15 (total) |
| 2. Other bovine: | 35 (total) |
| Ovine          |                                 |
| Fish           | 25 (total) |
| Crustaceans    | 50 (total) |
| Other species  | 25 (total) |

The project should be accomplished in a structured manner, that is following an Extensive Literature Search (ELS) process. The steps listed below should be followed and duly documented under the present Call for tender:

- Development of an ELS protocol, which will be subject to EFSA’s approval

1  EFSA-Q-2015-00435
2  https://ess.efsa.europa.eu/doi/doiweb/wg/681811
3  Poulsen HD, 1988. Zinc and copper as feed additives, growth factors or unwanted environmental factors. Journal of Animal and Feed Sciences, 7, 135–142.
4  Hedemann et al., 2006. Influence of dietary zinc and copper enzyme activity in intestinal morphology in weaned pigs. Journal of Animal Science 84, 3310–3320.
- Definition of Search terms and Boolean operators
- Databases to be consulted
- Criteria to select the papers
- Criteria for data extraction
- Assessment of the quality of the data
- Synthesis of the data

1.1.2. Terms of References

The purpose of the contract was to provide scientific assistance to the FEED Unit of EFSA in the completion of an extensive literature search on the effects of copper intake levels in the gut microbiota profile of target animals, in particular piglets. The procedure includes the provision of a report collecting, collating, analysing and synthesising the scientific data and information on the ELS topic. The specific objectives of the contract resulting from the present procurement procedure are as follows:

- Objective 1: Prepare the review protocol. The key issues of the review process should be detailed in advance. The protocol should cover background, review questions, objectives and inclusion criteria, search methodology, selection, data collection, methodological quality assessment, and data synthesis.

- Objective 2: Search and Selection. The search should be extensive and sensitive, in order to retrieve as many studies as possible that fit the inclusion criteria. The selection should be based on a first screening founded on titles and abstracts, and a second based on full-text reports.

- Objective 3: Data extraction and assessment. The relevant data shall be extracted in a structured manner, e.g. by means of Tables.

- Objective 4: Data synthesis and reporting. Provide a report with the synthesis of the data originated from the selected studies.

This contract/grant was awarded by EFSA to:
Contractor/Beneficiary: Bent Borg Jensen, Lyngbakken 2, 9500 Hobro, Denmark
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Contract/Grant number: NP/EFSA/FEED/2015/02
2. Data and Methodologies

2.1. Data

Published scientific papers were searched using two database platforms: Web of Science on the 4th of January 2016 (including the following databases: Web of ScienceTM Core Collection, BIOSIS Citation IndexSM, CAB Abstracts; Derwent Innovations OndexSM, FSTA® - the food science resources, KCI – Korean Journal Database, Medline®, Russian Science Citation Index and SciELO Citation Index) and CAB Abstracts on the 10th of March 2016. The searches were divided into three groups, based on the animal species/category, using the search terms and Boolean operators given below. Additional scientific papers were retrieved by consulting the reference list in relevant scientific papers.

As required in the Term of References, two other animal species/categories, apart from pigs (piglets in particular), were included in the ELS: chickens for fattening and dairy cows. The inclusion of these animal species/categories was based on: 1) a pre-pilot search, 2) different animal species/categories (such as (i) mammals vs. avian species, (ii) companion animals vs. food producing animals, and (iii) animal species/categories more sensitive to copper vs. animal species/categories less sensitive to copper) and 3) the used levels of copper in feed.

Search pigs and piglets

Search terms and Boolean operators (strings): TS=(copper OR CU OR copper sulphate) AND TS=(gut OR intestinal OR intestine or ileal or digestive tract or gastrointestinal tract or GIT) AND TS=(bacteria or microbiota or microflora or microorganism or microbial community) AND TS=(pig or piglets)

The searches in Web of Science resulted in 139 hits (shown in Appendix A3). The search in CAB Abstracts resulted in 79 hits of which one (P151 in Appendix A and Appendix B) was not among the hits from the search in Web of Science. Consulting the reference list of relevant papers from the search another 11 studies/papers were retrieved (P140 to P150 in Appendix A and Appendix B).

Search chickens

Search terms and Boolean operators (strings): TS=(copper OR CU OR copper sulphate) AND TS=(gut OR intestinal OR intestine or ileal or digestive tract or gastrointestinal tract or GIT) AND TS=(bacteria or microbiota or microflora or microorganism or microbial community) AND TS=(chickens or layer or hen or broiler)

The searches in Web of Science resulted in 71 hits (shown in Appendix A3). The search in CAB Abstracts resulted in 35 hits of which one (CH72 in Appendix A and Appendix B) was not among the hits from the search in Web of Science. No further studies/papers were found by consulting the reference lists from relevant papers from the search.

Search cows

Search terms and Boolean operators (strings): TS=(copper OR CU OR copper sulphate) AND TS=(gut OR intestinal OR intestine or ileal or digestive tract or gastrointestinal tract or GIT) AND TS=(bacteria or microbiota or microflora or microorganism or microbial community) AND TS=(cow or cattle or ruminant or bovine).

The searches in Web of Science resulted in 80 hits (shown in Appendix A3). The search in CAB Abstracts resulted in 34 hits of which all were included in the hits from the search in Web of Science. No further studies/papers were found by consulting the reference lists from relevant papers from the search.
2.2. Methodologies

For the general approach to complete this ELS the Contractor considered the Technical Manual for Performing Electronic Literature Searches in Food and Feed Safety\(^6\) and the EFSA guidance: Application of systematic review methodology to food and feed safety assessments to support decision making.\(^7\)

The inclusion criteria for the scientific papers were no limitation on timespan, no limitation on language and inclusion of Primary research and Review articles (the later to get a comprehensive view on the subject). The ELS protocol was primarily based on Primary research. Papers on foreign languages, where translation was not possible, were excluded.

Study selection

- The study had to contain a control diet and diet supplemented with one or several copper concentrations in the range of 10 to 1000 mg/kg.
- Studies including the effect of any sources of dietary copper on any aspect of the microbiota in the gastrointestinal tract or faeces (profile, activity, metabolites and pH) were selected.

The total number of references retrieved, the references rejected at the title and abstract screening and the references from which data was extracted are shown as a flow chart in Annexes A1 to A3. The list of Included and Excluded references are shown in Appendix B.

Data collection

Data collected was the effect of dietary copper in the range from 10 to 1000 mg/kg on any aspect on the microbiota in the gastrointestinal tract or faeces.

Copper content: Background content of feed, supplemented copper, concentration and source (data given in Table 3 for piglet/pigs and in Table 9 for broilers/layers).

Animals:

- Species/category and age (weight) of the animals (data given in Table 3 for piglet/pigs and in Table 9 for broilers/layers).
- Number of animals; animals per treatment; replicates (data given in Table 3 for piglet/pigs and in Table 9 for broilers/layers).

Parameters measured:

- Composition and activity of the microbiota in the gastrointestinal tract or faeces (profile, activity, metabolites and pH). An overview of the data obtained is given in Table 4 for piglets/pigs and in Table 10 for broilers/layers. The data obtained is narratively described in the text and an overview of the effect of copper concentrations and sources on the concentrations on bacterial populations is shown in Tables 5, 6 and 7 for piglets/pigs and in Table 11 for broilers/layer.
- If available, performance parameters (data given in Table 3 for piglet/pigs and in Table 9 for broilers/layers).

Results:

The results are narratively described in the text and shown in Tables 3, 4, 5, 6 and 7, for piglets/pigs and in Tables 9, 10 and 11 for broilers/layers.

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\(^6\) Glanville J, Wood H, Arber M, Varley D, Frampton G, Brazier H; Technical Manual for Performing Electronic Literature Searches in Food and Feed Safety. Supporting Publications 20YY:EN-NNNN. [49 p.]. Available online: www.efsa.europa.eu/publications

\(^7\) European Food Safety Authority; Application of systematic review methodology to food and feed safety assessments to support decision making. EFSA Journal 2010; 8(6):1637. [90 pp.]. doi:10.2903/j.efsa.2010.1637. Available online: http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/1637.pdf
Quality assessment

The study design (incl. e.g. the duration of the study), the conduction of the study and the description of the study was evaluated.

It was checked if the number of animals in each group in the studies were adequate.

It was checked if the methods used to describe the microbiota profiles were adequate.

It was checked if the statistical methods (incl. e.g. randomisation of the assignment of animals in treatment groups) used were adequate.

The methodological quality of the selected studies is captured in Table 2 for piglets/pigs and in Table 8 for broilers/layers.

Studies selected

Pigs and piglets

Of the 229 references found for pigs and piglets by the literature search, 28 were considered appropriate to be included in the ELS (Annex A1). 82 references were duplicates. Of the 119 excluded references, 50 were excluded when screening the title and abstract and 69 after consulting the full paper. The list of included and excluded references is provided in Appendix B.

Chickens

Of the 106 references found for chickens by the literature search, 17 were considered appropriate to be included in the ELS (Annex A2). 38 references were duplicates. Of the 51 excluded references, one full paper could not be retrieved, 18 were excluded when screening the title and abstract and 32 after consulting the full paper. It should be mentioned that 10 out of the 17 included papers were written in foreign languages (7 in Korean and 3 in Chinese) with English Abstracts and Table and Figure legends. The list of included and excluded references is provided in Appendix B.

Cows

Of the 114 references found for cows by the literature search, 5 were considered appropriate to be included in the ELS (Annex A3). 34 references were duplicates. Of the 75 excluded references, 46 were excluded when screening the title and abstract and 29 after consulting the full paper. A closer examination of the five studies first selected to be included in the ELS study on cows, revealed the papers not to be appropriate to be included in the ELS. One of the studies (Cow33) was published in Polish, two of the studies were dealing with antibiotic resistance (Cow2 and Cow31), one was dealing with the effect of copper on parasite control (Cow13) and the last one on the effect of copper on abomasum ulcers (Cow47). The list of included and excluded references is provided in Appendix B.
3. Results

3.1. Pigs and piglets

3.1.1. Study selection

Assessment of the methodological quality of the studies

Table 2: Assessment of the methodological quality of the piglet/pig studies

| Study ID | Country | Language | Study design | Feed formulation | Duration of experiment | Replications | Number of animals/replication | Microbial methods | Statistical methods | Overall quality | Remarks |
|----------|---------|----------|--------------|------------------|------------------------|--------------|-------------------------------|------------------|-------------------|----------------|---------|
| P5       | USA     | English  | OK           | Not given         | OK                     | OK           | OK                            | OK               | OK                | XXX            |         |
| P19      | Belgium | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            |         |
| P41      | Denmark | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            |         |
| P46      | China   | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            |         |
| P49      | Denmark | English  | OK           | Not given         | OK                     | Not given    | OK                            | Not given        | X                 | XXX            | *No statistics on microbiological data |
| P59      | Germany | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XX             |         |
| P69      | China   | Chinese  | OK           | Not given         | OK                     | Not given    | OK                            | OK               | OK                | XX             |         |
| P72      | China   | English  | OK           | OK                | OK                     | Not given    | OK                            | OK               | OK                | XXX            |         |
| P74      | France  | English  | OK           | OK                | OK                     | Not given    | OK                            | OK               | OK                | XXX            |         |
| P80      | Canada  | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            |         |
| P85      | USA     | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            | *Rate count of bacteria done on frozen samples |
| P103     | China   | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            |         |
| P116     | China   | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            |         |
| P123     | China   | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            |         |
| P124     | China   | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            |         |
| P130     | USA     | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            |         |
| P135     | China   | Chinese  | OK           | Not given         | OK                     | Not given    | OK                            | Not given        | X                 | X              |         |
| P140     | USA     | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            | *Microbial investigations done on faecal samples |
| P141     | USA     | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            |         |
| P142     | USA     | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            |         |
| P143     | USA     | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            |         |
| P144     | USA     | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            |         |
| P145     | Belgium | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            |         |
| P146     | Korea   | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            | *Only faecal pH and diarrhoea were measured |
| P147     | USA     | English  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            | *Data from before the experimental treatment were intake was included |
| P148     | China   | Chinese  | OK           | OK                | OK                     | OK           | OK                            | OK               | OK                | XXX            | *Three levels of Cu were included in the study but no control without Cu |

As shown in Table 2 the quality of the papers retrieved from the search on piglet/pig was generally judged to be good although some of them were rather old. Five out of 27 papers were written in foreign languages (1 in German and 4 in Chinese) with English Abstracts and Table and Figure legends.

3.1.2. Results

Type of copper sources used in the studies with pigs/piglets

Four types of copper sources were used in the research with pigs (Table 3): 1) inorganic copper, 2) organic bound copper, 3) clay bound copper and 4) copper-loaded nanoparticles.

Inorganic copper was used in 23 of the 28 studies. The sources were CuSO₄ (concentrations 26–283 mg Cu/kg feed, in studies P5, P19, P41, P49, P59, P69, P72, P74, P80, P85, P111, P123, P130, P135, P140, P141, P142, P143, P144, P145, P146, P147 and P149), CuO (concentrations 125–375 mg Cu/kg feed, in studies P59 and P142) and elemental copper (concentration 200 mg Cu/kg feed, in study P59).

Organic bound copper was used in 5 of the 28 studies. The sources were Cu-AA complex (cupric amino acid complex, concentration 90 mg Cu/kg feed, in study P85), Cu-Cit (cupric citrate, concentrations 33, 66 and 100 mg Cu/kg feed, in studies P140 and P141), Cu-Met (copper
methionate, concentrations 67 and 134 mg Cu/kg feed, in study P146) and Cu-Gly (copper glycinate, concentrations 13, 26 and 52 mg Cu/kg feed, in study P148).

Clay bound copper was used in 5 of the 28 studies. The sources were Cu-MM (copper-bearing montmorillonite from aluminosilicate clay). It was used in studies P46, P69, P103, P123 and P124 at concentrations from 36 to 49 mg Cu/kg.

Copper-loaded nanoparticles were used in 1 of the 28 studies. The source was copper loaded chitosan (P116 Mei et al., 2010 Piglets 25 (5 pens with 5 pigs) 28 days 7.5 kg 28 days Control 10 183 406 0.441).

Setup of the individual studies

| Study | Ref/Author | Tier | Type | Treatment | Source | Cu absorption/efficiency | Microbiota characteristics investigated |
|-------|------------|------|------|-----------|--------|-------------------------|--------------------------------------|
| P5    | Assfalk et al., 1980 USA | Growers | 9 pens with 1 pig | NG | 60 days | Control | 250 ppm Cu SO4 | 434 1375 0.222 |
| P18   | Dennis et al., 2006 Denmark | Growers | in vivo experiment | 10-40 kg | Control | 90 200 ppm Cu SO4 | 635 ppm Cu SO4 |
| P60   | Højberg et al., 2006 Denmark | Growers | 16-34 pens with 1 pig | 20 days 10.2 kg | Control | 25 45 ppm Cu SO4 | 250 ppm Cu SO4 |
| P61   | Huitink, 2004 China | Growers | 32 pens with 8 pigs | NG | 40 days | Control | 9 254 ppm Cu Cit | 0 ppm Zn |
| P65   | Jassow, 1996 Denmark | Growers | 6 pens with 1 pig | 30 days | Control | 25 45 ppm Cu | 0 ppm Zn |
| P85   | Aviotti et al., 1980 Growers | 9 pens with 1 pig | NG | 63 days | Control | 630 1571 0.372 |
| P96   | Kroger et al., 1977 Growers | NG | 70 days | 25 kg | Control | 630 1571 0.372 |
| P102  | Ma et al., 2007 China | Growers | 20 days 5.9 kg | Control | 15 200 ppm Cu CSN |
| P111  | Varel et al., 1987 Growers | Data not given | 25 kg to 100 kg | Control | 15 200 ppm Cu CSN |
| P116  | Wang et al., 2011 Piglets | 30 pens with 10 pigs | 21 days 7.2 kg | Control | 287 597 0.48 |
| P117  | Anderson et al., 2010 USA | Growers | 32 pens with 8 pigs | NG | 20.2 kg | Control | 9 672 1754 0.383 |
| P118  | Aviotti et al., 1980 Growers | Ex vivo experiment | 10 - 40 kg | Control | 31 170 276 |
| P119  | Armington et al., 2000 USA | Growers | 16 pens with 1 pig | 28 days 8.3 kg | Control | 31 170 276 |

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| Study Reference | Species | Study Design | Dose | Days | Fecal Odor Characteristics |
|-----------------|---------|-------------|------|------|----------------------------|
| Armstrong et al., (2004) | Piglets | 150 (5 pens with 30 piglets) | 6-18 days | 4.99 kg | Control - 379 |
| Bunch et al., 1961 | Piglets | 16 (4 pens of 4 piglets) | 13 days | 8.4 kg | Control - 328 |
| Bunch et al., 1961 | Piglets | 24 (6 pens of 4 piglets) | 20 days | 6.4 kg | Control - 328 |
| Fuller et al., 1960 | Growers | 5 (5 pens of 1 pig) | 8 weeks | NG | Control - 568 |
| Huang et al., (2010) | Growers | 20 (5 pens of 4 pigs) | 60 days | 21.5 kg | Control - 725 |
| Kellogg et al., 1964 | Growers | 12 (6 pens with 2 piglets) | 15 days | 4.2 kg | Control - 317 |
| Li et al., 2014 | Piglets | 60 (5 pens with 12 pigs) | 16 days | 9.1 kg | Control - 396 |
| Hawbaker et al., 1961 | Piglets | 16 (4 pens of 4 piglets) | 13 days | 8.4 kg | Control - 317 |
| Hawbaker et al., 1961 | Piglets | 24 (6 pens of 4 piglets) | 20 days | 6.4 kg | Control - 317 |
| Hawbaker et al., 1961 | Piglets | 32 (8 pens of 4 piglets) | 28 days | 6.4 kg | Control - 317 |
| Henderickx et al., 1982 | Growers | 25 (5 pens of 5 pigs) | 5 weeks | NG | Control - 568 |
| Huang et al., (2010) | Growers | 20 (5 pens of 4 pigs) | 60 days | 21.5 kg | Control - 725 |
| Kellogg et al., 1964 | Growers | 12 (6 pens with 2 piglets) | 15 days | 4.2 kg | Control - 317 |
| Li et al., 2014 | Piglets | 60 (5 pens with 12 pigs) | 16 days | 9.1 kg | Control - 396 |
| Hawbaker et al., 1961 | Piglets | 16 (4 pens of 4 piglets) | 13 days | 8.4 kg | Control - 317 |
| Hawbaker et al., 1961 | Piglets | 24 (6 pens of 4 piglets) | 20 days | 6.4 kg | Control - 317 |
| Hawbaker et al., 1961 | Piglets | 32 (8 pens of 4 piglets) | 28 days | 6.4 kg | Control - 317 |

| (x) | (y) | (z) |
|-----|-----|-----|
| (a) | values in red calculated from the amount of copper source added to the feed | number in black measured |
| (b) | values in bold and red significant different (p<0.05 or less) different from the control |
| NG | not given |

**Fecal pH and Diarrhea**

| Study Reference | Species | Study Design | Dose | Days | Fecal pH and Diarrhea |
|-----------------|---------|-------------|------|------|-----------------------|
| Huang et al., (2010) | Growers | 20 (5 pens of 4 pigs) | 60 days | 21.5 kg | Control - 725 |
| Kellogg et al., 1964 | Growers | 12 (6 pens with 2 piglets) | 15 days | 4.2 kg | Control - 317 |

**Fecal Microbiota**

| Study Reference | Species | Study Design | Dose | Days | Fecal Microbiota |
|-----------------|---------|-------------|------|------|-----------------|
| Huang et al., (2010) | Growers | 20 (5 pens of 4 pigs) | 60 days | 21.5 kg | Control - 725 |
| Kellogg et al., 1964 | Growers | 12 (6 pens with 2 piglets) | 15 days | 4.2 kg | Control - 317 |

**Conventional and Germ Free Pigs**

| Study Reference | Species | Study Design | Dose | Days | Fecal Microbiota |
|-----------------|---------|-------------|------|------|-----------------|
| Huang et al., (2010) | Growers | 20 (5 pens of 4 pigs) | 60 days | 21.5 kg | Control - 725 |
| Kellogg et al., 1964 | Growers | 12 (6 pens with 2 piglets) | 15 days | 4.2 kg | Control - 317 |

**In vitro Production of SCFA**

| Study Reference | Species | Study Design | Dose | Days | In vitro Production of SCFA |
|-----------------|---------|-------------|------|------|-----------------|
| Henderickx et al., 1982 | Growers | 25 (5 pens of 5 pigs) | 5 weeks | NG | Control - 568 |
| Huang et al., (2010) | Growers | 20 (5 pens of 4 pigs) | 60 days | 21.5 kg | Control - 725 |
| Kellogg et al., 1964 | Growers | 12 (6 pens with 2 piglets) | 15 days | 4.2 kg | Control - 317 |

**In vitro Growth of Lactobacilli and Streptococci**

| Study Reference | Species | Study Design | Dose | Days | In vitro Growth of Lactobacilli and Streptococci |
|-----------------|---------|-------------|------|------|----------------|
Microbial characteristics investigated in the studies

The microbial characteristics measured in each of the 28 selected studies with piglets/pigs are shown in Table 4. Eighteen of the studies were done with piglets (P41, P46, P49, P69, P72, P74, P80, P85, P103, P116, P123, P124, P135, P140, P141, P142, P147, P148 and P149) and 10 were done with growing pigs (P5, P19, P59, P111, P130, P143, P144, P145 and P146).

Table 4: Microbial characteristics investigated in the pig/piglet studies

| Study | Microbial characteristics investigated | Bifidobacteria | Clostridia | Lactic acid bacteria | Lactobacilli | Enterobacteria | Yeast | Coliforms | Lactic acid bacteria | Lactobacilli | Total aerobes | Total anaerobes | Urease activity | ATP concentration | β-Glucosidase activity | Deamination and decarboxylation | Odour quality | pH | Dry matter | Digesta content | No of species | Similarity | Effect in germ free pigs |
|-------|---------------------------------------|----------------|------------|---------------------|-------------|----------------|-------|------------|---------------------|-------------|---------------|---------------|----------------|-------------------|----------------------|-----------------------------|----------------|-----|-----------|-----------------|------------|-----------|-------------------|
| P41   | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P46   | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P49   | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P69   | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P72   | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P74   | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P80   | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P85   | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P103  | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P116  | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P123  | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P124  | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P135  | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P140  | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P141  | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P142  | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P147  | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P148  | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |
| P149  | Bifidobacteria                        | X              |           |                     |             |                |       |            |                     |             |               |               |                |                   |                       |                             |               |     |          |                 |           |          |                    |

Results from the studies

Effect of copper on microbial populations

*Bifidobacteria.* The effect of copper on the population of bifidobacteria was investigated in cecum content in one study with piglets (P74, Mei et al., 2009). Compared to the control fed piglets addition of 250 mg/kg Cu as CuSO4 had no significant effect on the population of bifidobacteria in caecal content.

*Clostridia.* The effect of copper on the population of clostridia was investigated in two studies with piglets (Song et al., 2013) and in two studies with growing pigs (P103 and P123) and in two studies with growing pigs (P46 and P59). In study P103 with piglets (Song et al., 2013) the population of clostridium was significantly reduced in small intestine (SI) as well as caecum content in pigs fed 36 mg/kg Cu as either Cu-CaMontmorillonite or Cu-NaMontmorillonite, compared to the control fed pigs. In agreement with that, it was found in study P123 (Xia et al., 2005) that supplementing piglet diets with 37 mg/kg Cu as CuMontmorillonite significantly reduced the population of clostridium in content from the SI as as well as content from the colon. However, although numerically lower, the effect in the SI was not significantly different from pigs fed copper free Montmorillonite. No effect was seen by on the population of *Clostridium* by addition 37 mg/kg Cu as CuSO4 to the diet neither in SI or colon content. In study P46 with growing
pigs (Hu et al., 2004) the population of Clostridium was significantly reduced in as well SI as caecum content in pigs fed 47 mg/kg Cu as Cu-Montmorillonite compared to the control fed pigs; however, although numerically lower, the effect was not significantly different from pigs fed copper free Montmorillonite. In study P59 (Kroger et al., 1997) addition of 200 mg/kg Cu as CuO or CuSO₄ to diets of growing pigs were shown significantly to reduce pigs containing Clostridium perfringens in gut content from the small intestine and colon. No effect was found of elementary copper. The effects of CuO and CuSO₄ addition were only seen if Cu was supplemented until the pigs were slaughtered at 100 kg live weight. If it was withdrawn from the feed at 80 kg live weight no effect was detected when the pigs were slaughtered at 100 kg. In conclusion low concentrations of copper as clay bound copper seem to reduce the population of Clostridium both in piglets and in growing pigs. Further high concentrations of inorganic copper as either CuSO₄ or CuO may reduce the population of Clostridium in growing pigs.

Coliform bacteria. The effect of copper on the population on coliform bacteria was investigated in 14 studies with piglets (P41, P49, P69, P72, P74, P80, P103, P116, P123, P124, P135, P142, P147, P148) and four studies with growing pigs (P5, P46, P143, P144). The results from the studies are summarised in Table 5. It may be concluded from the experiments that supplementing piglet (and growing pigs) diet with low additional copper amounts (below 50 mg/kg Cu) seems to inhibit the population of coliform bacteria in the gastrointestinal tract. Especially clay bound copper seems to be effective. When supplemented to the feed in amounts above 100 mg/kg copper it does not seem to have any effect on the population of coliform bacteria in the gastrointestinal tract of pigs.
Table 5: Effect of copper sources and concentration on populations of lactobacilli and coliform bacteria in gut content from the stomach, small intestine (SI), caecum or colon/faeces of piglets and growing pigs

| Study ID | Author | Animal category | Copper mg/kg Source | Lactobacilli Stomach SI Ceacum Colon | Coliform bacteria Stomach SI Ceacum Colon |
|----------|--------|-----------------|---------------------|--------------------------------------|-------------------------------------------|
| P116     | Wang   | Piglets         | 1.5 Cu-CSN          | ↑                                    | ↓ ↓ ↓                                     |
| P135     | Zhu    | Piglets         | 8 CuCS              | ↓                                    | ↓ ↓ ↓                                     |
| P135     | Zhu    | Piglets         | 16 CuCS             | ↓                                    | ↓ ↓ ↓                                     |
| P148     | Li     | Piglets         | 26 CuGly            | →                                    | ↓ ↓ ↓                                     |
| P103     | Song   | Piglets         | 36 Cu*CaMM          | ↓                                    | ↓ ↓ ↓                                     |
| P103     | Song   | Piglets         | 36 Cu*NaMM          | ↓                                    | ↓ ↓ ↓                                     |
| P123     | Xia    | Piglets         | 37 CuSO4            | ↓                                    | ↓ ↓ ↓                                     |
| P123     | Xia    | Piglets         | 37 Cu-MM            | ↓                                    | ↓ ↓ ↓                                     |
| P46      | Hu     | Growers         | 46 Cu-MM            | ↓                                    | ↓ ↓ ↓                                     |
| P124     | Xia    | Piglets         | 49 Cu-MM            | ↓                                    | ↓ ↓ ↓                                     |
| P69      | Ma     | Piglet          | 49 Cu-MM            | ↓                                    | ↓ ↓ ↓                                     |
| P135     | Zhu    | Piglets         | 51 CuSO4            | ↓                                    | ↓ ↓ ↓                                     |
| P148     | Li     | Piglets         | 52 CuGly            | ↓                                    | ↓ ↓ ↓                                     |
| P72      | Mei    | Piglets         | 110 CuSO4           | ↓                                    | ↓ ↓ ↓                                     |
| P46      | Bunch  | Piglets         | 125 CuO             | ↓                                    | ↓ ↓ ↓                                     |
| P49      | Jensen | Piglets         | 170 CuSO4           | ↓                                    | ↓ ↓ ↓                                     |
| P72      | Mei    | Piglets         | 185 CuSO4           | ↓                                    | ↓ ↓ ↓                                     |
| P41      | Hajberg| Piglets         | 190 CuSO4           | ↓                                    | ↓ ↓ ↓                                     |
| P74      | Mei    | Piglets         | 250 CuSO4           | ↓                                    | ↓ ↓ ↓                                     |
| P43      | Bunch  | Piglets         | 250 CuSO4           | ↓                                    | ↓ ↓ ↓                                     |
| P41      | Bunch  | Piglets         | 250 CuO             | ↓                                    | ↓ ↓ ↓                                     |
| P41      | Bunch  | Piglets         | 250 CuSO4           | ↓                                    | ↓ ↓ ↓                                     |
| P147     | Kellog | Piglets         | 250 CuSO4           | ↓                                    | ↓ ↓ ↓                                     |
| P144.2   | Hawbaker| Growers        | 250 CuSO4           | ↓                                    | ↑ ↑ ↑                                     |
| P144.3   | Hawbaker| Growers        | 250 CuSO4           | ↓                                    | ↑ ↑ ↑                                     |
| P144.4   | Hawbaker| Growers        | 250 CuSO4           | ↓                                    | ↑ ↑ ↑                                     |
| P5       | Avuotti| Growers         | 250 CuSO4           | ↓                                    | ↑ ↑ ↑                                     |
| P143     | Fuller | Growers         | 250 CuSO4           | ↓                                    | ↑ ↑ ↑                                     |
| P72      | Mei    | Piglets         | 260 CuSO4           | ↓                                    | ↓ ↓ ↓                                     |
| P80      | Namkung| Piglets         | 265 CuSO4           | ↓                                    | ↓ ↓ ↓                                     |
| P142.2   | Bunch  | Piglets         | 375 CuO             | ↓                                    | ↓ ↓ ↓                                     |

↑: Significant (p<0.05 or less) increase in the population
↓: Significant (p<0.05 or less) decrease in the population
→: No significant effect on the population

Enterococci. The effect of copper on the population density enterococci was investigated in one study with piglets. Dietary doses of 175 mg/kg Cu as CuSO₄ significantly reduced the counts of enterococci in stomach content of piglets while no effect was seen in content from the SI, caecum or colon (Study P41).

Lactic acid bacteria. The effect of copper on the population density of lactic acid bacteria was investigated in one study with piglets. Dietary doses of 175 mg/kg Cu as CuSO₄ significantly reduced the counts of enterococci in stomach content of piglets while no effect was seen in content from the SI, caecum or colon (Study P41).
**Lactobacilli.** The effect of copper on the population density and/or the composition of lactobacilli was investigated in 12 studies with piglets (P41, P49, P69, P72, P74, P80, P116, P123, P135, P142, P147 and P148) and in four studies with growing pigs (P46, P143, P144 and P145). The results from the studies are summarised in Table 5. In study P41 with piglets (Hejberg et al., 2005) dietary doses of 175 mg/kg Cu as CuSO₄ significantly reduce the counts of lactobacilli in stomach content while no effect was seen in content from the SI, caecum or colon. In study P49 (Jensen, 1998) the population of lactobacilli was reduced throughout the gastrointestinal tract in pigs fed 175 mg/kg Cu as CuSO₄. In study P69 (Ma et al., 2007) no effect of addition of 49 mg/kg Cu as CuMM was detected on the population of lactobacilli in content from the SI or colon. In study P72 (Mei et al., 2010) a numeric reduction in the population of lactobacilli was detected by addition of 100, 175 and 250 mg/kg Cu as CuSO₄ to piglet diets when the population density were enumerated using selectively cultivation, however if the population of lactobacilli were quantified by use of qPCR the reduction found for 175 and 250 mg/kg Cu as CuSO₄ were significant different from the results found in the control fed piglets. In study P74 (Mei et al., 2009) no significant effect of addition of 250 mg/kg Cu as CuSO₄ was found on the population of lactobacilli in cecal content. In study P80 (Namkung et al., 2006) no significant effects were detected in the population of lactobacilli in ileal or colon digesta by supplementing piglet feed with 250 mg/kg Cu as CuSO₄. In study P116 (Wang et al., 2011) the population of lactobacilli in content from the small intestine and caecum were significantly higher in piglets fed a diet supplemented with 1.5 mg/kg Cu as Cu-Chitosan nanoparticles. In study P123 (Xia et al., 2005) no effect on the population of lactobacilli was found in SI or colon digesta neither by supplementing the diet with 37 mg/kg Cu as CuSO₄ or CuMM. In study P135 (Zhu et al., 2011) the population of lactobacilli was reduced in caecum as well as colon content by addition of 8 or 16 mg/kg Cu as Cu-Chitosan or 51 mg/kg Cu as CuSO₄. In study P147 (Kellog et al., 1964) a significant reduction in the population of lactobacilli was found by feeding piglets with 250 mg/kg Cu as CuSO₄. In study P46 with growing pigs (Hu et al., 2004), the population of lactobacilli was not significantly affected neither in SI or caecum content in pigs fed 47 mg/kg Cu as Cu-Montmorillonite compared to the control fed pigs or to values from pigs fed copper free Montmorillonite. In study P143 (Fuller et al., 1960), it was found that the numbers of lactobacilli remained unaffected in fecal samples when growing pigs were fed 250 mg/kg Cu as CuSO₄ compared to the control fed pigs. However, the composition of the population of lactobacilli changed from a population dominated by *L. acidophilus* to one dominated by *L. brevis* and *L. cellobiosus*. In study P144 experiment 2, 3 and 4 (Hawbaker et al., 1961), a significant reduction of lactobacilli in fecal samples of growing pigs was found by supplementing the diet with 250 mg/kg CuSO₄ in experiment 2 and 4 while only a numerical reduction was found in experiment 3. In study P145 (Hendericks et al., 1982), it was concluded that CuSO₄ (concentration of Cu not given) strongly inhibited the growth of lactobacilli in *ex vivo* experiments. From the results it may be concluded that supplementing pig diets with copper concentrations above 170 mg/kg as CuSO₄ has a reducing effect on the population of lactobacilli in the gastrointestinal tract on pigs. The effect of Cu as CuO did not have the same effect.

**Salmonella.** The effect of copper on the population density of *Salmonella* spp. was investigated in two studies with piglets (P69 and P135). In both studies, the population of *Salmonella* spp. in gut content from piglets was significantly reduced by addition of copper to the diet. In study P69 (Ma et al., 2007), addition of 49 mg/kg Cu as CuMM significantly reduced the population of *Salmonella* spp. in content from the SI and the colon and in study P135 (Zhu et al., 2011) addition of 8 or 16 mg/kg Cu as Cu-Chitosan or 51 mg/kg Cu as CuSO₄ to the diet significantly reduced the population of *Salmonella* spp. in content from the caecum and the colon. In conclusion clay bound copper and copper as CuSO₄ seems to reduce the population of *Salmonella* spp. in piglet gut content.

**Staphylococci.** The effect of copper on the population of staphylococci was investigated in two studies with piglets (P142 and P147) and in one study with growing pigs (P144). In study P142 experiment 2 (Bunch et al., 1961), piglets were fed either a control diet or diets supplemented with 125, 250 or 375 mg/kg Cu as CuO and a diet supplemented with 250 mg/kg Cu as CuSO₄. The staphylococci counts varied with each copper level, resulting in a significant cubic regression. In study P142 experiment 3, no significant effect of either 250 mg/kg CuO or 250 mg/kg CuSO₄ was found on the population of staphylococci. In agreement with that, no effect on the population of staphylococci in fecal samples...
from piglets was found by supplementing the diet with 250 mg/kg in study P147 (Kellog et al., 1964). In the study with growing pigs (P144, Hawbaker et al., 1961) no significant effect on the population of staphylococci in fecal samples were detected in any of the three experiments carried out with 250 mg/kg Cu as CuSO₄. In conclusion the effect of dietary copper on the gastrointestinal populations of staphylococci in pigs seems to be non-existent or very week.

Streptococci. The effect of copper on the population density and composition of streptococci was investigated in two studies with piglets (P142 and P147) and four experiments with growing pigs (P111, P143, P144 and P145). With piglets no significant effect in the fecal counts of streptococci were found in study P142 experiment 2 and study P142 experiment 3 (Bunch et al., 1961) neither by feeding the piglets 125, 250 or 375 mg/kg Cu as CuO or by feeding them 250 mg/kg Cu as CuSO₄. There was, however, a numerical reduction in streptococci in both experiments by feeding 250 mg/kg Cu as CuSO₄. No significant reduction in the population of streptococci was found by feeding piglets with 250 mg/kg Cu as CuSO₄ in study P147 (Kellog et al., 1964). In study P111 (Varel et al., 1987) with growing pigs, 49 mg/kg Cu as CuSO₄ reduced the number of ureolytic organisms in fecal samples with a marked decrease in the Streptococcus spp., which made up 74% of the ureolytic bacteria in fecal samples from pigs fed the control diet. A marked fall in the number of streptococci in fecal samples was found in study P143 (Fuller et al., 1960) when growing pigs were fed 250 mg/kg Cu as CuSO₄ compared to pigs fed a control diet. The extent of the reduction varied but the counts of streptococci in the copper treated pigs were always lower than in the control pigs and the reduction could be as much as from 10⁹ to 10³ CFU/g faeces. In study P144 (experiments 2, 3 and 4), a significant reduction of streptococci in fecal samples of growing pigs was found in experiment 2 and 4 while only a numerical reduction was found in experiment 3 by supplementing the diet of growing pigs with 250 mg/kg CuSO₄. In study P145 (Hendericks et al., 1982), it was concluded that CuSO₄ (concentration of Cu not given) strongly inhibited the growth of streptococci in an ex vivo experiment with SI content from growing pigs. In conclusion the results strongly indicate that supplementation with 100 mg Cu/kg, or higher, as CuSO₄ significantly reduces the population of streptococci in the gastrointestinal tract of growing pigs. The effect of CuSO₄ on the population of streptococci in piglets is more questionable.

Total anaerobe bacteria. The effect of supplementary copper on the population of total anaerobic bacteria is shown in Table 6. No effect on the anaerobic population was observed if the amount of copper added to the diet was below 200 mg/kg copper. However, addition of 250 mg/kg Cu as CuSO₄ to the diet may reduce the population of total anaerobic bacteria in the colon, while copper as CuO does not seem to have any effect even at Cu concentrations of 375 mg/kg.
Table 6: Effect of copper source and copper concentration on the population of anaerobic bacteria in the gastrointestinal tract of piglets and growing pigs.

| Study ID | Author category | Copper mg/kg | Source | Total anaerobic bacteria |
|----------|-----------------|--------------|--------|-------------------------|
| P111     | Varel Piglets   | 49           | CuSO4  | →                       |
| P72      | Mei Piglets     | 110          | CuSO4  | →                       |
| P142.2   | Bunch Piglets   | 125          | CuO    | →                       |
| P72      | Mei Piglets     | 185          | CuSO4  | →                       |
| P41      | Højberg Piglets | 190          | CuSO4  | →                       |
| P142.2   | Bunch Piglets   | 250          | CuSO4  | ↓                       |
| P142.2   | Bunch Piglets   | 250          | CuO    | ↑                       |
| P142.3   | Bunch Piglets   | 250          | CuSO4  | ↓                       |
| P142.3   | Bunch Piglets   | 250          | CuO    | ↑                       |
| P144.2   | Hawbaker Growers| 250          | CuO    | ↑                       |
| P144.3   | Hawbaker Growers| 250          | CuSO4  | ↓                       |
| P144.4   | Hawbaker Growers| 250          | CuSO4  | ↓                       |
| P72      | Mei Piglets     | 260          | CuSO4  | →                       |
| P142.2   | Bunch Piglets   | 375          | CuO    | →                       |

→: No significant effect on the population
↓: Significant (p<0.05 or less) decrease in the population
↑: Significant (p<0.05 or less) increase in the population

**Total aerobe bacteria.** The effect of copper on the population of total aerobe bacteria was investigated in two studies with piglets (P142 and P147) and in one study with growing pigs (P144). In study P142 experiment 2 (Bunch et al., 1961), piglets were fed either a control diet or diets supplemented with 125, 250 or 375 mg/kg Cu as CuO and a diet supplemented with 250 mg/kg Cu as CuSO4. A significant reduction in the population of total aerobe bacteria in faecal samples was found in the piglets fed 250 mg/kg Cu as CuSO4. In contrast Cu as CuO resulted in a significant linear increase with increasing CuO supplementation in the population of total aerobic bacteria in faecal samples. In study P142 experiment 3, no significant effect of either 250 mg/kg CuO or 250 mg/kg CuSO4 was found on the population of aerobic bacteria in faecal samples. In agreement with that no effect on the population of aerobic bacteria in piglet faecal samples. In study P142 experiment 3, no significant effect of either 250 mg/kg CuO or 250 mg/kg CuSO4 was found on the population of total aerobic bacteria in piglet faecal samples. In conclusion the effect of copper concentration and source on the population on total aerobic bacteria seems to be very variable.

**Ureolytic bacteria.** The number of ureolytic bacteria was investigated in study P111 (Varel et al., 1987). Addition of copper sulfate to the diet at a concentration of 49 mg/kg copper significantly reduced the number of ureolytic bacteria compared to control fed pigs, in fecal samples collected after 3, 9 and 14 weeks of feeding the experimental diets, with a marked decrease occurring in Streptococcus spp., which make up 75% of the ureolytic isolates.

**Use of qPCR to quantify microbial populations**

Quantitative real-time polymerase chain reaction (qPCR) has been used in one investigation with piglets (P72, Mei et al., 2010) to detect the effect of copper supplementation on bacterial populations (lactobacilli, enterobacteria or total anaerobic bacteria) in gastrointestinal content. It was shown that
the population of lactobacilli in caecum content from piglets was significantly decreased in piglets fed diets supplemented with 175 or 250 mg/kg Cu as CuSO₄ while no effect was seen by a diet supplemented with 100 mg/kg Cu as CuSO₄. No effect was found of any of the CuSO₄ supplemented diets on the population of enterobacteria or total anaerobic bacteria. Use of traditional microbiological culturing methods did also show a reduction in the population of lactobacilli with increasing CuSO₄ addition; however these results were not significant.

**Effect of copper on community structure**

The microbial community was investigated in four studies with piglets (P41, P72, P80 and P85). In study P80 (Namkung et al., 2006), study P72 (Mei et al., 2010) and study P85 (Pérez et al., 2011) the microbial community was determined by isolating bacterial DNA, amplifying the V3 region of the 16S ribosomal DNA and performing denaturing gradient gel electrophoreses (DGGE). In study P41 (Højberg et al., 2005) the microbial community was determined by use of terminal restriction fragment polymorphism (T-RFLP) profiles of PCR amplified products of the 16S rRNA genes.

The results obtained from the microbial community structure studies are summarized in table 3.1.4. In all studies the number of bands per sample was measured and in study P80 and P85 the similarity indices for bands between samples were calculated. The number of bands is indicative for the number of bacterial species within a sample; the similarity coefficient is indicative of how similar the bacteria within a treatment group are.

**Table 7:** Effect of copper sources and copper concentrations on microbial diversity and microbial similarity obtained from microbial community structure studies.

| Study ID | Author | Animal category | Copper mg/kg | Source | Species | Similarity | Species | Similarity | Species | Similarity |
|----------|--------|-----------------|--------------|--------|---------|------------|---------|------------|---------|------------|
| P72      | Mei    | Piglets         | 110          | CuSO₄   | ↓       |            |         |            |         |            |
| P85      | Pérez  | Piglets         | 250          | CuAA    | →       |            |         |            |         |            |
| P85      | Pérez  | Piglets         | 247          | CuSO₄   | ↓       |            |         |            |         |            |
| P72      | Mei    | Piglets         | 250          | CuSO₄   | ↓       |            |         |            |         |            |
| P41      | Højberg| Piglets         | 247          | CuSO₄   | →       |            |         |            |         |            |
| P85      | Pérez  | Piglets         | 252          | CuSO₄   | →       |            |         |            |         |            |
| P85      | Pérez  | Piglets         | 247          | CuSO₄   | ↓       |            |         |            |         |            |
| P80      | Namkung| Piglets         | 265          | CuSO₄   | ↓       |            |         |            |         |            |

↑: Significant (p<0.05 or less) increase  
↓: Significant (p<0.05 or less) decrease  
→: No significant effect

No effect of copper concentration or sources was found in study P85 neither on the number of species nor on the similarity of the microbiota in faecal samples. However in studies P41, P72 and P80 it was shown that supplementing piglet diets with high amounts of copper (100 to 250 mg/kg) as CuSO₄ affect the number of bacterial species in ileal (P80), in caecal (P41 and P72) and in colonic (P80) digesta. Furthermore, in study P80 addition of 250 mg/kg Cu as CuSO₄ significantly reduced the degree of similarity of the microbiota in ileal samples compared to pigs fed a diet without supplementary copper addition. As also pointed out by the authors in study P85, the combination of faecal samples and the microbial methods used in their study may not have been sensitive enough to detect differences among the dietary treatments. In study P80, the similarity analysis further shows that the piglets did restore a diverse microbiota 2 weeks after withdrawal from the high Cu diet, but the community structure still seems to be different from that in the control fed pigs. In general, the community structure studies strongly suggest that supplementing piglet diets with 100 to 250 mg/kg Cu as CuSO₄ change the microbial community in the small intestine, the caecum and the colon.
Effect of copper on gut content characteristics

Digesta content. The amount of gastrointestinal content was investigated in study P41 (Højberg et al., 2005). The addition of high concentration of CuSO₄ (175 mg/kg Cu as CuSO₄) to piglet diets had no significant influence on digesta content in any part of the gastrointestinal tract compared to piglets fed a control diet.

Dry matter. The dry matter content in gastrointestinal digesta was investigated in piglets in study P41 (Højberg et al., 2005). In contrast to high concentrations of ZnO (addition of 2500 mg/kg Zn as ZnO to the diet) —which significantly increase the dry matter content in the stomach and decrease the dry matter content in caecum and colon—, high concentration of CuSO₄ (175 mg/kg Cu as CuSO₄) had no significant influence on the dry matter content in any part of the gastrointestinal tract (stomach, SI, caecum and colon).

pH. The effect of copper on faecal pH and pH of digesta were investigated in three studies with piglets (P41, P49 and P135) and one study with growing pigs (P146). In study P41 (Højberg et al., 2005) and P47 (Jensen 1998) no effect of feeding piglets diets supplemented with either 175 mg/kg (P41) or 170 mg/kg of Cu (P47) as CuSO₄ was found on pH in any segments of the gastrointestinal tract (stomach, SI, caecum and colon) compared to piglets fed control diets. In study P135 (Zhu et al., 2011) a significant effect on pH in caecum and colon content was detected by feeding copper as Cu-Chitosan (8 and 16 mg/kg Cu) or as CuSO₄ (51 mg/kg Cu) to piglets compared to piglets fed a control diet. In study P146 (Huang et al., 2010) the effect of feeding copper supplemented diets to growing pigs on pH in faecal samples taken at the beginning of the experiment and after three and five weeks on the experimental diets were investigated. Four different copper supplemented diets were used: either 67 or 134 mg/kg Cu as CuSO₄ or 67 or 134 mg/kg Cu as CuCitrate. Apart from a small but significant decrease in pH in faecal samples taken after 5 weeks from the pigs fed the diet supplemented with 67 mg/kg Cu as CuSO₄ (pH 5.89 in faecal samples from the control fed pigs versus 5.79 in the copper fed pigs), no effect on pH was detected. In general the effect of supplementing pig diet with copper on faecal or gastrointestinal pH seems to be limited.

Diarrhoea. The effect of copper on diarrhoea incidence was investigated in three studies with piglets (P103, P116 and P124) and in one study with growing pigs (P146). In all studies addition of supplementary copper to the diet had a significant effect on the incidences of diarrhoea. In study P103 (Song et al., 2013), piglets were fed 36 mg/kg Cu as either Cu-CaMontmorillonite or Cu-NaMontmorillonite. Both copper sources significantly reduce the incidences of diarrhoea compared to the control fed pigs. In study P116 (Wang et al., 2011), significantly lower diarrhea incidence was observed in piglets fed a diet supplemented with 1.5 mg/kg Cu as Cu-Chitosan nanoparticles compared to the control fed piglets. However, although numerical lower, the incidence of diarrhoea was not significantly lower than in piglets fed copper free Chitosan nanoparticles. In study P124 (Xia et al., 2004), supplementation of the diet with 47 mg/kg Cu as Cu-Montmorillonite decreased the mean diarrhoea incidence from 19.2% in the control fed piglets to 5.4% in the copper fed piglets. In study P146 (Huang et al., 2010), diets supplemented with either 67 or 134 mg/kg Cu as CuSO₄ or 67 or 134 mg/kg Cu as CuCitrate all reduced the incidence of diarrhoea compared to the control fed pigs. However the results for the pigs fed the diet supplemented with 67 mg/kg Cu as CuSO₄ was not statistically different from the control fed pigs. In conclusion supplementation of pig diets with additional copper seems to reduce diarrhoea incidences.

Odour quality. The effect of dietary copper on the faecal odour characteristics was investigated in two experiments with piglets (P140 and P141) and in one experiment with growing pigs (P140). In study P140 (Armstrong et al., 2000), piglets weaned at 18 to 22 days of age were fed a control diet and five experimental diets supplemented either 66 mg/kg, 225 mg/kg Cu as CuSO₄, 33 mg/kg Cu as Cu Citrate, 66 mg/kg Cu as Cu Citrate or 100 mg/kg Cu as Cu Citrate. Two experiments were carried out: one where an antibiotic was included in the all diets tested (50 mg/kg Mecadox) and one without antibiotic in the diets. In both, experiment faecal samples were taken at day 28 for measurement of the odour characteristics. In the study with antibiotic the pigs were kept on the experimental diets through the growing-finishing phase and samples taken after 131 days for measurements of odour characteristics. In samples taken after 28 days from the piglets fed the diets with antibiotics addition
of 225 mg/kg Cu as CuSO₄ and 66 and 100 mg/kg Cu as Cu Citrate significantly reduced two of the all three odour characteristics measured (odour intensity and irritation intensity) while no significant effect were found on the third characteristic (odour quality). No significant effect of diets with 66 mg/kg Cu as CuSO₄ or 33 mg/kg Cu as CuSO₄ was found on any of the odour characteristics measured. In samples taken after 28 days from the piglets fed the diets without antibiotics, odour intensity was significantly reduced in piglets fed 33, 66 and 100 mg/kg Cu as Cu Citrate while no significant effects were found for piglets fed 66 or 225 mg/kg Cu as CuSO₄. None of the experimental diets affected irritation intensity. However, 225 mg/kg Cu as CuSO₄ and 33, 66 and 100 mg/kg Cu as Cu Citrate all significantly improved odour quality. No effect of 66 mg/kg CuSO₄ was found on odour quality. In samples taken after 131 days from the pig fed the diets with antibiotics odour intensity and irritation intensity were significantly reduced by all experimental diets except the diet supplemented with 33 mg/kg Cu as Cu Citrate which had no effect on odour intensity and irritation intensity. Odour quality was significantly improved in faecal samples from all pigs fed copper supplemented diets compared to faecal samples from the control pigs. In contrast to the above mentioned results no effect of adding copper (62 or 125 mg/kg as Cu as CuSO₄ or 15, 31, 62 or 62 mg/kg Cu as Cu Citrate) to antibiotic supplemented diets were found in study P141 (Armstrong et al., 2004) on either odour intensity, irritation intensity or odour quality of faecal samples from piglets (weaned at 16 to 18 days) after 40 days feeding the experimental diets. In conclusion supplementing pig diet with 66 or 100 mg/kg as Cu Citrate or 250 mg/kg as CuSO₄ may improve odour characteristics of swine waste. The mechanism of action was believed to be attributed to an antibiotic-like function of copper on the microbiota in the gastrointestinal tract.

**Effect of copper on microbial metabolites**

**Short Chain Fatty Acids (SCFA) concentration.** The effect of copper on SCFA in gastrointestinal content of piglets was investigated in two studies (P41 and P72). In study P41 (Højberg et al., 2005), no effect was found in SCFA (acetate, propionate and butyrate, isobutyrate, valerate and isovalerate) concentrations in content from the stomach SI, caecum or colon in piglets fed diets with added 175 mg/kg Cu as CuSO₄ compared to a control diet without addition of CuSO₄, both diets were fed with or without addition of 2500 mg/kg ZnO. In accordance with that, it was found in study P72 (Mei et al., 2010) that the addition of 100 or 175 mg/kg Cu as CuSO₄ had no effect on SCFA concentrations in caecum content of piglets; however, at a concentration of 250 mg/kg Cu as CuSO₄ a significant increase in all the SCFA’s measured (acetate, propionate and butyrate) was detected.

**Lactate.** Microbial production of lactate was investigated in two studies with piglets (P41 and P103). Addition of 2500 mg/kg Zn as ZnO to the diets in study P41 significantly reduced the concentration of lactate in digesta in the stomach and SI. However in the caecum and colon, where lactate is usually not detectable, a significant accumulation of lactate was observed in the piglets receiving the high ZnO diet. In contrast, the addition of 175 mg/kg Cu as CuSO₄ to the feed had no significance on the concentration of lactate in any segments of the gastrointestinal tract, but counteracted the accumulation of lactate found in the high ZnO fed piglets in caecum and colon. In study P103 (Song et al., 2013), the concentration of D-lactate was measured in plasma samples. D-lactate is produced by the microbiota in the gastrointestinal tract and absorbed to the blood. Mammals do not have the enzymatic system to metabolise D-lactate so plasma D-lactate may be a measurement of the microbial activity in the gastrointestinal tract. A significant reduction in plasma D-lactate was found in study P103 in pigs fed 36 mg/kg Cu as either Cu-CalMontmorillonite or Cu-NaMontmorillonite compared to the control fed pigs, indicating a reduced fermentation in the gastrointestinal tract in the piglets fed the copper supplemented diets.

**Succinate.** The concentration of succinate in gastrointestinal content was investigated in study in study P41 (Højberg et al., 2005). Addition of 2500 mg/kg Zn as ZnO to the diets in study P41 significantly reduced the concentration of succinate in digesta in the stomach and SI. However in the caecum and colon, where succinate is usually not detectable, a significant accumulation of succinate was observed in the piglets receiving the high ZnO diet. In contrast to Zn, the addition of 175 mg/kg Cu as CuSO₄ to the feed had no significance on the succinate concentration in any segments of the...
Effect of copper intake on gut microbiota (pigs/piglets, chickens, dairy cows)

gastrointestinal tract, but counteracted the accumulation of succinate in caecum and colon found in the high ZnO fed piglets.

Absorption of ammonia. Effect of copper on ammonia absorption was investigated in growing pigs in study P130 (Yen and Nienaber, 1993). The addition of 250 mg/kg Cu as CuSO₄ to the diet significantly reduced the net absorption of ammonia to the portal vein compared to pigs fed a diet without the addition of extra copper. As concluded by the author, that may reflect decreased urease activity and ammonia production by the gastrointestinal microbiota in the pigs receiving the copper supplemented diet.

Effect of copper on microbial activity

Deamination and decarboxylation of amino acids. The effect of copper on microbial formation of ammonia by deamination of amino acids and production of amines (histamine, putrescine, cadaverine, tyramine and phenylethylamine) by decarboxylation of amino acids in the SI has been investigated using ex vivo incubations in study P19 (Dierick et al., 1986) using SI content from growing pigs. Both processes were severely decreased if 98 mg/kg Cu as CuSO₄ was added to the incubations, indicating that copper has a sparing action on microbial degradation of amino acids in the small intestine, leaving more amino acids available for absorption.

Urease activity. Effect of copper on urease activity has been investigated in two studies, one study with piglets (study P41) and one study with growing pigs (study P111). In the study with piglets (P41, Højberg et al., 2005) urease activity was investigated in content from the stomach, SI, caecum and colon in piglets fed diets either added 175 mg/kg Cu as CuSO₄ or control diets without the addition of extra CuSO₄. Both diets were fed with or without addition of 2500 mg/kg ZnO. The urease activity was below the detection limit in stomach and SI content, highest urease activity was found in the colon. Addition of CuSO₄ to the diets had no significant effect of the urease activity. In contrast to piglets, a significant effect (p<0.10) of 49 mg/kg Cu as CuSO₄ was found on urease activity in fecal samples from growing pigs in study P111 (Varel et al., 1987).

ATP concentrations. Bacterial activity as determined by the concentration of adenosine triphosphate (ATP) was measured in study P41 and study P49. In study P41 (Højberg et al., 2005) no significant effects were found on ATP concentration in any of the gastrointestinal segment investigated (stomach, SI caecum and colon) by addition of 175 mg/kg Cu as CuSO₄ to piglet diets compared to a control diet without additional addition of CuSO₄. Similar results were found in study P49 (Jensen, 1989), where no effect of the addition of 170 mg/kg CuSO₄ to a diet containing 12 mg/kg Cu was found on the ATP concentrations in content from the stomach, three segments from the SI, the caecum and three segments from the colon.

Microbial enzyme activity. The effect of copper on microbial enzyme activity of β-glycosidase and β-glucuronidase has been investigated in one study (P123, Xia et al., 2005). No significant effect was found by the addition of 37 mg/kg Cu as CuSO₄ to the diets on either β-glycosidase or β-glucuronidase activity in SI or colon content. Addition of 37 mg/kg Cu as Cu-Montmorillonite reduced the activity of both enzymes in SI as well as colon content compared to the control fed pigs. However apart from the activity of β-glucuronidase in the colon content, the effects were not significantly different from the activities found in pigs fed copper free Montmorillonite.

Effect of copper in germ free pigs

Feeding high copper diets to germ free pigs has been investigated in one study with growing pigs (P149, Shurson et al., 1990). Germ free pigs tended to have higher average daily gain (ADG) and average daily feed intake (ADFI) than conventionally reared pigs. Addition of 250 mg/kg Cu as CuSO₄ to the feed tended to reduce ADG and ADFI in germ-free pigs while it increases ADG and ADFI in conventionally reared pigs. The high copper diets did not appear to make organ weight or intestinal characteristics in conventionally reared pigs different than those of the germ free pigs. High copper diets seem to reduce intestinal cell turnover in germ free pigs while it tended to accelerate intestinal turnover in conventionally reared pigs, perhaps via an interaction with the gut microbiota.
3.1.3. Overall effect of copper on the pig/piglet gut microbiota

**Microbial activity:** No effect of Cu as CuSO\(_4\) at 175 mg/kg feed on ATP and urease activity in piglets. Significant effect of Cu as CuSO\(_4\) on urease activity in growing pigs.

**Microbial metabolites:** No effect on SCFA, lactate or succinate concentrations in the gastrointestinal tract of inorganic or organic bound copper at concentrations below 175 mg/kg, 250 mg/kg Cu as CuSO\(_4\) on increase the SCFA concentrations.

**Clostridium**. It may be concluded from the experiments that supplementing piglet and growing pigs with low additional copper amounts (below 50 mg/kg Cu) seems to inhibit the population of coliform bacteria in the gastrointestinal tract. Especially clay bound copper seems to be effective. When supplemented to the feed in amounts above 100 mg/kg copper, it doesn’t seem to have any effect on the population of coliform bacteria in the gastrointestinal tract of pigs.

**Lactobacilli.** From the results it may be concluded that supplementing pig diets with copper concentrations above 170 mg/kg as CuSO\(_4\) has a reducing effect on the population of lactobacilli in the gastrointestinal tract of piglets as well as growing pigs. The effect of Cu as CuO did not affect the population of lactobacilli in piglets at high concentrations (250 and 375 mg/kg feed). Diets with copper concentration below 125 mg/kg feed seem to have marginal effect on the population of lactobacilli in the gastrointestinal tract, both in piglets and in growing pigs.

**Staphylococci:** In conclusion the effect of dietary copper on the gastrointestinal populations of staphylococci in pigs seems to be non-existent or very weak.

**Streptococci:** In conclusion the results with growing pigs strongly indicate that the addition of 100 mg Cu/kg, or higher, as CuSO\(_4\) significantly reduces the population of streptococci in the gastrointestinal tract of growing pigs. The effect of CuSO\(_4\) on the population of streptococci in piglets is more questionable.

In general, the studies on community structure strongly suggest that supplementing piglet diets with 100 to 250 mg/kg Cu as CuSO\(_4\) change the microbial community in the small intestine, as well as in the caecum and the colon.

In conclusion, supplementing pig diets with 66 or 100 mg/kg as Cu Citrate or 250 mg/kg as CuSO\(_4\) may improve odour characteristics of swine waste. The mechanism of action was believed to be attributed to an antibiotic-like function of copper on the microbiota in the gastrointestinal tract.

In general, the effect of supplementing pig diets with copper on faecal or gastrointestinal pH seems to be limited.

The overall conclusion from the studies with piglets and growing pigs is that copper even at low concentrations (<50 mg/kg feed) may affect the microbiota in the gastrointestinal tract. Especially the population of clostridia and coliform bacteria seems to be affected by low concentrations of copper. In particular, copper bound clay minerals seem to have an effect on the populations of coliform bacteria and clostridia. At higher concentrations (>170 mg/kg feed) Cu as CuSO\(_4\) reduce the population of lactobacilli in piglet as well as growing pigs. In growing pigs, the addition of Cu as CuSO\(_4\) reduces the population of streptococci in colonic and fecal samples, the population of ureolytic bacteria in the colon of which streptococci make up 74%, the urease activity in the colon, and decarboxylation and deamination of amino acids in the small intestine. However, no effect of Cu as CuSO\(_4\) on the population of streptococci and on urease activity was seen in piglets. Supplementing piglet diets with 100 to 250 mg/kg Cu as CuSO\(_4\) significantly affects the community structure of the microbiota in the small intestine, the caecum and the colon.
3.2. Chickens

3.2.1. Study selection

Assessment of the methodological quality of the studies

Table 8: Assessment of the methodological quality of the chicken studies.

| Study ID | Country | Language | Study design | Feed formulation | Duration of experiment | Replications | Number of animals/replication | Microbial methods | Statistical methods | Overall quality | Remarks |
|----------|---------|----------|--------------|------------------|-----------------------|--------------|-------------------------------|------------------|-------------------|-----------------|---------|
| CH2      | Turkey  | English  | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | XX              | * Samples for plate count of bacteria were stored on ice before treatment. |
| CH5      | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | No statistics on microbial data. |
| CH14     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | Concentration of Cu in diet not given. |
| CH15     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH16     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH18     | Korea   | English  | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH20     | China   | Chinese  | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH23     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH28     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH30     | China   | Chinese  | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH33     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH34 (Exp. 1) | Korea | Korean | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH34 (Exp. 2) | Korea | Korean | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH40     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH42 (ex vivo) | USA | English | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH42 (in vivo) | USA | English | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH43     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH44     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH45     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH46     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH47     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH48     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH49     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH50     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH51     | Korea   | Korean   | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH56     | USA     | English  | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH64     | China   | English  | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH65     | China   | English  | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |
| CH67     | China   | Chinese  | OK           | OK               | OK                    | OK           | OK                            | OK               | OK                | X               | |

X: Acceptable
XX: Quite good
XXX: Good
XXXX: Very good
XXXXX: Excellent

As shown in Table 8 the quality of the papers retrieved from the search on chickens was generally judged to be low. Ten out of 17 papers were written in foreign languages (7 in Korean and 3 in Chinese) with English Abstracts and Table and Figure legends.

3.2.2. Results

Type of copper sources used in the studies with chickens

Four types of copper sources were used in the research with chickens (Table 9): 1) inorganic copper, 2) organic bound copper, 3) clay bound copper and 4) copper-loaded nanoparticles.

Inorganic copper was used in 13 of the 17 studies. The sources were CuSO₄ (concentrations 37–375 mg Cu/kg feed, in 13 studies).

Organic bound copper was used in 8 of the 17 studies. The sources were Cu-soy proteinate (concentrations 50–100 mg Cu/kg feed, in studies CH2, CH14, CH15, CH16, and CH40), Cu-Met (concentrations 50–100 mg Cu/kg feed, in studies CH16, CH33 and CH40), Cu-fish meal (concentration 200 mg Cu/kg feed, in study CH33), starch Cu complex (concentration 200 mg Cu/kg feed, in study CH33), starch methionine Cu complex (concentration 200 mg Cu/kg feed, in study CH34), and Na-alginate Cu complex (concentration 200 mg Cu/kg feed, in study CH34).

Clay bound copper was used in 2 of the 17 studies. The source was copper-bearing montmorillonite from aluminosilicate clay (Cu-MMT). It was used in study CH64 and CH65 at concentrations from 25 to 37 mg Cu/kg feed.

Copper-loaded nanoparticles were used in 2 of the 17 studies. The source was copper-Montmorillonite nanomaterial (concentrations 34–68 mg Cu/kg feed, in study CH30) and copper silicate nanoparticles (concentration 20 mg Cu/kg feed, in study CH51).
Effect of copper intake on gut microbiota (pigs/piglets, chickens, dairy cows)

Setup of the individual studies

Table 9: Setup of the individual studies

| Study | Ref | Author | Country | Category | No/treatment | Diet | ppm Cu | Source | ADG | ADFI | G/F | Microbial characteristics investigated |
|-------|-----|--------|---------|----------|--------------|------|-------|--------|-----|-----|-----|---------------------------------------|
| C15  | C15 | Byun et al., 2011 | South Korea | Broiler | 4 groups of 35 chicks | <18 days | NS | NS | Control | 44.5 | 78.6 | 0.56 | Microbial enzyme activity in gut content |
| C16  | C16 | Byun et al., 2012 | South Korea | Broiler | 4 groups of 35 chicks | 6 weeks | NS | NS | Control | 47.6 | 84.4 | 0.58 | Microbial enzyme activity in gut content |
| C17  | C17 | Byun et al., 2013 | South Korea | Broiler | 4 groups of 35 chicks | 6 weeks | NS | NS | Control | 44.5 | 78.6 | 0.56 | Microbial enzyme activity in gut content |
| C18  | C18 | Byun et al., 2014 | South Korea | Broiler | 4 groups of 35 chicks | 6 weeks | NS | NS | Control | 47.6 | 84.4 | 0.58 | Microbial enzyme activity in gut content |
| C19  | C19 | Byun et al., 2015 | South Korea | Broiler | 4 groups of 35 chicks | 6 weeks | NS | NS | Control | 44.5 | 78.6 | 0.56 | Microbial enzyme activity in gut content |
| C20  | C20 | Byun et al., 2016 | South Korea | Broiler | 4 groups of 35 chicks | 6 weeks | NS | NS | Control | 47.6 | 84.4 | 0.58 | Microbial enzyme activity in gut content |
| C21  | C21 | Byun et al., 2017 | South Korea | Broiler | 4 groups of 35 chicks | 6 weeks | NS | NS | Control | 44.5 | 78.6 | 0.56 | Microbial enzyme activity in gut content |

Microbial characteristics investigated in the studies

The microbial characteristics measured in each of the 17 selected studies with chickens are shown in Table 10. Fifteen of the studies were done with broilers (CH2, CH5, CH14, CH15, CH16, CH18, CH30, CH33, CH34, CH42, CH43, CH51, CH56, CH64, CH65 and CH67) and 2 were done with laying hens (CH15 and CH40).
Table 10: Microbial characteristics investigated in the studies with chickens

| Microbial characteristics investigated | Study |
|--------------------------------------|-------|
|                                     | CH2   |
|                                      | CH5   |
|                                      | CH14  |
|                                      | CH15  |
|                                      | CH16  |
|                                      | CH30  |
|                                      | CH33  |
|                                      | CH40  |
|                                      | CH41  |
|                                      | CH42  |
|                                      | CH43  |
|                                      | CH51  |
|                                      | CH56  |
|                                      | CH64  |
|                                      | CH65  |
|                                      | CH67  |
| Plate count of microbial populations: |       |
| Bifidobacterium                      | X     |
| Campylobacter                        | X     |
| Clostridia                           | X     |
| Enterococci                          | X     |
| Lactobacilli                         | X     |
| Salmonella                           | X     |
| Staphylococci                        | X     |
| Streptococci                         | X     |
| Total anaerobes                      | X     |
| Total aerobes                        | X     |
| Community structure                  |       |
| % of species                         |       |
| Similarity                           |       |
| Gut content characteristics          |       |
| Faecal moisture content              | X     |
| pH                                   | X     |
| Ammonia emission                     |       |
| Gizzard erosion                      | X     |
| Microbial metabolites                |       |
| Ammonia concentration                |       |
| Microbial activity                   |       |
| β-Glucosidase activity               | X     |
| β-Glucosidase activity               | X     |

Results from the studies

Effect of copper on microbial populations

*Bifidobacteria.* The effect of copper on the population density of bifidobacteria was investigated in four studies with chickens (CH30, CH64, CH65 and CH67). In study CH30 (Ma et al., 2006), no significant effect was found on the population density of bifidobacteria in content from the small intestine or caecum from broilers by addition of 34 or 68 mg/kg copper as Copper Montmorillonite Nanomaterial to the diet. Similar results were found in study CH64 (Xia et al., 2004) by addition of 37 mg/kg copper as Copper Montmorillonite or as CuSO₄ and in study CH65 (Xu et al., 2003), by addition of 25 mg/kg copper to the diet as Copper Montmorillonite to the diet. In study CH67 (Zhang et al., 2009), a significant increase in the population of bifidobacteria in content from the cecum was found by addition of 8 or 150 mg/kg copper as CuSO₄ to the diet both in broilers slaughtered after 4 and 7 weeks feeding the treatment diets. In contrast to that, addition of 225 mg/kg copper to the diet as CuSO₄ resulted in a significant reduction in the population of bifidobacteria in cecal content in broilers slaughtered after 4 weeks while no effect was found in broilers slaughtered after 7 weeks. In all studies the population density of bifidobacteria were determined by counting the bacteria on selective media but several studies has shown that no really good selective media exist for the enumeration of bifidobacteria.

*Campylobacter.* The effect of copper on the population density of campylobacter was investigated in study CH2 (Aydin et al., 2010). No significant effect of an addition of 250 mg/kg copper as CuSO₄ to the diet was found on the population density of Campylobacter in ileal content.

*Clostridia.* The effect of copper on the population density on clostridia in content from the small intestine was investigated in 6 studies with broilers (CH14, CH16, CH30, CH33, CH64 and CH65) and in two studies with layers (CH15 and CH40). The effect of copper concentration and copper sources on populations of clostridia in chicken gut content is shown in Table 11. A significant reduction in the population of clostridia in SI content from broilers were found by the addition of 100 mg/kg Cu as Cuproteinate in study CH14 (Kim et al., 2014a), by addition of 34 or 68 mg/kg Cu as Cu-Montmorillonite-Nanomaterial to the diet in study CH30 (Ma et al., 2006), by addition of 37 mg/kg Cu to the diet as Cu-Montmorillonite in study CH64 (Xia et al., 2004), and by addition of 25 mg/kg Cu to the diet as Cu-Montmorillonite in study CH65 (Xu et al., 2003), while no significant effect on the population density of clostridia in SI content from broilers were found in study CH16 (Kim et al., 2011) by addition of neither 50 or 100 mg/kg Cu as Cu-methionine nor by addition of 50 or 100 mg/kg Cu as...
Cu-proteinate. Similarly, no effect on the population of clostridia was found in study CH33 (Min et al., 1994) by the addition of 200 mg/kg Cu as either CuSO₄, Cu-methionine or Cu-fish meal to the diet; or in study CH64 by the addition of 37 mg/kg Cu as CuSO₄. In the studies with layers a significant reduction of the population of clostridia were found in both investigations (CH15 and CH40). In study CH15 (Choi et al., 1989) the population of clostridia was reduced by the addition of 50 and 100 mg/kg Cu whatever it was added to the diet as CuSO₄ or Cu-proteinate, and in study CH40 (Paik et al., 2008), by addition of 50 and 100 mg/kg Cu whatever it was added as Cu-methionine or Cu-proteinate. The effect of copper on the density of clostridia in content from the caecum was investigated in two studies (CH64 and CH65). In study CH64 (Xia et al., 2004) a significant reduction in the population of clostridia was found by the addition of 37 mg/kg Cu as Cu-Montmorillonite to the diet while no effect was found by the addition of 37 mg/kg Cu as CuSO₄ to the diet. In study CH65 (Xu et al., 2003) no effect on the population of clostridia in caecum content was found by the addition or 25 mg/kg Cu-Montmorillonite to the diet. Supplementing chicken (broilers and layers) diets with additional copper seems to reduce the population of clostridia in the gastrointestinal tract of the birds, even at low copper concentrations. In particular, clay bound copper seems to be effective in reducing the population of clostridia.
Table 11: Effect of copper source and concentration on populations of clostridia, lactobacilli and coliform bacteria in gut content from the gizzard, small intestine (SI) and caecum of chickens.

| Study ID | Animal category | Copper mg/kg | Source | Lactobacilli | Coliforms | Clostridia |
|----------|-----------------|-------------|--------|--------------|-----------|------------|
| CH67     | Broilers        | 8           | CuSO₄  | ↑            | ↓         | ↑          |
| CH67     | Broilers        | 8           | CuSO₄  | ↑            | ↓         | ↓          |
| CH51     | Broilers        | 20          | CuSN   | ↑            | ↓         | ↓          |
| CH65     | Broilers        | 25          | CuMM   | → → → →      | → → ↓     | →          |
| CH30     | Broilers        | 34          | CuMMN  | → → → →      | ↓ → ↓     | ↓          |
| CH67     | Broilers        | 37          | CuSO₄  | → → → →      | ↓ → ↓     | ↓          |
| CH67     | Broilers        | 37          | CuMM   | → → → →      | ↓ → ↓     | ↓          |
| CH30     | Broilers        | 68          | CuMMN  | → → → →      | ↓ → ↓     |
| CH15     | Layers          | 50          | CuSO₄  | ↑            | →         | ↓          |
| CH15     | Layers          | 50          | CuPro  | ↑            | →         | ↓          |
| CH16     | Broilers        | 50          | CuPro  | →            | →         | ↓          |
| CH40     | Layers          | 50          | CuPro  | ↑            | →         | ↓          |
| CH40     | Layers          | 50          | CuMet  | (↑)          | →         | (↓)        |
| CH16     | Broilers        | 50          | CuMet  | ↑            | →         | ↓          |
| CH15     | Layers          | 100         | CuSO₄  | ↑            | →         | ↓          |
| CH14     | Broilers        | 50          | CuPro  | →            | →         | ↓          |
| CH16     | Broilers        | 100         | CuPro  | ↑            | →         | ↓          |
| CH40     | Layers          | 100         | CuPro  | ↑            | →         | ↓          |
| CH16     | Broilers        | 100         | CuMet  | ↑            | →         | ↓          |
| CH15     | Layers          | 100         | CuPro  | →            | →         | ↓          |
| CH15     | Layers          | 50          | CuSO₄  | ↑            | →         | ↓          |
| CH14     | Broilers        | 50          | CuPro  | →            | →         | ↓          |
| CH16     | Broilers        | 100         | CuPro  | →            | →         | ↓          |
| CH40     | Layers          | 100         | CuPro  | ↑            | →         | ↓          |
| CH16     | Broilers        | 100         | CuMet  | ↑            | →         | ↓          |
| CH15     | Layers          | 125         | CuSO₄  | →            | →         | ↓          |
| CH67     | Broilers        | 150         | CuSO₄  | ↑            | →         | ↓          |
| CH65     | Broilers        | 150         | CuSO₄  | ↑            | →         | ↓          |
| CH42     | Broilers        | 187         | CuSO₄  | → → → →      | → → → →   |
| CH42     | Broilers        | 187         | TCuC   | → → → →      | → → → →   |
| CH18     | Broilers        | 200         | CuSO₄  | → → → →      | → → → →   |
| CH18     | Broilers        | 200         | CuSO₄  | → → → →      | → → → →   |
| CH18     | Broilers        | 200         | CuSO₄  | → → → →      | → → → →   |
| CH18     | Broilers        | 200         | CuSO₄  | → → → →      | → → → →   |
| CH33     | Broilers        | 200         | CuSO₄  | → → → →      | → → → →   |
| CH34,1   | Broilers        | 200         | CuSO₄  | → → → →      | → → → →   |
| CH34,1   | Broilers        | 200         | CuSO₄  | → → → →      | → → → →   |
| CH34,1   | Broilers        | 200         | CuSO₄  | → → → →      | → → → →   |
| CH33     | Broilers        | 200         | CuMet  | → → → →      | → → → →   |
| CH33     | Broilers        | 200         | CuFM   | (↑)          | → → → →   |
| CH34,2   | Broilers        | 200         | CuStarch/Met | → (↑) → → → | → (↑) → → → |
| CH34,2   | Broilers        | 200         | CuStarch/Met | → → → → → | → → → → → |
| CH34,2   | Broilers        | 200         | CuStarch/Met | → → → → → | → → → → → |
| CH34,2   | Broilers        | 200         | CuStarch/Met | → → → → → | → → → → → |
| CH34,2   | Broilers        | 200         | CuStarch/Met | → → → → → | → → → → → |
| CH34,2   | Broilers        | 200         | CuAlg   | → (↑) → → → | → (↑) → → → |
| CH67     | Broilers        | 225         | CuSO₄  | ↓ → → → →   |
| CH67     | Broilers        | 225         | CuSO₄  | ↓ → → → →   |
| CH5      | Broilers        | 250         | CuSO₄  | ↓ → → → →   |
| CH2      | Broilers        | 250         | CuPro  | → → → → →   |
| CH5      | Broilers        | 375         | CuSO₄  | ↓ → → → →   |
| CH5      | Broilers        | 375         | CuSO₄  | ↓ → → → →   |

- ↑: Significant (p<0.05 or less) increase in the population
- ↓: Significant (p<0.05 or less) decrease in the population
- →: No significant effect on the population
- BD: Below detection limit
**Coliform bacteria.** The effect of copper on the population density of coliform bacteria in content from the gizzard was investigated in three investigations (CH18, *Kim et al.*, 1993 and CH34 experiments 1 and 2, *Min et al.*, 1993). As shown in Table 11, no effect of neither copper concentration nor copper source was found. The concentrations and sources of copper tested were: 200 mg/kg Cu as either CuSO₄·H₂O or CuSO₄·5H₂O (study CH18 and CH34 experiments 1 and 2), 200 mg/kg Cu as a Cu-starch complex (CH34 experiment 1), 200 mg/kg Cu as a Cu-starch/methionine complex (CH34, experiments 1 and 2) and 200 mg/kg Cu as a Na-alginate Cu complex.

The effect of copper on the population density of coliform bacteria in SI content was investigated in 12 experiments with broilers and in 2 experiments with layers (Table 11). A significant reduction in the population of coliform bacteria was found in study CH5 (*Choi et al.*, 1989) by the addition of 125, 250 or 375 mg/kg Cu as CuSO₄ to the diet, while no effect of an addition of 200 mg/kg Cu as CuSO₄ was found in studies CH18 (*Kim et al.*, 1993), CH33 (*Min et al.*, 1994), CH34 experiments 1 and 2 (*Min et al.*, 1993); and of 37 mg/kg Cu as CuSO₄ in study CH64. Furthermore, no effect was found in layers (CH15, *Kim et al.*, 2014b) fed diets containing 50 or 100 mg/kg Cu as CuSO₄. In study CH18 (*Kim et al.*, 1993) a significant reduction in the population of coliform bacteria in broiler SI content was found by the addition of 100 mg/kg Cu as Cu-methionine to the diet while it had no effect at a concentration of 50 mg/kg. No effect was found by the addition of 200 mg/kg Cu as Cu-methionine in study CH33 (*Min et al.*, 1994) and no effects were found in layers at concentrations of 50 and 100 mg/kg (CH40, *Paik et al.*, 2008). Copper as Cu-protonate was shown to reduce the population of coliform bacteria in study CH16 (*Kim et al.*, 2011) when it was added to the diet at a concentration of 100 mg/kg Cu while it had no effect at a concentration of 50 mg/kg. No effect of Cu-protonate was found in study CH2 (*Aydin et al.*, 2010) at a concentration of 250 mg/kg Cu or in study CH14 (*Kim et al.*, 2014a) at a concentration of 100 mg/kg Cu. Cu-propionate at concentrations of 50 and 100 mg/kg Cu had no effect on the density of coliform bacteria in SI content from layers neither in study CH15 nor in study CH40 (*Paik et al.*, 2008). A significant reduction in the population of coliform bacteria in SI content from broilers were found in study CH30 (*Ma et al.*, 2006) by addition of 34 or 68 mg/kg Cu as Cu-Montmorillonite-Nanomaterial to the diet and in study CH51 (*Shi et al.*, 2013) by addition of 37 mg/kg Cu as Cu-Montmorillonite at no effect was found by Cu-Montmorillonite at a concentration of 25 mg/kg Cu in study CH65 (*Xu et al.*, 2003). No effect was found on coliform bacteria of concentrations of 200 mg/kg Cu as a Cu-fish meal complex (study CH33, *Min et al.*, 1994), of 200 mg/kg Cu as a Cu-starch complex (Study CH34 experiment 1, *Min et al.*, 1993), of 200 mg/kg Cu as a Cu-starch/methionine complex (CH34 experiments 1 and 2) or of 200 mg/kg Cu as a Na-alginate Cu complex (CH34 experiment 2).

Two *ex vivo* experiments (CH42, *Pang et al* 2009a and CH43, *Pang et al.*, 2009b) have been conducted where small intestinal content was incubated for 24 hours with or without addition of copper. In both experiments it was found that 250 mg/kg as CuSO₄ reduced the growth of coliform bacteria, while 125 mg/kg Cu as CuSO₄ or tribasic copper chloride at concentrations of 125 mg/kg and 250 mg/kg had no effect on the growth of coliform bacteria.

The effect of copper on the population of coliform bacteria in caecal content has been investigated in seven studies with broilers (CH18, CH34 experiment 1, CH34 experiment 2, CH51, CH64, CH65 and CH67). A summary of results from the experiments are shown in Table 11. No effect on the population of coliform bacteria were found by feed concentrations of 200 mg/kg Cu as CuSO₄ in study CH18 (*Kim et al.*, 1993), CH34 experiments 1 or 2 (*Min et al.*, 1993) and by concentrations of 37 mg/kg Cu as CuSO₄ in study CH64 (*Xia et al.*, 2004). A significant decrease in the population of coliform bacteria was found in study CH67 (*Zhang et al.*, 2009) by the addition of 150 mg/kg Cu as CuSO₄ to the diet while addition of Cu as CuSO₄ to the diet at a concentration of 225 mg/kg Cu resulted in a significant increase in the population of coliform bacteria. Supplemeting 20 mg/kg Cu as Cu-silicated nanoparticles to the diet resulted in a significant reduction in the population of coliform bacteria (CH51, *Shi et al.*, 2013) the same was the case if 37 mg/kg Cu as Cu-Montmorillonite was added to the diet (CH64, *Xia et al.*, 2004), while addition of 25 mg/kg Cu as Cu-Montmorillonite had no significant effect on the population of coliform bacteria (CH65, *Xu et al.*, 2003). No effect was found on the population of coliform bacteria of cecum content by supplementing feed with 200 mg/kg Cu as a Cu-fish meal complex (CH33, *Min et al.*, 1994), or with 200 mg/kg Cu as a Cu-starch complex (CH34 experiment 2).
Effect of copper intake on gut microbiota (pigs/piglets, chickens, dairy cows)

In general the effect of copper on the gastrointestinal populations of coliform bacteria seems to be rather weak, in most cases where an effect was seen, the concentrations were higher than 200 mg/kg Cu; the exception may be copper bound clay particles, especially copper bound nanoparticles that seems to have an effect at concentrations below 50 mg/kg Cu (CH30 and CH51). That high concentrations of copper may reduce the population of coliform bacteria in gut content from chickens is in agreement with the *ex vivo* experiment studies (CH42 and CH43) that show that Cu as CuSO4 inhibit the growth of coliform bacteria at a concentration of 250 mg/kg Cu but not at a concentration of 125 mg/kg Cu.

**Enterococci.** The effect of copper on the population of enterococci has only been investigated in a single experiment (CH2, Aydin et al., 2010). Supplementation of the diet with Cu-proteinate corresponding to a Cu concentration at 250 mg/kg Cu had no significant effect on the population of enterococci in broiler ileal content.

**Lactobacilli.** The effect of copper concentration and copper sources on populations of lactobacilli in gut content is shown in Table 11.

The population lactic acid bacteria (LAB) in content from the gizzard was investigated in three experiments with broilers: CH18 (Kim et al., 1993), CH34 experiment 1 and CH34 experiment 2 (Min et al., 1993). No effect of either copper concentration or copper source was found (Table 11).

The effect of copper on the population density of lactobacilli in SI content was investigated in 12 experiments with broilers and in 2 experiments with layers. A significant reduction in the population of lactobacilli was found in study CH5 (Choi et al., 1989) by addition of 250 or 375 mg/kg Cu as CuSO4 to the diet, while no effect was found by addition of 125 mg/kg Cu as CuSO4. No effect of the addition of 200 mg/kg Cu as CuSO4 was found in studies CH18 (Kim et al. 1993), CH33 (Min et al., 1994), CH34 experiments 1 and 2 (Min et al., 1993); and of 37 mg/kg Cu as CuSO4 in study CH64 (Xia et al., 2004). In the studies with layers (CH15, Kim et al., 2014b) the population of lactobacilli was significantly increased when the diet was supplemented with either 50 or 100 mg/kg Cu as CuSO4. Cu-methionat at a Cu concentration of 200 mg/kg Cu had no effect on the population of lactobacilli (CH33, Min et al., 1994) while at Cu concentrations of 100 mg/kg, it had a significant stimulating effect on the population of lactobacilli in broiler SI content (CH16, Kim et al., 2011). Only a tendency to an increase (CH16) was found when it was added to a diet at a concentration of 50 mg/kg Cu. In layers fed a diet supplemented with 100 mg/kg Cu as Cu-methionat a significant increase in the population of lactobacilli was observed (CH40, Paik et al., 2008), while at a concentration of 50 mg/kg it resulted in a tendency to an increase (CH40). Addition of Cu-propionate corresponding to Cu amount of 100 mg/kg to broiler diets resulted in an increase in the population in study CH40 while no effect was seen in study CH14 (Kim et al., 2014a). At an addition corresponding to a Cu concentration of 50 mg/kg no effect was found of Cu-propionate on the population of lactobacilli. In layers addition of Cu-propionate corresponding to an addition of 100 mg/kg Cu resulted either in a significant reduction in the population of lactobacilli (study CH15) or to a tendency to an increase (study CH40). Both in study CH15 and CH40, an increase was detected in the population of lactobacilli when layer diets were supplemented with 50 ppm Cu as Cu-propionate. No effect on the population of lactobacilli in SI content from broilers were found in study CH30 by addition of 34 or 68 mg/kg Cu as Cu-Montmorillonite-Nanomaterial to the diet; in study CH67 (Zhang et al., 2009) by addition of 37 mg/kg Cu as Cu-Montmorillonite; and in study CH65 with Cu-Montmorillonite at a concentration of 25 mg/kg Cu.

Two *ex vivo* experiments (CH42 *ex vivo* and CH43, Pang et al 2009a and b) have been conducted where small intestinal content was incubated for 24 hours with or without addition of copper. In study CH42 *ex vivo* it was found that 125 mg/kg as CuSO4 increase the growth of lactobacilli, while 250 mg/kg Cu as CuSO4, 250 mg/kg Cu as tribasic copper chloride and 125 mg/kg Cu as tribasic copper chloride had no effect. In study CH43 the growth of lactobacilli was neither affected by copper concentration or copper source (125 and 250 mg/kg Cu as either CuSO4 or tribasic copper chloride).
The population lactobacilli in content from the caecum of broilers was investigated in seven experiments (Table 11): CH18, CH34 experiment 1, CH34 experiment 2, CH51, CH64, CH65 and CH67. In experiment CH18, no effects of supplementing feed with 200 mg/kg Cu as CuSO₄ were found on the population of lactobacilli in cecal content. The same was the case in study 34 experiments 1 and 2 when feed was supplemented with 200 mg/kg Cu-starch, Cu starch/met and Cu-NaAlginate; in experiment CH64, for supplementation with 37 mg/kg CuSO₄; and in experiments CH64 and CH65 for supplementation with 37 and 24 mg/kg Cu-Montmorillonite. In study CH51, addition of 20 mg/kg Cu as Cu-Montmorillonite-Nanomaterial to broiler fed stimulated the population of lactobacilli in cecal content. The same was found in study CH67 when it was added at concentrations of 8 mg/kg and 150 mg/kg Cu as CuSO₄ to broiler diets, while Cu as CuSO₄ at a concentration of 225 mg/kg resulted in a significant reduction in the population of lactobacilli.

From the investigation of copper concentrations and sources on the population of lactobacilli in gastrointestinal content it may be concluded that copper concentrations at or below 150 mg/kg seems to stimulate the population of lactobacilli in chicken gut content, while it does not seem to have any effect at concentrations from 187 to 200 mg/kg but has an inhibitory effect at concentrations above 225 mg/kg. In agreement with that, the ex vivo experiments (CH42, Pang et al., 2009a and CH43, Pang et al. 2009b) show that 125 mg/kg Cu as CuSO₄ stimulated the growth of lactobacilli in broiler SI content. However no inhibitory effect on the growth was seen by a copper concentration of 250 mg/kg CuSO₄. That no effect of tribasic copper chloride (TBCC) was seen in the ex vivo experiment is proper due to the low solubility of TBCC in water (CH42).

Salmonella. The effect of copper on the population of Salmonella spp. has only been investigated in a single experiment (CH2, Aydin et al., 2010). Copper supplementation with Cu-proteinate corresponding to a Cu concentration at 250 mg/kg Cu had no significance on the population density of Salmonella spp. in broiler ileal content.

Staphylococci. The effect of copper on the population of staphylococci has only been investigated in a single experiment (CH2, Aydin et al., 2010). Copper supplementation with Cu-proteinate corresponding to a Cu concentration of 250 mg/kg Cu had no significant effect on the population density of staphylococci in broiler ileal content.

Streptococci. The effect of copper on the population of streptococci was investigated in three experiments with broilers from two studies (CH5 and CH34 experiment 1 and 2). In study CH5 (Choi et al., 1989) the population of streptococci were higher after 1 week but lower after 3 weeks in content from the SI of broilers fed either 125, 250 or 375 mg/kg Cu as CuSO₄ compared to the control fed birds, while no effect were seen of any of the Cu concentrations after 5 or 7 weeks on the experimental diets. In study CH34 experiment 1 (Min et al., 1993) the effect of the addition of 200 mg/kg Cu as CuSO₄, Cu-starch or Cu-starch/methionine to diets to broilers on the population of streptococci in content from the gizzard, upper and lower SI and cecum was investigated. No effect of any of the added copper sources was found on the population of streptococci in any of the investigated GIT segments. In study CH34 experiment 2, the effect of the addition of 200 mg/kg Cu as CuSO₄, a Cu-starch/methionine complex, a Cu-starch/methionine complex (added to a diet where the methionine was reduced with the amount of methionine added with the Cu-starch/methionine complex) and a Na-alginate Cu complex to diets of broilers on the population of staphylococci in content from the gizzard, upper and lower SI and cecum was investigated. No effect of any of the added copper sources was found on the population of streptococci in any of the investigated GIT segments. In general supplementing broiler diets with additional copper dose not seems to have any effect on the population of streptococci in the gastrointestinal tract.

Total aerobe bacteria. The effect of copper on the population of total anaerobic bacteria was investigated in three studies (CH30, CH51 and CH65). No effect was found in study CH30 (Ma et al., 1994) by the addition of 34 or 68 mg/kg Cu as Cu-Montmorillonite-Nanomaterial to the diet to broilers neither on the population of aerobic bacteria in ileal content nor on the population of aerobic bacteria in cecal content. A significant decrease in the population of aerobic bacteria in cecal content was found in study CH51 (Shi et al., 2013) by addition of 20 mg/kg Cu as Cu Silicat Nanoparticles to broiler feed. No effect was found in study CH65 (Xu et al., 2003) on the population of aerobic bacteria.
in ileal and cecal content by addition of 24 mg/kg Cu as Cu-Montmorillonite to broiler feed neither in birds slaughtered after 21 days on the diet or in birds slaughtered after 42 days on the diet.

Total anaerobe bacteria. The effect of copper on the population of total anaerobic bacteria was investigated in six studies (CH2, CH16, CH30, CH51, CH64 and CH65). No effect was found in study CH2 (Aydin et al., 2010) on the population of anaerobic bacteria in broiler ileal content by addition of 250 mg/kg Cu as CuSO4 to the diet. An increase in the population of anaerobic bacteria in content from the SI was found in study CH16 (Kim et al., 2011) by addition of 100 mg/kg of either Cu-methionite or Cu-protonate to the diet of broilers while no effect was found by addition of 50 mg/kg Cu as either Cu-methionite or Cu-protonate to the diet. No effect was found in study CH30 (Ma et al., 2006) by addition of 34 or 68 mg/kg Cu as Cu-Montmorillonite-Nanomaterial to the diet to broilers neither on the population of anaerobic bacteria in ileal content nor on the population of anaerobic bacteria in cecal content. A significant increase in the population of anaerobic bacteria in cecal content was found in study CH51 (Shi et al., 2013) by addition of 20 mg/kg Cu as Cu Silicat Nanoparticles to broiler feed. No effect was found in study CH64 (Xia et al., 2004) on the population of anaerobic bacteria in ileal and cecal content by addition of neither 37 mg/kg Cu as CuSO4 or as Cu-Montmorillonite to broiler feed. Likewise no effect was found in study CH65 (Xu et al., 2003) on the population of anaerobic bacteria in ileal and cecal content by addition of 24 mg/kg Cu as Cu-Montmorillonite to broiler feed neither in birds slaughtered after 21 days or in birds slaughtered after 42 days on the diet.

Effect of copper on community structure

The microbial community was investigated in two studies (CH42 and CH56). In both studies the microbial community was determined by isolating bacterial DNA, amplifying the V3 region of the 16S ribosomal DNA and performing denaturing gradient gel electrophoreses (DGGE). In both studies the number of bands per sample and the similarity indices for bands between samples were calculated. The number of bands is indicative for the number of bacterial species within a sample whereas the similarity coefficients indicate how similar the bacteria within a treatment group are. In study CH56 (Thompsen et al., 2008) broilers were fed either a control diet, the control diet supplemented with 30 mg/kg bacitracin or the control diet supplemented with 250 mg/kg Cu as CuSO4 from day 21 to day 42. At day 42, six birds per treatment group were slaughtered and the bacterial community structure analysed in samples of ileal content and samples of ileal mucosa. Amplicon profiles from the ileal content revealed that dietary treatment had no effect on band numbers or similarity coefficients. Likewise, band numbers of the mucosa-associated bacteria were not affected by dietary treatments. The comparison similarity coefficients, however, revealed that the mucosa-associated bacterial communities of birds fed 250 mg/kg Cu as CuSO4 had higher similarity indices than either the control or bacitracin fed broilers. The high similarity coefficient observed in birds fed copper might indicate that addition of high concentrations of copper may select for copper resistant bacteria. In study CH42 (Pang et al., 2009a) broilers were fed either a control diet, a diet supplemented with 188 mg/kg Cu as CuSO4 or a diet supplemented with 188 mg/kg Cu as tribasic copper chloride (TBCC) from hatch to day 35. On day 34 eight birds per treatment were slaughtered and the bacterial community structure analysed in samples of ileal content and samples of ileal mucosa. Neither Cu supplementation at 187.5 mg/kg nor Cu sources affected the number of predominant bacterial species (DGGE band number) in ileal content (mean = 9.6) or ileal mucosa (mean = 8.6). Furthermore, neither Cu supplementation at 187.5 mg/kg nor Cu sources (CuSO4 or TBCC) affected the similarity coefficient of the microbial community structure in ileal content. However supplementation of the diet with 187.5 mg/kg Cu as TBCC increased the similarity coefficient of ileal mucosal bacteria, while supplementing the diet with 187.5 mg/kg Cu as CuSO4 did not affect the similarity coefficient of ileal mucosal bacteria. This shows that the mucosa associated microbiota was more similar between individual birds for the TBCC fed broilers, compared to the control and the CuSO4 fed birds. In conclusion the effect of copper supplementation of broiler diets seems to have little effect on the community structure of the microbiota in the small intestine. No effect of supplementing broiler diets with either 188 or 250 mg/kg Cu as CuSO4 or 188 mg/kg Cu as TBCC were found on band numbers (number of bacterial species) neither in digesta samples nor in mucosal samples. Moreover, no effect of copper...
supplementation was found on population similarity in digesta samples, while 250 mg/kg Cu as CuSO₄ affected the population similarity in mucosa samples in study CH56 but no effect of 188 mg/kg Cu as CuSO₄ was found on the population similarity in mucosal samples in study CH42. In study CH42, however, an effect of 188 mg/kg Cu as TBCC was found on the population similarity in mucosal samples.

**Effect of copper on gut content characteristics**

**Fecal moisture content.** Fecal moisture content was investigated in one study (CH5, Choi et al., 1989). It was measured each week during the six weeks of the experiment. The moisture content was significantly higher at week 1 in the chickens fed 125 and 250 mg/kg Cu as CuSO₄ and numerically higher in the group fed 375 mg/kg Cu as CuSO₄ compared to the control group (71.0, 74.3, 74.5 and 73.3 % for the control group and the groups fed diets supplemented with 125, 250 and 375 mg/kg Cu as CuSO₄ respectively). No effect on the moisture content of any of the copper concentrations investigated was found from week 2 to 6.

**pH.** The effect of copper on pH of gastrointestinal or fecal content was investigated in five experiments with broilers (CH2, CH33, CH34 experiment 1, CH34 experiment 2 and CH51). In study CH2 (Aydin et al., 2010), no effect of 250 mg/kg Cu as Cu-proteinate was found on pH of ileal content in broilers slaughtered at day 42. In study CH43 experiment 1 (Min et al., 1993), the addition of 200 mg/kg Cu as either CuSO₄, a Cu-starch complex or a Cu-starch/methionine complex to broiler diets had no effect on pH of content from gizzard, upper SI, lower SI or fecum taken from broilers slaughtered at day 42. In study CH33 (Min et al., 1994), supplementation of broiler diets with 200 mg/kg Cu as CuSO₄, Cu-methionine or Cu-fish meal significantly reduced the pH in content from the gizzard (4.03, 3.79, 3.56 and 3.66 for the control group and the three Cu supplemented groups, respectively) from broilers slaughtered at day 42. No effects of any of the copper sources were found on pH in content from the upper or lower SI or the caecum. In study CH43 experiment 2 (Pang et al., 2009), supplementation of broiler diets with 200 mg/kg Cu as either CuSO₄, a Cu-starch/methionine complex, a Cu-starch/methionine complex (added to a diet where the methionine was reduced with the amount of methionine added with the Cu-starch/methionine complex) and a Na-alginate Cu complex significantly reduced the pH in content from the gizzard (4.02, 3.45, 3.27, 3.60, 3.38 for the control group and the four Cu supplemented groups respectively) from broilers slaughtered at day 42. No effect of any of the copper sources was found on pH in content from the upper or lower SI or the caecum. In study CH51 (Shao et al., 2012), supplementation of broiler diets with 20 mg/kg Cu as Cu silicate nanoparticles significantly reduce the pH of content from the caecum from birds slaughtered at day 21 (7.60 and 7.27 for the control fed and the copper fed birds, respectively). No effect of copper supplementation were found on pH of ileal content from the caecum or on pH of fecal samples taken throughout the experimental period of 50 days. In conclusion the effect of supplementing broiler diets seems to reduce pH in gizzard content while the effect on the rest of the gastrointestinal tract seems to be marginal.

**Ammonia emission.** Effect of copper on ammonia emission was investigated in study CH51 (Shi et al., 2013). Supplementation of broiler diets with 20 mg/kg Cu as Cu silicate nanoparticles significantly reduce the ammonia emission at day 19, 25, 31, 37, 43 and 49 to about half of the emission found for the control fed birds.

**Gizzard erosion.** The effect of copper on gizzard erosion was investigated in five experiments with broilers (CH5, CH18, CH33, CH34 experiment 1, and CH34 experiment 2). In study CH5 (Choi et al., 1989), gizzard erosion was investigated in broilers fed four different diets (a control and three diets supplemented with 125 mg/kg, 250 mg/kg and 375 mg/kg Cu as CuSO₄). Gizzard erosion was measured in three birds per treatment after 1, 3 and 5 weeks on the experimental diet. Gizzard erosion indices were scored from 0 (normal) to 4 (severe). The incidence of gizzard erosion increased as the level of copper in the diet increased and with time on the experimental diets. After 5 weeks on the experimental diet the gizzard erosion indices were 0.0, 1.3, 2.7 and 3.7 for the birds fed the...
Control diet and the birds fed 125, 250 and 375 mg/kg Cu as CuSO₄, respectively. In study CH18 (Kim et al., 1993) gizzard erosion was investigated in broilers in a 2 x 3 factorial experiment including 2 levels of added fat (0% and 4%) and 3 levels of added copper (no addition, 200 mg/kg Cu as CuSO₄·H₂O and 200 mg/kg Cu as CuSO₄·5H₂O). Again increasing gizzard erosion indices were evident in birds fed the copper supplemented diets; both in the birds fed the low and the high fat diet. In experiment CH33 (Min et al., 1993), gizzard erosion was investigated in birds fed a control diet and three diets supplemented with three different copper sources (200 mg/kg Cu as CuSO₄, 200 mg/kg Cu as Cu-methionine and 200 mg/kg Cu as Cu-fish meal). Gizzard erosion was measured as described for study CH5 in four birds per treatment after 42 days on the experimental diets. Again severe gizzard erosion was observed in the birds fed the copper supplemented diets (0.0, 3.2, 3.4 and 3.0 for the control fed birds and the birds fed 200 mg/kg Cu as CuSO₄, Cu-methionine and Cu-fish meal, respectively). In study CH34 experiments 1 and 2 (Min et al., 1993), gizzard erosion was investigated for five sources of copper all added to the diets to increase the Cu concentration with 200 mg/kg. The five copper sources were CuSO₄, a Cu-starch complex, a Cu-starch/methionine complex, a Cu-methionine complex and a Na-Alginate Cu complex. Regardless of copper sources, severe gizzard erosion was observed in all broilers fed copper supplemented diets in all four studies where it was investigated. In conclusion, supplementation of broiler diets with copper at concentrations above 125 mg/kg strongly increases gizzard erosion.

**Effect of copper on microbial metabolites**

Ammonia concentration. Effect of copper on ammonia concentration in fecal samples was investigated in study CH51 (Shi et al., 2013). Supplementation of broiler diets with 20 mg/kg Cu as Cu silicate nanoparticles had no effect on ammonia concentration in fecal samples neither on day 21 nor on day 50.

**Effect of copper on microbial activity**

Microbial enzyme activity. The effect of copper on microbial enzyme activity of β-glucosidase and β-glucuronidase has been investigated on two studies with broilers (CH30 and CH65). In study CH30, broilers were fed a control diet and two experimental diets supplemented with either 34 or 68 mg/kg Cu as Cu-Montmorillonite-Nanomaterial (Cu-MMN). The diets were fed to the broilers for 42 days. Eight birds from each treatment were slaughtered at day 21 and day 42 and the enzyme activity measured in content from the SI and the caecum. The activities of β-glucosidase and β-glucuronidase in the small intestine of birds fed on the diet supplemented with both concentrations Cu-MMN were significantly lower than the control, both for the birds slaughtered at day 21 and for the birds slaughtered at day 42. Similarly both enzyme activities were reduced in the Cu-MMN fed birds compared to the control fed birds in caecal content at day 21 but not at day 42. In study CH65 (Xu et al., 2003), broilers were fed a control diet and two experimental diets supplemented with either 1 g/kg Montmorillonite (MM) or 1 g/kg Cu-Montmorillonite (Cu-MM) (resulting in an addition of 25 mg/kg Cu to the feed). The diets were fed to the broilers for 42 days. Eight birds from each treatment were slaughtered at day 21 and day 42 and the enzyme activity measured in content from the SI and the caecum. The activities of β-glucosidase and β-glucuronidase in the small intestine of birds fed on the diet supplemented with Cu-MM were significantly lower than the control both for the birds slaughtered at day 21 and for the birds slaughtered at day 42. The caecal β-glucuronidase was not significantly affected by Cu-MM. The enzyme activities in the MM fed birds were somehow between the control fed birds and the birds fed the copper enriched MM (Cu-MM).

3.2.3. Overall effect of copper on the chicken gut microbiota

Clostridia: Supplementing chicken (broilers and layers) diets with additional copper seems to reduce the population of clostridia in the gastrointestinal tract of the birds, even at low concentrations of copper. Especially clay bound copper seems to be effective in reducing the population of clostridia.
**Coliform bacteria:** In general the effect of copper on the gastrointestinal populations of coliform bacteria seems to be rather weak; in most cases where an effect was seen, the concentrations was higher than 200 mg/kg Cu, the explanation may be copper bound clay particles, especially copper bound nanoparticles seem to have an effect at concentrations below 50 mg/kg Cu (CH30 and CH51). That high concentrations of copper may reduce the population of coliform bacteria in gut content from chickens is in agreement with the *ex vivo* experiment studies (CH42 and CH43) that show that Cu as CuSO4 inhibit the growth of coliform bacteria at a concentration of 250 mg/kg Cu but not at a concentration of 125 mg/kg Cu.

**Lactobacilli:** From the investigation of copper concentrations and sources on the population of lactobacilli in gastrointestinal content it may be concluded that copper concentrations at or below 150 mg/kg seem to stimulate the population of lactobacilli in chicken gut content, while it does not seem to have any effect at concentrations from 187 to 200 mg/kg but has an inhibitory effect at concentrations above 225 mg/kg. In agreement with that, the *ex vivo* experiments (CH42 and 43) show that 125 mg/kg Cu as CuSO4 stimulated the growth of lactobacilli in broiler SI content. However no inhibitory effect on the growth was seen by a copper concentration of 250 mg/kg CuSO4. That no effect of tribasic copper chloride (TBCC) was seen in the *ex vivo* experiment is properly due to the low solubility of TBCC in water (CH42).

**Streptococci.** Supplementing broiler diets with additional copper does not seem to have any effect on the population of streptococci in the gastrointestinal tract.

**Aerobe bacteria:** Only three studies could be found and copper supplementation showed no effect (the effect in study CH51 may be an effect of the nanoparticles rather than an effect of copper).

**Total anaerobe bacteria:** Copper supplementation had no effect in most studies (only in one study with high copper concentration).

**Ex vivo studies.** 250 mg/kg Cu as CuSO4 inhibit the growth of coliform bacteria in chicken SI content. No effect of 250 mg/kg CuSO4 on growth of lactobacilli. No effect on growth of either coliform bacteria or lactobacilli by TBCC (properly due to low water solubility).

**pH.** Supplementing broiler diets with copper seems to reduce pH in gizzard content while the effect on the rest of the gastrointestinal tract seems to be marginal.

**Community structure.** The effect of copper supplementation of broiler diets seems to have little effect on the community structure of the microbiota in the small intestine. Neither Cu supplementation at 187.5 mg/kg or 250 mg/kg nor Cu sources (CuSO4 or TBCC) affected the number of predominant bacterial species (DGGE band number) in ileal content or ileal mucosa or affected the similarity coefficient of the microbial community structure in ileal content. In study CH42, it was found that supplementation of the diet with 187.5 mg/kg Cu as TBCC increase the similarity coefficient of ileal mucosal bacteria while supplementing the diet with 187.5 mg/kg Cu as CuSO4 had no effect. In contrast to that, it was found in study CH56 that supplementing broiler diets with 250 mg/kg Cu as CuSO4 increase the similarity coefficient of the mucosa associated microbiota.

**Gizzard erosion.** Supplementation of broiler diets with copper at concentrations above 125 mg/kg strongly increase gizzard erosion.

### 3.3 Dairy cows

#### 3.3.1 Study cows

Of the 114 references found for cows by the literature search, 5 were in the first phase considered appropriate to be included in the ELS. However, a closer examination of the five studies revealed the papers not to be appropriate to be included in the ELS. One of the studies was published in Polish, two of the studies were dealing with antibiotic resistance, one was dealing with the effect of copper on parasite control and the last one on the effect of copper on abomasal ulcers.
4. Conclusions

Piglets and pigs

Of the 229 references found for pigs and piglet by the literature search, 28 were considered appropriate to be included in the ELS. In total 34 different characteristics related to the gastrointestinal microbiota were investigated in the selected studies. The number of characteristics investigated in the individual studies varied from 1 to 15. The number of studies in which each characteristic was studied varied from 1 to 18. The characteristic studied could be divided in 8 themes: Plate count of bacteria (21 studies), qPCR of bacterial populations (1 study), microscopic counts of bacteria (1 study), community structure analyses (4 studies), gut content characteristics (9 studies), microbial metabolites (4 studies), microbial activity (5 studies) and studies in germ free pigs (1 study). The copper concentrations used in the studies varied from 1.5 to 375 mg/kg feed. Four types of copper sources were used in the studies: inorganic bound copper (23 studies at concentrations from 26-375 mg/kg feed), organic bound copper (5 studies at concentrations from 13-134 mg/kg feed), copper bound to clay minerals (5 studies at concentrations from 36-49 mg/kg feed) and copper-loaded nanoparticles (1 study at a concentration of 1.5 mg/kg).

Microbial activity: No effect of Cu as CuSO\(_4\) at 175 mg/kg feed was observed on ATP and urease activity in piglets. Significant effect of Cu as CuSO\(_4\) on urease activity in growing pigs

Microbial metabolites: No effect in piglets on SCFA, lactate or succinate concentrations in the gastrointestinal tract of Cu as CuSO\(_4\) at supplemental concentrations below 175 mg/kg. 250 mg/kg Cu as CuSO\(_4\) increases the SCFA concentrations in piglets.

It may be concluded from the experiments that supplementing pig diets with low additional copper amounts (below 50 mg/kg Cu) seems to inhibit the population of coliform bacteria in the gastrointestinal tract of as well piglets as growing pigs. In particular, clay bound copper seems to be effective. When supplemented to the feed in amounts above 100 mg/kg copper, the effect on the population of coliform bacteria in the gastrointestinal tract of piglets as well as pigs was marginal.

Supplementing pig diets with copper concentrations above 170 mg/kg as CuSO\(_4\) has a reducing effect on the population of lactobacilli in the gastrointestinal tract of as well piglets as growing pigs. The effect of Cu as CuO did not affect the population of lactobacilli at high concentrations (250 and 375 mg/kg feed). Diets with copper concentration below 125 mg/kg feed seem to have marginal effect on the population of lactobacilli in the gastrointestinal tract both in piglets and in growing pigs.

The results with growing pigs strongly indicate that the addition of 100 mg Cu/kg, or higher, as CuSO\(_4\) significantly reduces the population of streptococci in the gastrointestinal tract of growing pigs. The effect of CuSO\(_4\) on the population of streptococci in piglets is more questionable.

Community structure studies strongly suggest that supplementing piglet diets with 100 to 250 mg/kg Cu as CuSO\(_4\) affects the community structure of the microbiota in the small intestine, the caecum and the colon.

The overall conclusion from the studies with piglets and growing pig is that copper, even at low concentrations (<50 mg/kg feed), may affect the microbiota in the gastrointestinal tract. Especially the population of clostridia and coliform bacteria seems to be affected of low concentrations of copper, and especially copper bound clay minerals seem to have an effect. At higher concentrations (>170 mg/kg feed) Cu as CuSO\(_4\) reduces the population of lactobacilli in piglets as well as in growing pigs.

In growing pigs CuSO\(_4\) reduces the population of streptococci in colonic and fecal samples, the population of ureolytic bacteria in the colon of which streptococci make up 74%, the urease activity in the colon, and decarboxylation and deamination of amino acids in the small intestine. No effect of Cu as CuSO\(_4\) on the population of streptococci and on urease activity was seen in piglets. Supplementing piglet diets with 100 to 250 mg/kg Cu as CuSO\(_4\) significantly affected the community structure of the microbiota in the small intestine, the caecum and the colon.
Chickens

Of the 106 references found for chickens by the literature search, 17 were considered appropriate to be included in the ELS. In total 20 different characteristics related to the gastrointestinal microbiota were investigated in the selected studies. The number of characteristics investigated in the individual studies varied from 1 to 16. The number of studies in which each characteristic was studied varied from 2 to 8. The characteristic studied could be divided in 5 themes: Plate count of bacteria (16 studies), community structure analyses (2 studies), gut content characteristics (6 studies), microbial metabolites (1 study) and microbial activity (2 studies). The copper concentrations used in the studies varied from 8 to 375 mg/kg feed. Four types of copper sources were used in the studies: inorganic bound copper (13 studies at concentrations from 8-375 mg/kg feed), organic bound copper (8 studies at concentrations from 50-200 mg/kg feed), copper bound to clay minerals (2 studies at concentrations from 25-37 mg/kg feed) and copper-loaded nanoparticles (1 study at a concentration from 34-68 mg/kg).

From the plate count investigations of bacterial population it can be concluded that supplementing chicken (broilers and layers) diets with additional copper seems to reduce the population of clostridia in the gastrointestinal tract of the birds, even at low concentrations of copper. Especially clay bound copper seems to be effective in reducing the population of clostridia.

In general the effect of copper on the gastrointestinal populations of coliform bacteria seems to be rather week, in most cases where an effect were seen the concentrations was higher than 200 mg/kg Cu, the exception may be copper bound clay particles, especially copper bound nanoparticles seems to have an effect at concentrations below 50 mg/kg Cu. That high concentrations of copper may reduce the population of coliform bacteria in gut content from chickens is in agreement with the ex vivo experiment studies that show that Cu as CuSO₄ inhibits the growth of coliform bacteria at a concentration of 250 mg/kg Cu but not at a concentration of 125 mg/kg Cu.

From the investigation of copper concentrations and sources on the population of lactobacilli in gastrointestinal content it may be concluded that copper concentrations at or below 150 mg/kg seems to stimulate the population of lactobacilli in chicken gut content, while it does not seem to have any effect at concentrations from 187 to 200 mg/kg. However, it has an inhibitory effect at concentrations above 225 mg/kg. In agreement with that, the ex vivo experiments show that 125 mg/kg Cu as CuSO₄ stimulated the growth of lactobacilli in broiler SI content. However, no inhibitory effect on the growth was seen by a copper concentration of 250 mg/kg CuSO₄.

In conclusion the effect of supplementing broiler diets with copper at concentrations of 200 mg/kg either as inorganic or organic bound copper seems to reduce pH in gizzard content while the effect in the rest of the gastrointestinal tract seems to be marginal.

In conclusion the effect of copper supplementation of broiler diets seems to have little effect on the community structure of the microbiota in the small intestine. Neither Cu supplementation at 187.5 mg/kg or 250 mg/kg nor Cu sources (CuSO₄ or TBCC) affected the number of predominant bacterial species (DGGE band number) in ileal content or ileal mucosa or affected the similarity coefficient of the microbial community structure in ileal content. In study CH42, it was found that supplementation of the diet with 188 mg/kg Cu as TBCC increase the similarity coefficient of ileal mucosal bacteria while supplementing the diet with 188 mg/kg Cu as CuSO₄ had no effect. In contrast to that, it was found in study CH56 that supplementing broiler diets with 250 mg/kg Cu as CuSO₄ increase the similarity coefficient of the mucosa associated microbiota.

Supplementation of broiler diets with copper at concentrations above 125 mg/kg strongly increases gizzard erosion.

The overall conclusion from the studies with broilers is that copper even at low concentrations (<50 mg/kg feed) may affect the microbiota in the gastrointestinal tract. Especially the population of clostridia seems to be affected by low concentrations of copper. In particular, copper bound clay minerals seem to have an effect. At higher concentrations (>200 mg/kg feed) inorganic or organic...
bound copper also seems to affect the population of lactobacilli, and coliform bacteria, to reduce the pH in gizzard content and to produce severe gizzard erosion.

**Dairy cows:**

Of the 114 references found for cows by the literature search, 5 were in the first phase considered appropriate to be included in the ELS. However, a closer examination of the five studies revealed the papers not to be appropriate to be included in the ELS. One of the studies was published in Polish, two of the studies were dealing with antibiotic resistance, one was dealing with the effect of copper on parasite control and the last one on the effect of copper on abomasal ulcers.

5. **General remarks**

While implementing this extensive literature search some suggestions and remarks were raised:

- The quality of the papers retrieved from the search on chickens was generally judged to be low. 10 out of 17 papers were written in foreign languages (7 in Korean and 3 in Chinese) with English Abstracts and Table legends.
- A closer examination of the five studies first selected to be included in the ELS study on cows, revealed the papers not to be appropriate to be included in the ELS. One of the studies (Cow33) was published in Polish, two of the studies were dealing with antibiotic resistance (Cow2 and Cow31), one was dealing with the effect of copper on parasite control (Cow13) and the last one on the effect of copper on abomasal ulcers (Cow47).
References of included studies

Alphabetic list of references included in the ELS for pigs and piglets (* ID number according to Annex A1)

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Hu CH, Xia MS, Xu ZR and Xiong L, 2004. Effect of copper-bearing montmorillonite on growth performance and digestive function of growing pigs. *Asian-Australasian Journal of Animal Science* 17, 1575-1581. P46*

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Jensen BB, 1998. The impact of feed additives on the microbiology of the gut in young pigs. *Journal of Animal Feed Science* 7, 45-64. P49*

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Ma YL, Guo T and XuZR, 2007. Effect of Cu (II)-exchange montmorillonite on diarrhea incidence, intestinal microflora and mucosa morphology of weaning pigs. *Chinese Journal of Veterinary Science* 27, 279-283. P69* (In Chinese)
Effect of copper intake on gut microbiota (pigs/piglets, chickens, dairy cows)

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Choi Y and Paik IK, 1989. The effect of supplementing copper sulfate on the performance of broiler chickens. *Korean Journal of Animal Nutrition and Feedstuffs* 13, 193-200. CH5* (In Korean)

Kim CH; Kang HK; Bang HT; Kim JH; Jong H; Choi HC; Paik IK; Moon HK, 2014. Effects of dietary supplementation of copper-sulfate and copper-soy proteinate on the performance and small intestinal microflora in laying hens. *Korean Journal of Poultry Science* 41, 241-247. CH15* (In Korean)
Effect of copper intake on gut microbiota (pigs/piglets, chickens, dairy cows)

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Alphabetic list of references included in the ELS for cows (* ID number according to Annex A3)

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**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| ADFI         | Average daily feed intake |
| ADG          | Average daily gain |
| ATP          | Adenosine triphosphate (used to measure microbial activity in the GIT) |
| BW           | Body weight |
| CFU          | Colony forming units |
| Cu           | Copper |
| CuAA         | Cupric amino acid |
| CuAlg        | Copper Na-alginate complex |
| Cu*CaMM      | Copper exchanged Ca-montomorillonite |
| CuCit        | Copper citrate |
| CuCS         | Copper loaded chitosan |
| CuCSN        | Copper-loaded chitosan nanoparticles |
| CuFM         | Copper fish meal complex |
| CuGly        | Copper glycinate |
| CuMe         | Copper methionine |
| CuMM         | Copper loaded montmorillonite |
| CuMMN        | Copper loaded montmorillonite nanoparticles |
| Cu*NaMM      | Copper exchanged Na-montmorillonite |
| TCuC         | Tribasic cobber chloride |
| CuPro        | Copper proteinate |
| CuSP         | Copper soy proteinate |
| CuSN         | Copper loaded silicate nanoparticles |
| CuStarch     | Starch copper complex |
| DGGE         | Denaturing gradient gel electrophoresis (molecular biology technique for profiling of microbial communities) |
Effect of copper intake on gut microbiota (pigs/piglets, chickens, dairy cows)

| Acronym | Definition |
|---------|------------|
| EFSA    | European Food Safety Authority |
| G/F     | Growth to feed ratio (kg weight gain/kg feed) |
| GIT     | Gastro intestinal tract |
| ppm     | Parts per million |
| PCR     | Polymerase Chain Reaction, a relatively simple and inexpensive tool used to copy pieces of DNA billions of times. PCR is used every day to diagnose diseases and to identify bacteria and viruses. |
| qPCR    | Quantitative PCR (PCR method to quantify microbial populations) |
| SCFA    | Short chain fatty acids, also referred to as volatile fatty acids (VFAs) are fatty acids with an aliphatic tail of less than six carbon atoms. Short-chain fatty acids are produced by microbial fermentation in the GIT. |
| SI      | Small intestine |
| SLR     | Systematic literature review |
| T-RFLP  | Terminal Restriction Fragment Length Polymorphism (molecular biology technique for profiling of microbial communities) |
| TBCC    | Tribasic copper chloride |
Appendix A – Flow charts of included/excluded studies

Flow chart showing how the studies from the pig search were included or excluded.

| Literature Search on Pigs |  |
|--------------------------|--|
| Web of Science | 139 |
| CAB Abstracts | 79 |
| Additional references from reference lists of relevant scientific papers | 11 |
| **Total** | **229** |
| Included | 28 |
| Excluded | 119 |
| Excluded in Title and Abstract screening | 50 |
| Excluded when reading full paper | 69 |
| **Duplicates** | **82** |

Flow chart showing how the studies from the chicken search were included or excluded.

| Literature Search on Chickens |  |
|-----------------------------|--|
| Web of Science | 71 |
| CAB Abstracts | 35 |
| **Total** | **106** |
| Included | 17 |
| Excluded | 51 |
| Full paper could not be retrieved | 1 |
| Excluded in Title and Abstract screening | 18 |
| Excluded when reading full paper | 32 |
| **Duplicates** | **38** |

Flow chart showing how the studies from the cow search were included or excluded.

| Literature Search on Cows |  |
|--------------------------|--|
| Web of Science | 80 |
| CAB Abstracts | 34 |
| **Total** | **114** |
| Included | 5 |
| Excluded | 80 |
| Excluded in Title and Abstract screening | 46 |
| Excluded when reading full paper | 29 |
| **Duplicates** | **34** |
Appendix B — Lists of included/excluded references

Alphabetic list of references included in the ELS for pigs and piglets (* ID number according to Annex A1)

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Jones C and Bitz DM, Patent. Microbial decomposition of organic cpds.|comprises a mixt. of magnetic field-treated gastrointestinal microorganism culture and micronutrients. EP0804543-A; WO9622359-A; WO9646457-A; EP804543-A1; BR9510296-A; US5811276-A; US5843427-A; MX9705494-A1; JP2002514889-W. Cow35*

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Effect of copper intake on gut microbiota (pigs/piglets, chickens, dairy cows)

| Paper ID | Title/Authors | Year | Journal | Key Animal | Status | Paper Category | Decision |
|----------|---------------|------|---------|------------|--------|----------------|----------|
| 104      | Anonymous 2012 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 105      | Anonymous 2012 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 106      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 107      | Anonymous 2012 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 108      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 109      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 110      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 111      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 112      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 113      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 114      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 115      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 116      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 117      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 118      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 119      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 120      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 121      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 122      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 123      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 124      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 125      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 126      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 127      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 128      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 129      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 130      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 131      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 132      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 133      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 134      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 135      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 136      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 137      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 138      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 139      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 140      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 141      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 142      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 143      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 144      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 145      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 146      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 147      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 148      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 149      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 150      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 151      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 152      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 153      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 154      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 155      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 156      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 157      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 158      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |
| 159      | Anonymous 2015 | YES  | NO      | Pig        | NO     | NO             | Rejected |

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Annex A2 - Flow chart showing how the studies from the chicken search were included or excluded.

### Annex A2. Flow chart showing how the studies from the chicken search were included or excluded.

| Title                        | Year | Journal                  | Fate | Publ. | Rets. | Author(s) | Title | Fate | Publ. |
|------------------------------|------|--------------------------|------|-------|-------|-----------|-------|------|-------|
| Effect of copper intake on gut microbiota (pigs/piglets, chickens, dairy cows) |      |                          |      |       |       |           |       |      |       |

#### Comments
- Papers from search in CABI abstracts not included in the search in Web of Science.
- Papers from search in CABI abstracts not included in the search in Web of Science.
## Annex A3 - Flow chart showing how the studies from the dairy cow search were included or excluded.

### Table: Inclusion and Exclusion Criteria

| Study | Type of paper | Year | Journal | Abstract | Accept | Copper | Microbial | Remarks |
|-------|---------------|------|---------|----------|--------|--------|-----------|---------|
| 1.     |                |      |         |          |        |        |           |         |
| 2.     |                |      |         |          |        |        |           |         |
| 3.     |                |      |         |          |        |        |           |         |
| 4.     |                |      |         |          |        |        |           |         |
| 5.     |                |      |         |          |        |        |           |         |
| 6.     |                |      |         |          |        |        |           |         |
| 7.     |                |      |         |          |        |        |           |         |
| 8.     |                |      |         |          |        |        |           |         |
| 9.     |                |      |         |          |        |        |           |         |

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### Flow Chart

- **Inclusion Criteria**
  - Adult dairy cows
  - Copper intake
  - Gut microbiota

- **Exclusion Criteria**
  - Other species or animals
  - Other nutrient intake
  - Other conditions

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**Notes:**
- **Accepted** studies met all inclusion criteria.
- **Rejected** studies did not meet all inclusion criteria.
- **In Progress** studies are ongoing.
- **Not evaluated** studies were not selected for evaluation.

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