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Dried microalgae – an overview about microbiological standards and values

Getrocknete Mikroalgen – ein Überblick über mikrobiologische Normen und Werte

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Summary

The market for foods made from and with microalgae has been growing steadily for years. Microalgae are cultivated in various systems, then subjected to a drying process and marketed. Currently, there are no legally binding microbial limits or guideline values for these products, which makes risk assessment difficult. Previously used microbiological guidelines, the specifications of various manufacturers, as well as national specifications of different organizations and countries were compared. In addition, the microbiological status of 19 commercially available microalgae products from *Chlorella* spp., *Arthrospira platensis* and *Nannochloropsis gaditana* was analyzed. Data were collected on total aerobic mesophilic plate count, coagulase positive staphylococci, *Bacillus cereus,* Enterobacteriaceae, sulfite reducing clostridia, *Escherichia coli,* yeasts and molds. The specifications and guidelines differ both in the number of parameters required and in the specified limit values, and the specifications often name more parameters than the guidelines. The samples examined show a wide variation in microbiological quality, especially in the total aerobic mesophilic plate count. The microbiological values were mostly below the maximum values defined in the specifications. The only pathogen detected was *Bacillus cereus* in two samples with more than 3.0 log cfu/g.

Keywords: Microalgae, microbiology, novel food, microbiological standards

Zusammenfassung

Der Markt für Lebensmittel aus und mit Mikroalgen wächst seit Jahren stetig. Mikroalgen werden in verschiedenen Systemen kultiviert, anschließend einem Trocknungsverfahren unterzogen und vermarktet. Derzeit fehlen für diese Produkte allgemein gültige mikrobielle Grenz- oder Richtwerte, was eine Risikobeurteilung erschwert. Die Spezifikationen verschiedener Hersteller sowie nationale Vorgaben verschiedener Organisationen und Länder wurden verglichen um einen Überblick bisher genutzter mikrobiologischer Analysen- und Grenzwerte zu erhalten. Zudem wurden 19 kommerziell erhältliche Mikroalgenprodukte aus *Chlorella* spp., *Arthrospira platensis* und *Nannochloropsis gaditana* auf ihre mikrobiologische Beschaffenheit untersucht. Es wurden Daten zur aeroben mesophilen Gesamtkeimzahl, Koagulase- positiven Staphylokokken, *Bacillus cereus,* Enterobacteriaceaen, sulfitreduzierende Clostridien, *Escherichia coli,* Hefen und Schimmelpilze erhoben. Die Spezifikationen und Vorgaben unterscheiden sich sowohl in der Anzahl der geforderten Parameter als auch bei den angegebenen Grenzwerten und die Spezifikationen benennen häufig mehr Parameter als die offiziellen Vorgaben. Die untersuchten Proben weisen, insbesondere bei der aeroben, mesophilen Gesamtkeimzahl eine breite Streuung der mikrobiologischen Beschaffenheit auf. Die mikrobiologischen Werte lagen zumeist unter den in den Spezifikationen festgelegten Höchstwerten. Als Pathogene wurden nur präsumtive *Bacillus cereus* in zwei Proben mit über 3.0 log KbE/g nachgewiesen.

Schlüsselwörter: Mikroalgen, Mikrobiologie, neuartige Lebensmittel, mikrobiologische Standards

Introduction

Microalgae are a phylogenetic heterogeneous group of microorganisms and specified by their ability for oxygenic photosynthesis and their growth in aquatic environments. They are a large and diverse group with high potential and growing importance for the global food market. Microalgae can synthesize a wide range of different nutritional beneficial ingredients, for example, protein, omega-3-fatty acids or vitamins. Their reputation as "superfood" led to them being used in a variety of products. The dry matter production increased nine-fold from 1999–2011 (1.000 to 9.000 tons) (Enzing et al., 2014). In 2021, there was already a global production of 15.000 tons of spirulina biomass and 10.000 tons of *Chlorella* sp. (Acién Fernández et al., 2021). The global market volume has reached $ 977.3 million in 2020, and a compound annual growth rate of 5.4 % has been predicted for the period 2020 – 2028 (Kumar and Deshmukh, 2021). Even though microalgae can be extracted for their high-value compounds, like astaxanthin and omega-3-fatty acids, the bulk of microalgae is marketed as either whole or powdered dried biomass (Pulz and Gross, 2004; Enzing et al., 2014). Most of these dried algae are sold as powder, tablets, or capsules (Pulz and Gross, 2004; van der Voort et al., 2015), but the number of food products that use microalgae as ingredients is growing. The range extends from pure microalgae as an additive for beverages to finished microalgae preparations such as noodles, smoothies, chocolate, or ice cream (Koyande et al., 2019).

As microalgae are natural products, it is nearly impossible to produce them on commercial scale under aseptic conditions. Bacteria can also be beneficial for microalgal growth, e. g. for accumulating Vitamin B12 (Croft et al., 2005). On the other hand, microalgae grown in open systems are potentially susceptible to contamination with human pathogens like *Salmonella* spp. This is particularly critical if pure microalgae are consumed in smoothies or juices. Previously inactivated pathogens could be reactivated due to rehydration (Beuchat et al., 2013).

In 2017, a CEN/TC Committee (CEN/TC Committee 454 "Algae and Algae Products") was established, responsible for developing and establishing uniform standards regarding microalgae throughout Europe in cooperation with the CEN/TC Committee 463 ("Microbiology of the food chain"). It also works on harmonizing examination methods and establishing definitions. A new standard concerning the terms and definitions regarding microalgae, the technical report CEN/TR 17559:2022 "Algae and algae products – Food and feed applications: General overview of limits, procedures, and analytical methods" and the draft of the standard "Algae and algae products – Methods of sampling and analysis – Sample treatment", prEN 17605:2020, were published in 2020 and 2022. The CEN/TR 17559:2022 recommends the evaluation and harmonization of specific thresholds in the national guidelines, highlighting the importance of microbiological specifications for this product group. In Annex D of CEN/TR 17559:2022 values for yeast, molds and total aerobic counts (TAC) were recommended for determining the safety of novel algae and algae food products (CEN, 2022; CEN, 2020).

Dried microalgae are neither explicitly considered in the regulation (EC) No 2073/2005 nor in the national guidelines of the "Deutsche Gesellschaft für Hygiene und Mikrobiologie" (DGHM). Nevertheless, there are two guidelines for dried microalgae provided by the the Centre d'Étude et de Valorisation des Algues (CEVA) and Naturland e. V. (Ullmann, 2017). The fact that national guidelines exist in Germany and France is probably due to the fact that Germany, France and Spain are the largest microalgae producers in Europe (Araújo et al., 2021).

Additionally, if new microalgae are approved as novel foods microbiological specifications are part of the application. So far, only *Tetraselmis chuii* and *Odontella aurita* as whole microalgae have been accepted as novel food (Commission of the European Communities, 2017).

Outside of Europe, standards for food-grade spirulina and ready-to-eat algae products have been published in India and China, respectively (Bureau of Indian Standards, 1990; National Health and Family Planning Commission of People's Republic of China, 2014). India and China are both important global players in microalgae production and produce a significant amount of spirulina, which explains the presence of standards in these countries (Haoujar et al., 2022).

These standards are an exception and do not give answers for the wide variety of algae products that are available today. There is a need for more studies concerning the microbiological status of dried microalgae. Only a few research groups examined the microbiological quality of commercial microalgae products (Čabarkapa et al., 2022; Martelli et al., 2021; Sánchez-Parra et al., 2020; Abdelsalam et al., 2018; Hoekstra et al.,2011; Görs et al., 2010). These investigations were carried out on a limited number of samples and microalgae species. Additionally, a few research groups investigated the microbiological quality of different dried microalgae produced in a laboratory setting (Pereira et al., 2019; Seghiri et al., 2019; Sultan et al., 2014; Jittanoonta et al., 1999; Mahadevaswamy and Venkataraman, 1981).

Our study aims to generate more data regarding the microbiological quality of microalgae and to discuss which microbiological parameters should be relevant for the novel product group. Therefore, we summarize the current situation regarding microbiological guidelines for microalgae and specifications given by producers. Additionally, commercially available dried microalgae were microbiologically examined.

Materials and Methods

Specifications and standards

18 specifications of commercially available microalgae products originating from 11 suppliers were analyzed (Table 1). Two guidelines and three standards for microalgae were acquired from Naturland e. V., the CEVA, the Bureau of Indian Standards (BIS), the National Health and Family Planning Commission of the People's Republic of China (NHFPC) and the CEN/TC Committee 454 (Table 2). Not every specification corresponds to microalgae products tested. Five producers did not supply product specifications. Therefore 12 additional specifications from seven different producers were included in the analysis.

Microalgae samples

19 samples of commercially available algae powders (n=16), capsules (n=2) and flakes (n=1) from nine suppliers were purchased from online mail-order companies, with six originating from organic and 13 from commercial cultivation (Table 3). Ten of the 19 samples consist of *Chlorella* sp.,

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TABLE 1: *Microbiological specifications given for dried microalgae products by the producers (log cfu/g).*

No	Producer	Product	TAC	*S. aureus*	Molds	Yeasts	Entero-bacteri-aceae	Coliforms	*E. coli*	*Salmonella* spp.	*L. mono-cytogenes*	Presump-tive *B. cereus*	Sulfit-reducing anaerobes	*C. perfringens*
1	1	*Aphanizomenon flos-aquae*	< 5.0	< 2.0	< 3.0 combined		/	< 3.0	< 2.0	n.d.	/	/	/	/
2	2	*Aphanizomenon flos-aquae*	< 5.0	< 2.0	< 3.0 combined		/	< 2.0	n.d.	n.d.	/	/	/	/
3	3	*Arthrospira platensis*	< 3.0	n.d.	n.d.	< 1.4	/	/	n.d.	n.d.	/	/	/	/
4	4	*Arthrospira platensis*	< 6.0	/	/	/	/	/	/	/	/	/	/	/
5	5	*Arthrospira platensis*	< 5.0	/	< 2.5	< 2.5	/	/	< 0.5	n.d. in 25 g	/	/	/	/
6	6	*Arthrospira platensis*	< 5.0	n.d. in 25 g	< 2.5 combined		/	< 1.0	n.d. in 25 g	n.d. in 25 g	/	/	/	/
7	7	*Arthrospira platensis*	< 5.0	n.d. in 25 g	< 5.0 combined		< 3.0	< 3.0	< 1.0	n.d. in 25 g	/	/	/	/
8	1	*Arthrospira platensis*	< 5.0	/	≤ 4.0	/	≤ 3.0	/	n.d. in 1 g	n.d. in 25 g	/	/	/	/
9	8	*Chlorella pyrenoidosa*	< 3.0	< 1.0	< 2.0	< 2.0	< 2.0	/	< 1.0	n.d. in 25 g	n.d. in 25 g	< 2.0	/	/
10	6	*Chlorella pyrenoidosa*	< 5.0	n.d. in 25 g	< 2.7 combined		/	< 1.0	n.d. in 25 g	n.d. in 25 g	/	/	/	/
11	9	*Chlorella pyrenoidosa*	< 5.0	< 0.7	2.0	< 2.0	< 1.0	/	< 1.0	n.d. in 25 g	n.d. in 25 g	< 1.0	/	< 1
12	8	*Chlorella sorokiniana*	< 4.7	< 1.0	< 2.0	< 2.0	< 2.0	< 1.0	< 1.0	n.d. in 25 g	n.d. in 25 g	< 2.0	/	/
13*	4	*Chlorella vulgaris*	< 6.0	/	Gv: 2.0 Cv: 3.0	< 5.0	Gv: 4.0 Cv: 5.0	/	Gv: 1.0 Cv: 2.0	n.d.	/	/	/	/
14	5	*Chlorella vulgaris*	< 5.0	/	< 4.0	< 6.0	/	/	< 2.0	n.d.	/	/	/	/
15	8	*Chlorella vulgaris*	< 3.0	< 1.0	/	/	< 2.0	< 1.0	< 1.0	n.d. in 25 g	n.d. in 25 g	< 2.0	/	/
16	10	*Chlorella vulgaris*	< 7.0	/	< 3.0	< 3.0	/	< 3.0	n.d.	n.d. in 25 g	/	/	/	/
17	2	*Chlorella vulgaris*	< 5.0	n.d.	< 1.8	<1.6	/	/	n.d.	n.d.	/	/	/	/
18	11	*Tetraselmis chuii*	< 3.0	< 1.0	< 2.0 combined		< 1.0	/	/	n.d. in 25 g	/	/	/	/

*DGHM guidance/critical values for dried mushrooms; n.d. not detectable; Gv: Guidance value; Cv: Critical value

eight samples of *Arthrospira platensis* (widely known as spirulina algae) and one sample of *Nannochloropsis gaditana* which is not approved as novel food. The algae were cultivated in Europe (n=6), Asia (n=5), Africa (n=1), and America (n=1). In six samples the cultivation area could not be traced. Two samples were cultivated in closed photobioreactors and four samples in open ponds, for the other samples, no cultivation information was available.

Microbiological analysis

Samples were analyzed for total aerobic mesophilic plate count (TAC), coagulase positive staphylococci *(Staphylococcus (S.) aureus),* presumptive *Bacillus (B.) cereus,* sulfite-reducing clostridia, Enterobacteriaceae, *Escherichia (E.) coli,* yeasts and molds following the microbiological standard procedures listed in Table 4. 10 g of each sample were mixed with 190 ml buffered peptone water (Carl Roth GmbH + Co. KG, Karlsruhe, Deutschland) and homogenized in a Stomacher 400 Circulator (Seward Limited, Worthing, West Sussex, Großbritannien) at 260 rpm for one minute. Capsules were opened under sterile conditions before mixing them with buffered peptone water. The resulting suspension was serially diluted with buffered peptone water and 100 µl of each dilution was plated in duplicate on the selected media (Table 4).

Additionally, 1.0 ml of the first dilution was spread onto three agar plates to lower the detection limit. Samples were prepared, incubated and evaluated according to the respective norms and results were expressed as colony forming units (cfu) (Table 4). Suspicious isolates were confirmed following the methods specified in Table 4.

The limit of detection (LOD) for all parameters is 1.3 log cfu/g; that for quantification (LOQ) has been determined according to the respective norms (Table 4). The LOQ for TAC is 1.3 log cfu/g, for yeasts and molds 2.3 log cfu/g, for Enterobacteriaceae 2.5 log cfu/g and for presumptive *B. cereus,* 1.9 log cfu/g and values below 2.5 log cfu/g are estimated.

TABLE 2: *Microbiological guidelines and standards given for microalgae products (log cfu/g).*

No	Group	Product	TAC	*S. aureus*	Molds	Yeasts	Entero-bacteri-aceae	Coliforms	*E. coli*	Salmonella *spp.*	*L. mono-cytogenes*	Presump-tive *B. cereus*	Sulfit-reducing anaerobes	*C. perfringens*
1	Naturland e. V.	all microalgae	< 5.0	/	/	< 4.0	< 3.0	/	n.d. in 1 g	n.d. in 1 g	/	/	/	/
2	CEVA	all micro- and macroalgae	< 5.0	< 2.0	/	/	/	< 1	/	n.d. in 25 g	/	/	< 2.0	n.d. in 1 g
3	Bureau of Indian Standards	food-grade spirulina	/	/	/	/	/	n.d. in 0.1 g (*Shigella* n.d. in 1 g)	n.d. in 1 g	n.d. in 1 g	/	/	/	/
4	NHFPC	ready-to-eat algae products	/	m = 2.0 M = 3.0 (n=5; c=1)	/	/	/	/	/	n.d. in 25 g	/	/	/	/
5	CEN/TC Committee 454	all microalgae	3.0 – 7.0	/	< 2.0 combined		/	/	/	/	/	/	/	/

n.d. not detectable; n =number of units in the sample; m = guidance value; M = critical value; c =maximum number of samples that can be > m but < M

Statistical analysis

Statistical analysis was carried out using the graph pad prism software (GraphPad Prism Version 4.00). The data were prepared by setting raw data values below LOD to 19.0 cfu/g (1.28 log cfu/g) and raw data values below LOQ to LOQ - 1.0 cfu/g (1.28 log cfu/g / 2.48 log cfu/g) for all analyses. This approach was chosen to minimize the underestimation of microbiological risks.

For descriptive analysis, mean cfu values and standard deviation for the different groups (Figure 1) were calculated from the raw data and log transformed afterwards.

Raw data was used for statistical analysis. The datasets were grouped according to their species and type of cultivation (commercial or organic) and checked for normal distribution using the Kolmogorov-Smirnov Test. Variances were compared by F-Test for significant differences. When normal distribution and homogeneous variances were given, the groups were compared using an unpaired, two-tailed t-test. If the distribution was not normal or variances were heterogeneous, the groups were compared using an unpaired two-tailed t-test with Welch's correction.

TABLE 3: *Analysed microalgae samples.*

No	Species	Preparation	Origin	Cultivation	Organic / Conventional
1	*Chlorella pyrenoidosa*	powder	Taiwan	not specified	conventional
2	*Chlorella pyrenoidosa*	powder	Taiwan	not specified	organic
3	*Chlorella pyrenoidosa*	powder	China	not specified	organic
4	*Chlorella pyrenoidosa*	powder	Asia	open pond	organic
5	*Chlorella pyrenoidosa*	powder	not specified	not specified	conventional
6	*Chlorella vulgaris*	powder	Germany	closed photobioreactor	conventional
7	*Chlorella pyrenoidosa*	powder	USA	not specified	conventional
8	*Chlorella sorokiniana*	capsule	not specified	not specified	conventional
9	*Chlorella sorokiniana*	powder	Europe	closed photobioreactor	conventional
10	*Chlorella vulgaris*	capsule	not specified	not specified	conventional
11	*Arthrospira platensis*	powder	Germany	open pond	conventional
12	*Arthrospira platensis*	powder	Greece	not specified	organic
13	*Arthrospira platensis*	powder	Burkina Faso	not specified	conventional
14	*Arthrospira platensis*	flakes	South India	not specified	conventional
15	*Arthrospira platensis*	powder	not specified	not specified	conventional
16	*Arthrospira platensis*	powder	not specified	not specified	organic
17	*Arthrospira platensis*	powder	Austria	open pond	conventional
18	*Arthrospira platensis*	powder	not specified	not specified	organic
19	*Nannochloropsis gaditana*	powder	Netherlands	open pond	conventional

Results

Specifications of microalgae products

The 18 microbiological specifications given by producers of dried microalgae (Table 1) vary in their chosen parameters and the accepted values.

All companies specified TAC in a wide range of 3.0 to 7.0 log cfu/g. 10 out of 18 declare a TAC < 5.0 log cfu/g. All but one company list a limit for *Salmonella* spp., demanding them to be non-detectable. In 16 of 18 specifications, a limit for *E. coli* is specified, generally as non-detectable in the product. 16 specifications include yeasts and molds, either combined (between < 2.0 log cfu/g and < 5.0 log cfu/g) or as separated values for yeasts and molds (< 1.4 log cfu/g to < 6.0 log cfu/g for yeasts, non-detectable to < 4.0 log cfu/g for molds). All but five specifications limit Enterobacteriaceae and coliforms or one of these parameters. With the exception of one specification (giving < 5.0 log cfu/g as a warning value), the range for these parameters starts at < 1.0 to < 3.0 log cfu/g. 12 of the 18 specifications define limits for *S. aureus* ranging from non-detectable to < 2.0 log cfu/g; the most frequent value (n= 5) given was non-detectability. For presumptive *B. cereus* and *Listeria (L.) monocytogenes* only two companies specified a limit of < 1.0 log cfu/g to < 2.0 log cfu/g or not detectable in 25 g, respectively. Sulfite-reducing clostridia and *Clostridium (C.). perfringens* are not suggested as a parameter by any of the companies.

Guidelines and standards for microalgae products

The guidelines and standards differ in limits and parameters (Table 2). Naturland e. V. microbiological guidelines include TAC, yeasts, Enterobacteriaceae, *E. coli* and *Salmonella* spp. (Ullmann, 2017). In France, the CEVA proposes microbiological guidelines for microalgae that do not include yeasts, molds, or *E. coli*. They are the only ones considering sulfite-reducing clostridia and *C. perfringens* (Ullmann, 2017).

In China, ready-to-eat algae products are included in the National Food Safety Standard of Pathogen Limits for Food. In India, a standard for food-grade spirulina, but not

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for any other algae, has been established. The Chinese standard includes *S. aureus, Salmonella* spp. and *Vibrio (V.) parahaemolyticus. Salmonella* spp. should be undetectable, which is consistent with other investigated standards. A m value of 2.0 log cfu/g and a M value of 3.0 log cfu/g is provided for *S. aureus* and *V. parahaemolyticus*. The standard for food-grade spirulina considers Enterobacteriaceae, *Shigella* sp., *E. coli* and *Salmonella* spp. and specifies them as non-detectable (Bureau of Indian Standards, 1990; National Health and Family Planning Commission of the People's Republic of China, 2014).

The informative Annex D of CEN/TR 17559:2022 suggests a limit of yeast and molds < 2.0 log cfu/g and a TAC range of 3.0–7.0 log cfu/g. (CEN, 2022).

TABLE 4: *Selected methods and media for microbiological analysis.*

Parameter	Media	Methods in accordance with:
Sample preparation	Buffered peptone water (Carl Roth GmbH + Co. KG, Karlsruhe, Deutschland)	DIN EN ISO 6887-1, except that for the serial dilution, 0.5 ml of the initial dilution was mixed with 4.5 ml peptone water
Total aerobic mesophilic plate count	Plate count agar (PC) (sifin diagnostics GmBH, Berlin, Germany)	EN ISO 4833-2:2013
Staphylococcus aureus	Baird Parker agar (BP) (Oxoid Limited, Hampshire, Great Britain)	DIN EN ISO 6888-1:1999, in contrast to ISO norm plating of 1.0 ml initial dilution on three plates was not done in duplicate and incubation was done at 37°C
Sulfite-reducing clostridia	Tryptose sulfite cycloserine agar (TSC) (sifin diagnostics GmBH, Berlin, Germany)	„Amtliche Sammlung von Untersuchungsverfahren nach §64 LFGB (ASU) L06.00-39", differing from this approach samples were plated and incubated anaerobically. Additionally, 1.0 ml of initial dilution was plated.
Yeasts and molds	Yeast glucose chloramphenicol agar (YGC) (sifin diagnostics GmBH, Berlin, Germany)	„Amtliche Sammlung von Untersuchungsverfahren nach §64 LFGB (ASU) L01.00-37", differing from this approach samples were plated. Additionally, 1.0 ml of initial dilution was plated.
Bacillus cereus	Polymyxin pyruvate egg yolk mannitol bromothymol blue Agar (PEMBA) (Oxoid Limited, Hampshire, Great Britain)	EN ISO 7932:2004, in contrast to ISO norm PEMBA agar was used. Biochemical methods were replaced by identification using microflex LT/microflex LRF MALDI-TOF-MS (Bruker Daltonics GmbH, Billerica, Massachusetts).
Enterobacteriaceae	Violet red bile glucose agar (VRBD) (sifin diagnostics GmBH, Berlin, Germany)	EN ISO 21528-2017, in contrast to DIN norm samples were plated and incubated anaerobically. Additionally, 1.0 ml of initial dilution was plated.
ß-Glucuronidase-positive *Escherichia coli*	Tryptone bile X glucuronide agar (TBX) (sifin diagnostics GmBH, Berlin, Germany)	DIN ISO 16649-2: 2020-12, in contrast to DIN norm plates were incubated at 42°C and samples were plated, to allow for differentiation of grown colonies.

Microbiological analysis of 19 commercial products

The TAC of all samples ranged between 2.5 to 6.6 log cfu/g. Yeasts were mostly below the LOD (n=14) or LOQ (n=5). Mold counts fell below LOD in eight microalgae samples and below LOQ in nine microalgae samples. The remaining two samples showed mold counts of up to 3.2 log cfu/g. Enterobacteriaceae were detected in four (21 %) samples below LOQ and presumptive *B. cereus* in seven samples (37 %). The count for the latter ranged from below LOQ to 4.0 log cfu/g. *E. coli, S. aureus* and sulfite-reducing clostridia were not detected.

The microalgae were grouped according to their species (Figure 1) and cultivation and their microbiological load was compared. The mean TAC in *Chlorella* products was 4.9 log cfu/g, 5.7 log cfu/g in spirulina and 3.9 log cfu/g in *Nannochloropsis* products. Yeasts could only be found in spirulina algae even though the counts were below LOQ. Growth of molds could be proven in 20 % of Chlorella products, in all spirulina products as well as in the tested *Nannochloropsis* sample, even though most of these were below LOQ. 10 % of *Chlorella* and 25 % of spirulina products were positive for Enterobacteriaceae (below LOQ). *B. cereus* could be found in one sample of Chlorella, in all except two samples of spirulina and in the *Nannochloropsis* product. There were no statistically significant ($P > 0.05$) differences between organically and conventionally cultivated microalgae. *Chlorella* sp. and spirulina showed no statistically significant ($P > 0.05$) differences for TAC, Enterobacteriaceae and *B. cereus* while counts of yeasts and molds differ significantly ($P < 0.05$).

Figure 2 demonstrates the percentages of samples that are within certain log ranges. The TAC values in the majority of samples (74 %) are within a log range of < 1.0 log cfu/g to 4.0 log cfu/g and all samples were below 7.0 log cfu/g. Regarding yeasts and Enterobacteriaceae, none of the samples exceeded the LOQ and for molds and *B. cereus* the majority of samples (89.5 %) were below LOQ as well.

Discussion

The microbiological load of microalgae products depends on the species, the cultivation method and the post-harvest processing (Ullmann, 2017). Microalgae are cultivated with or without the addition of carbohydrates and in open or closed systems, all of which can affect the mic-

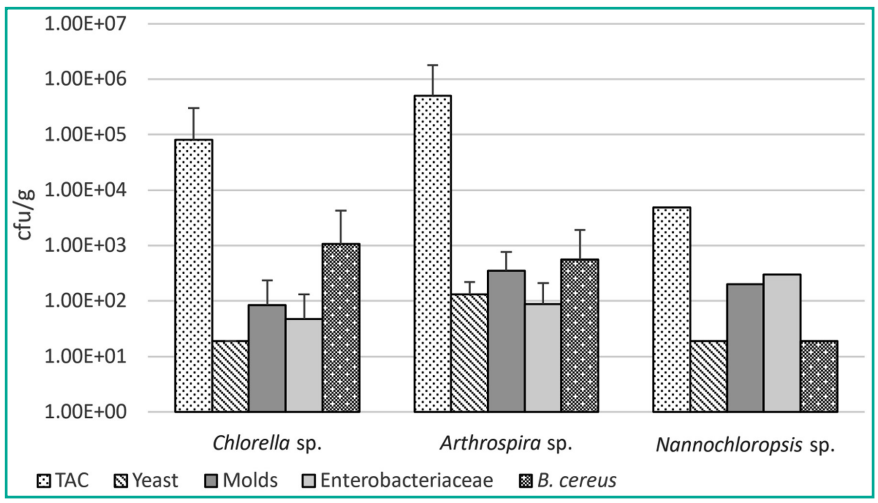

FIGURE 1: *Microbial load of Chlorella sp. (n = 10), Arthrospira sp. (n = 8) and Nannochloropsis sp. (n = 1) in microalgae products.*

robiological community and microbial load (Araújo et al., 2021). Producing microalgae in an axenic culture without any microbiological contamination is difficult, as the microalgae have higher productivity in the presence of symbiotic bacteria (Fuentes et al., 2016; Berthold et al., 2019). Aseptic cultivation is not always possible because microalgae, e. g. *Chlorella,* rely on symbiosis with bacteria to accumulate vitamin B12. Vitamin B12-rich *Chlorella* products can thus only be produced in microalgae-bacteria co-culture (Croft et al., 2005).

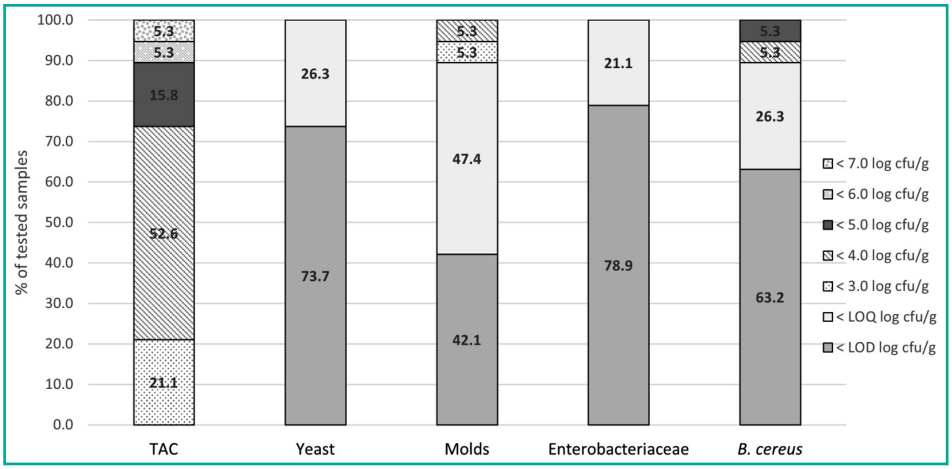

FIGURE 2: *Percentage distribution of microbiological values in dried microalgae products (n=19).*

Most microalgae are produced in open systems, which are susceptible to contamination with microorganisms from the environment (Enzing et al., 2014). During the growth and reproduction of microalgae, the contamination with bacteria can be influenced by the used growth medium. Spirulina, for example, grows best in a high alkalinity medium with pH 8–11 (Oliveira et al., 1999; Hu, 2004). High salinity or alkalinity can prevent the growth of some contaminants. It has been shown that a pH of 10 can stop the growth of *E. coli, B. cereus,* and *Salmonella* spp. in a culture medium (Kim et al., 2018). Therefore, these parameters could make microalgae cultures less susceptible to contaminations as shown for *Synechocystis* sp. at a pH of 11 (Touloupakis et al., 2016). Another point of contamination occurs during the harvest of microalgae. The microalgae suspension is centrifuged for separation from their medium. The concentrated slurry is collected and after an optional step of cell disruption, the microalgae are dried by spray, drum or sun drying. As the algae slurry is transferred and sometimes stored before drying, this period is a potential risk for microbiological contamination.

Microalgae are sold mostly as powder or tablets, with low water activities ranging from 0.2 to 0.4 depending on the technology and protocol used for biomass drying (Santos Buelga et al., 2013; Silva et al., 2020). The low water activity prevents or lowers the microbial growth in products (Lin, 1985). Knowledge about the influence of production, harvest and drying on the bacterial load can help to estimate which microbiological criteria are applicable for dried microalgae. However, given that these production parameters are not harmonized, studying commercial products and comparing these studies to existing specification is important and will be carried out in the following paragraphs to gain an overview of the market situation and to determine which criteria might be applicable. Microalgae produced in laboratory settings will not undergo all production processes, therefore our discussion will focus only on commercially available products to improve the comparability.

Total aerobic plate count

TAC is included and used as an indicator for inadequate manufacturing practice in all analyzed microalgae product specifications, as well as in Annex D of CEN/TR 17559:2022 and the guidelines published by Naturland e. V., and CEVA (Table 1 and 2). Guidance values of products that are comparable to microalgae, like dried fruit powders, recommend a value of 5.0 log cfu/g (DGHM, 2020). 56 % of the reviewed specifications meet this value as well, 17 % of even recommend higher values of log 6.0 cfu/g and log 7.0 cfu/g as a limit comparable with data in Annex D of CEN/TR 17559:2022 Overall guidelines, standards and specifications suggest a TAC limit of < 3 log cfu/g – < 7 log cfu/g. In our study, about 90 % of the products complied with the limit of ≤ 5.0 log cfu/g, and all with the limit of ≤ 7.0 log cfu/g (Figure 2). The mean TAC in *Chlorella* sp. products was 4.9 log cfu/g (2.5 log cfu/g – 5.9 log cfu/g) and in spirulina products 5.7 log cfu/g (3.9 log cfu/g – 6.6 log cfu/g). The high variance in TAC is consistent with published results of other research groups who reported TAC ranging from less than 1 log cfu/g to more than 6 log cfu/g in 33 spirulina and *Chlorella* sp. products (Čabarkapa et al., 2022; Martelli et al., 2021; Abdelsalam et al., 2018; Görs et al., 2010). The high variance in TAC is correlated with the high variance of production conditions as well as a combination of cultivation and post-harvest treatment and cannot be explained by a single factor. However, based on the 33 data sets for algae products, it can be assumed that a count of ≤ 7.0 log cfu/g is generally reachable through good hygienic practice and could be applied as a specification. Our study also delivers TAC data for commercially available *Nannochloropsis* sp. The TAC of 3.9 log cfu/g is lower than that for *Chlorella* sp. and spirulina products. The reason could be either the low sample size or the difference between cultivation in fresh water (e. g. *Chlorella* sp. and spirulina) and seawater (*Nannochloropsis* sp.).

Yeasts and molds

Specifications for yeasts and molds (separately or combined) are very common among the microalgae producers. Annex D of CEN/TR 17559:2022 states that yeast and molds should be < 2.0 log cfu/g in novel algae and algae food products as higher values could result from unsatisfactory hygienic practices (CEN, 2022).

Another recommendation proposes a microbiological specification of < 3.0 log cfu/g for yeast and molds in *Nannochloropsis* products (Zanella and Vianello, 2020). This value correlates with our data for all tested microalgae as only one of the samples had counts of more than 3.0 log cfu/g (Figure 2) as well as with the data of Čabarkapa et al. (2022) and Martelli et al. (2021). They found yeasts and molds in one out of four samples at 2.15 log cfu/g, which would be beneath our LOQ. Therefore, it is possible that the value suggested by Zanella and Vianello (2020) is applicable to other microalgae species as well.

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Yeasts, as cause for food spoilage, have rarely been investigated in dried algae because they are not problematic at low water activities in dried microalgae products (Beuchat et al., 2013). Nevertheless, ten out of 18 of the specifications and the guidelines of Naturland e. V. have set limits separately for yeasts and molds with a wide range from < 1.4 to < 5 log. Half of them specified less than 2.0 log cfu/g for yeasts (Table 1, Table 2).

In our study yeasts were below LOQ in all samples and below LOD in 14 out of 19 analyzed products (Figure 2). Only one other group investigated yeasts in commercial spirulina products and all samples (n=10) were free of yeasts (Abdelsalam et al., 2018). These findings could potentially support the opinion, that yeasts are not a relevant indicator for product quality in commercial dried microalgae: However, this would have to be validated in further studies.

Besides being a cause of food spoilage molds can have human pathogenic potential through the production of mycotoxins (Puri et al., 2019; Beuchat et al., 2013). Nine specifications list molds as a separate parameter (not detectable to < 4 log cfu/g) but only two studies are published for microalgae products. One of them detected fungi hyphae microscopically in ten algae products but did not quantify them whereas the other one detected no molds in ten spirulina products (Sánchez-Parra et al., 2020; Abdelsalam et al., 2018). In our study molds were detectable in 11 samples. This could indicate that molds do pose a problem in microalgae production and should be monitored, even though there is a difference in the prevalence of molds in spirulina and *Chlorella* sp. products.

Enterobacteriaceae, coliforms and *E. coli*

Coliforms as part of the Enterobacteriaceae group serve as an indicator for fecal contamination. 13 of 18 microalgae product specifications include values for either Enterobacteriaceae or coliforms.

The values range from 1.0 to 5.0 log cfu/g for Enterobacteriaceae and coliforms, a very high value compared to similar products like milk, whey and dried fruit powders. The regulation (EC) No 2073/2005 specifies a limit of 1.0 log cfu/g for milk and whey powders (Commission of the European Communities, 2005). The DGHM values for dried fruit powders propose a guidance value of 2.0 log cfu/g and a critical value of 3.0 log cfu/g for Enterobacteriaceae (DGHM, 2020). Zanella and Vianello (2020) even suggest that Enterobacteriaceae should not be detectable in 10 g of microalgae product, as is the norm for follow-on formula in powder form (Commission of the European Communities, 2005); the Indian Standard states that coliforms should not be detectable in 0.1 g of spirulina (Bureau of Indian Standards, 1990).

In our investigation, Enterobacteriaceae were detected (< LOQ) in four samples (Figure 2). Otherwise, none of the studies done on commercial microalgae products (*Chlorella* sp. (n=20) and spirulina (n=16)) detected Enterobacteriaceae or coliforms (Čabarkapa et al. 2022; Martelli et al., 2021; Abdelsalam et al., 2018; Hoekstra et al., 2011; Görs et al., 2010). However, the spirulina biomass in our study, in which Enterobacteriaceae were detected had been labeled as having been processed below 42 °C and as described by Mahadevaswamy and Venkataraman (1981) and Lin (1985) the drying step is essential to reduce Enterobacteriaceae in microalgae. The drying temperature could not be determined for the *Chlorella* sp. products, but due to the diversity in post-harvest processing in microalgae products further investigations are needed to decide whether the specifications for milk and whey powders are actually applicable.

Also *E. coli,* as a potential human pathogen and fecal indicator, should be controlled in microalgae products and is listed in 16 out of 18 specifications. Seven of these specifications demand *E. coli* to be non-detectable, the remaining nine accept values up to 2.0 log cfu/g similar to the DGHM values for dried fruit powders (guidance value of 1.0 log cfu/g, critical value of 2.0 log cfu/g) (DGHM, 2020). These values are in line with those given for ready to eat fruit and vegetables by the regulation (EC) No 2073/2005 (2.0 log cfu/g as m and a limit of 3.0 log cfu/g as M).

In our study, *E. coli* was not detectable in any of the tested microalgae (< 1.3 log cfu/g), similar to the investigations of Čabarkapa et al. (2022) and Abdelsalam et al. (2018) with commercial spirulina (n=11) and *Chlorella* sp. (n=1) products.

As microalgae products are not likely to be fed to infants or young children, it does not seem necessary to demand a non-detectability for *E. coli.*

Staphylococcus aureus

Parameters for *S. aureus* exist in 13 of the 21 microalgae specifications ranging from not detectable to 2.0 log cfu/g. While growth and toxin production in the finished microalgae product is not possible due to the low water activity, already produced toxins will persist. Therefore, toxin content and detection of *S. aureus* might not correlate (Bergdoll and Lee Wong, 2006; Beuchat et al., 2013). A possible approach could be to use values similar to those given for milk powder in the regulation (EC) No 2073/2005 (m value of 1.0 log cfu/g; M value of 2.0 log cfu/g); non-detectability of staphylococcus enterotoxin would have to be proven at a concentration of *S. aureus* above 5.0 log cfu/g. Reactivation and toxin production is possible after rehydration (Fung and Vanden Bosch, 1975), but as microalgae products are not intended to be rehydrated and left at room temperature for a longer period, this could be prevented by education of the consumers. In our study, all samples were negative as well as in the studies of Čabarkapa et al. (2022) and Abdelsalam et al. (2018) who studied twelve commercial algae products.

B. cereus and *C. perfringens*

B. cereus and *C. perfringens* are able to sporulate and survive long times in low water activity foods (Beuchat et al., 2013). They are part of the normal flora of soil and can be easily transmitted into the microalgae biomass through dust (Labbe and Juneja, 2006; Schraft and Griffiths, 2006). *C. perfringens* grows best in anaerobic conditions, which are mostly given in large pond systems, with lacking ventilation (Ullmann, 2017). As *C. perfringens* and *B. cereus* are present as spores in the dried microalgae biomass, it is difficult to define critical values because the number of viable microorganisms contributing to the colony-forming units might not correspond to the number of spores (National Research Council Subcommittee on Microbiological Criteria, 1985). Furthermore, as not all isolates of *C. perfringens* and *B. cereus* are able to produce toxins, the analysis of colony-forming units might not be sufficient to identify a risk to human health and further analysis could be necessary (Messelhäusser et al., 2007; Bundesinstitut für Risikobewertung, 2020).

B. cereus is only commented on in four specifications; *C. perfringens* in one specification and in the guidelines of CEVA (Table 1; Table 2). As microalgae are not like-

ly to be fed to infants, parameters from regulation (EC) No 2073/2005 should not be taken as a basis. Alternatively, the approach of the "Arbeitskreis der auf dem Gebiet der Lebensmittelhygiene und der Lebensmittel tierischer Herkunft tätigen Sachverständigen" (ALTS) (2020) which differentiates the accepted limit of presumptive *B. cereus* based on detection of toxin genes and the ability of the food to promote cereulid production could be used.

While vegetative cells of *C. perfringens* were not detected in any of our tested samples, vegetative cells of presumptive *B. cereus* were found above the limit of detection in 37 % of the samples (Figure 2). Presumptive *B. cereus* have only been examined by Martelli et al. (2021) in two commercial spirulina products with 2.2–2.4 log cfu/g. Our findings demonstrate that monitoring in microalgae products is advisable. This is further emphasized as *B. cereus* was reported in an organic *Chlorella* product at 5.0 log cfu/g in 2018, resulting in entries in the Rapid Alert System for Food and Feed (RASFF) (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, 2022). In regards to *C. perfringens,* Sánchez-Parra et al. (2020) found bacteria with endospores in one of ten commercial microalgae products (spirulina, *Chlorella* sp. and *Aphanizomenon flos-aquae*). In accordance with these results, Hoekstra et al. (2011) detected *Clostridium* spp. endospores up to 7.3 log spores/g in spirulina tablets (n=3). In contrast, Čabarkapa et al. (2022) and Abdelsalam et al. (2018) did not find anaerobic, sulfite-reducing bacteria in eleven spirulina and one *Chlorella* sp. product respectively. *Nannochloropsis* sp. biomass or products have not been investigated for *C. perfringens* in other studies. The reason for the absence of *C. perfringens* in our study and that of Čabarkapa et al. (2022), Abdelsalam et al. (2018) could be that the chosen method would only have detected vegetative cells.

Salmonella spp. and *Listeria* spp.

Salmonella is restricted by most product specifications for microalgae as being not detectable in 25 g of microalgae product (Table 1), which is reasonable as microalgae products could potentially become contaminated with *Salmonella* and studies showed that a low a_w correlates to lower infectious doses (Beuchat et al., 2013). The same requirements are laid down in regulation (EC) No 2073/2005 for a number of foods which include milk and whey powders as well as ready to eat fruits, vegetables and sprouts (Commission of the European Communities, 2005). *L. monocytogenes* is mostly transmitted in wet environments and present in most aqueous ecosystems, which means that a microalgae plant could be a possible spreading ground (Pagotto et al., 2006). Only two manufacturers have included *L. monocytogenes* in their specification (not detectable in 25 g). On the other hand, the regulation (EC) No 2073/2005, which sets 2.0 log cfu/g as a limit for ready-to-eat foods, which do not promote growth of *L. monocytogenes* could be applicable for microalgae (Commission of the European Communities, 2005). As *Salmonella* and *Listeria* are routinely screened in food safety control the status of both bacteria in microalgae is well known, monitored and for *Listeria* even regulated by regulation (EC) No 2073/2005. Therefore, we did not investigate these parameters in our samples.

Salmonella were not detected in any of the studies focusing on dried microalgae biomass (n=8) (Čabarkapa et al., 2022; Pereira et al., 2019; Sultan et al., 2014). Martelli et al. (2021) investigated spirulina products and could not detect *L. monocytogenes,* but up to 2.8 log cfu/g of *Listeria* spp.. However, *Salmonella* spp. were found in *Chlorella* sp. powder products in 2013, 2014, 2015 and 2019, leading to five entries in the RASFF (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, 2022), demonstrating that monitoring these parameters in the interest of consumer protection is necessary.

Conclusion

This study gives insight into the microbiological status of microalgae powders and the specifications to discuss potential parameters applicable for the control of dried microalgae products. The specifications of producers considered in the study vary greatly, but all specifications detail TAC and the majority of them include food hygiene parameters e. g. Enterobacteriaceae, yeasts and molds. Regarding pathogens most specifications include *Salmonella,* while other pathogens e. g. *B. cereus* and *Listeria* are only included in a minority of the specifications.

Standards and guidelines vary as well and can be either stricter or less specific than the specifications without a clear trend.

The microbiological quality of the products we examined was overall satisfactory, with most of them displaying a TAC below 5.0 log cfu/g and counts for yeasts and molds, Enterobacteriaceae and *B. cereus* mostly below LOQ, while all of the other tested parameters were below LOD. Therefore, our samples complied with the given specifications with few exceptions regarding *B. cereus* and some specifications that defined counts below our LOD or LOQ. Based on the current state of knowledge, it seems reasonable to request that *L. monocytogenes* should be regulated due to the production in an aquatic system. The same applies to *Salmonella, E. coli* and Enterobacteriaceae, especially when microalgae are produced in open pond systems. Drying procedures can be effective in reducing microbiological load, but their effectiveness and the correct storage conditions should be controlled and regulated using the TAC and mold count of the finished product. As microalgae are likely to be rehydrated for consumption, it is possible that enterotoxin producing bacteria are reactivated and limits for *S. aureus, B. cereus* and *C. perfringens* should be also established. Additionally, it is important to educate the consumers that dried microalgae should be consumed directly after rehydration. In addition, only a limited number of studies investigating the microbiological load of commercial microalgae products are available, which makes it challenging to set harmonized limits. Therefore, further examinations are needed and values evaluated in this paper can therefore only serve as suggestions.

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Conflict of interest

The authors declare no conflict of interest.

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SUPPLEMENT 1: *Microbiological values of the different microalgae samples (log cfu/g).*

No	Species	TAC	Yeasts	Molds	Enterobacteriaceae	*B. cereus*
1	*Chlorella pyrenoidosa*	2.48	1.28*	1.28*	1.28*	1.28*
2	*Chlorella pyrenoidosa*	3.56	1.28*	1.28*	1.28*	1.28*
3	*Chlorella pyrenoidosa*	3.43	1.28*	1.28*	1.28*	1.28*
4	*Chlorella pyrenoidosa*	4.06	1.28*	2.30**	1.28*	1.28*
5	*Chlorella pyrenoidosa*	4.50	1.28*	2.70	2.48**	4.03
6	*Chlorella vulgaris*	5.87	1.28*	1.28*	1.28*	1.28*
7	*Chlorella pyrenoidosa*	2.60	1.28*	1.28*	1.28*	1.28*
8	*Chlorella sorokiniana*	2.73	1.28*	1.28*	1.28*	1.28*
9	*Chlorella sorokiniana*	2.48	1.28*	1.28*	1.28*	1.28*
10	*Chlorella vulgaris*	3.68	1.28*	1.28*	1.28*	1.28*
11	*Arthrospira platensis*	3.93	2.30**	3.16	2.48**	1.28+
12	*Arthrospira platensis*	4.93	2.30**	2.30**	2.48**	3.61
13	*Arthrospira platensis*	3.96	1.28*	2.30**	1.28*	1.28*
14	*Arthrospira platensis*	3.49	2.30**	2.30**	1.28*	1.90**
15	*Arthrospira platensis*	6.59	2.30**	2.30**	1.28*	1.90**
16	*Arthrospira platensis*	4.00	1.28*	2.30**	1.28*	1.90**
17	*Arthrospira platensis*	3.90	2.30**	2.30**	1.28*	1.90**
18	*Arthrospira platensis*	3.90	1.28*	2.30**	1.28*	1.90**
19	*Nannochloropsis gaditana*	3.88	1.28*	2.30**	2.48**	1.28*

* cfu/g is below LOD and was set to 1.90E*01 cfu/g; ** cfu/g is below LOQ and was set to LOQ – 1 cfu/g

SUPPLEMENT 2: *Limits of detection and quantification.*

Parameter	Limit of Detection (log cfu/g)		Limit of Quantification (log cfu/g)	
	LOD	Shown in diagrams as	LOQ	Shown in diagrams as
TAC	1.30	1.28		
Yeasts	1.30	1.28	2.3	2.3
Moulds	1.30	1.28	2.3	2.3
Enterobacteriaceae	1.30	1.28	2.48	2.48
Presumptive *B. cereus*	1.30	1.28	1.90 / 2.48*	1.90 / Estimate
coagulase positive Staphylococci	1.30	1.28	2.48*	Estimate
β-Glucuronidase-positive *Escherichia coli*	1.30	1.28	2.48*	Estimate
Sulfite reducing Clostridia	1.30	1.28	2.48*	Estimate

*values below this limit can only be estimated